Chapter 1

Introduction
1.1 History and epidemiology of *Acinetobacter* spp.

The Gram-negative bacteria classified as members of the genus *Acinetobacter* have a long history of taxonomic change. These bacteria have been classified in more than 10 genera, the best known of which are *Achromobacter, Alcaligenes, Bacterium, Herellea, Mima, Neisseria, Micrococcus*, and *Moraxella* (Juni, 1978; Brisou, 1957; Schaub and Hauber, 1948; De Bord, 1939). However, the taxonomic proposals for these organisms have emerged and *Bergey’s Manual of Systematic Bacteriology* (Juni, 1984) has classified the genus *Acinetobacter* in the family *Neisseriaceae* with one species, *Acinetobacter calcoaceticus*. This “species” has often been subdivided in the literature into two subspecies, *anitratus* (formerly *Herellea vaginicola*) and *lwoffii* (formerly *Mima polymorpha*), but this arrangement has never been formally approved by taxonomists (Juni, 1978; Henriksen, 1973). More recent taxonomic developments have resulted in the proposal that members of the genus should be classified in the new family *Moraxellaceae*, which includes *Moraxella, Acinetobacter, Psychrobacter*, and related organisms (Rossau et al., 1991). This family constitutes a discrete phylometric branch in superfamily II of the *Proteobacteria* on the basis of 16S rRNA studies and DNA-DNA hybridization assays (Rossau et al., 1989; Van Landschoot et al., 1986).

Delineation of species within the genus *Acinetobacter* is still the subject of much research. Traditionally, a microbial species has been considered to be a group of strains that show a high degree of similarity in terms of their phenotypic properties. Phenotypic identification of individual species is complex and time-consuming (Gerner-Smidt et al., 1991). However, using the formal molecular definition of a microbial species proposed by Wayne *et al.* (1987); it states that a species should include strains of approximately 70% or greater DNA-DNA relatedness and 5°C or less divergence values (ΔTm). To date, more than 20 separate genomic species (DNA-DNA homology groups) have been recognized
within the genus by different research groups on the basis of DNA hybridization studies.
Based on the taxonomic recommendation that only genomic groups readily distinguishable
by phenotypic methods and containing more than 10 strains should be given names, seven
Acinetobacter genomic species have been given formal species names as listed in
Table 1.1. The seven Acinetobacter genomic species listed are namely, Acinetobacter
baumannii, Acinetobacter calcoaceticus, Acinetobacter johnsonii, Acinetobacter Iwoffii,
Acinetobacter junii, Acinetobacter haemolyticus and Acinetobacter radioresistens. Among
these genomic species, Acinetobacter baumannii and Acinetobacter calcoaceticus have
been shown to have an extremely close relationship (Tjernberg and Ursing, 1989) and are
referred together as the Acinetobacter calcoaceticus-Acinetobacter baumannii complex
(Gerner-Smidt et al., 1991) mostly based on the phenotypic characteristic of glucose
acidifying. However, among the members of the genus Acinetobacter, A. baumannii is the
most commonly reported species associated with hospital outbreaks and nosocomial
infections (Seifert et al., 1993).

Certain genomic species described previously (Tjernberg and Ursing, 1989a; Bouvet and Jeanjean, 1989), had some minor discrepancies in the numbering systems. To avoid further confusion, it is current practice to add the suffix BJ or TU (Table 1.1) to denote the genomic species delineated by the two studies. Since many of the strains studied in DNA-DNA hybridization studies have been derived from hospital sources, and the most common habitats of these organisms are soil and water, it seems clear that many naturally occurring genomic species of Acinetobacter have yet to be delineated and that the current taxonomic listing is incomplete (Nemec et al., 2000). However, there have been many reports of Acinetobacter in the scientific and medical literature that still do not use the latest taxonomy or use inadequate identification methods. Although phenotypic identification is problematical, various molecular methods have been developed in an
attempt to provide a rapid identification method suitable for routine taxonomic and epidemiological use.

Table 1.1: Formally recognized genomic species of *Acinetobacter*.

<table>
<thead>
<tr>
<th>Genomic species number</th>
<th>Genomic species name</th>
<th>Type strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>ATCC 23055</td>
</tr>
<tr>
<td>2</td>
<td><em>Acinetobacter baumannii</em></td>
<td>CIP 70.34</td>
</tr>
<tr>
<td>3</td>
<td>Not named</td>
<td>ATCC 19004</td>
</tr>
<tr>
<td>13TU</td>
<td>Not named</td>
<td>ATCC 17903</td>
</tr>
<tr>
<td>4</td>
<td><em>Acinetobacter haemolyticus</em></td>
<td>ATCC 17906</td>
</tr>
<tr>
<td>5</td>
<td><em>Acinetobacter johnsonii</em></td>
<td>ATCC 17908</td>
</tr>
<tr>
<td>6</td>
<td>Not named</td>
<td>ATCC 17979</td>
</tr>
<tr>
<td>7</td>
<td><em>Acinetobacter johnsonii</em></td>
<td>ATCC 17909</td>
</tr>
<tr>
<td>8</td>
<td><em>Acinetobacter lwofii</em></td>
<td>ATCC 15309</td>
</tr>
<tr>
<td>9</td>
<td>Not named</td>
<td>ATCC 9957</td>
</tr>
<tr>
<td>10</td>
<td>Not named</td>
<td>ATCC 17924</td>
</tr>
<tr>
<td>11</td>
<td>Not named</td>
<td>ATCC 11171</td>
</tr>
<tr>
<td>12</td>
<td><em>Acinetobacter radioresistens</em></td>
<td>IAM 13186</td>
</tr>
<tr>
<td>13BJ</td>
<td>Not named</td>
<td>ATCC 17905</td>
</tr>
<tr>
<td>14</td>
<td>Not named</td>
<td>Bouvet 382</td>
</tr>
<tr>
<td>15BJ</td>
<td>Not named</td>
<td>Bouvet 240</td>
</tr>
<tr>
<td>15TU</td>
<td>Not named</td>
<td>Tjernberg 151a</td>
</tr>
<tr>
<td>16</td>
<td>Not named</td>
<td>ATCC 17988</td>
</tr>
<tr>
<td>17</td>
<td>Not named</td>
<td>Bouvet 942</td>
</tr>
</tbody>
</table>

*aNumerous published reports refer to *Acinetobacter* isolates that cannot be identified with any of the genomic species listed above. Such new isolates have not yet been formally grouped or given species names. (Adapted from Bergogne and Towner, 1996).

*Acinetobacters* are ubiquitous in nature and have been isolated frequently in animal and human hosts (Henriksen, 1976). Several studies during the 1960s and 1970s reported isolation of these organisms from the skin of healthy individuals at rates of 0.8–20% for glucose-acidifying *Acinetobacters* (*Acinetobacter anitratus*), and 0–33.6% for glucose-non-acidifying *Acinetobacters* (*Acinetobacter lwofii*) (Al Khoja and Darrell, 1979; Rosenthal, 1974; Somerville and Noble, 1970; Taplin and Zaias, 1963). However, up to date, several studies from the year 1999 to 2007 have reported increased rates of skin colonization as high as 13.5% to 40% for healthy ambulatory volunteers and up to 75% for
hospitalized patients (Marchaim et al., 2007; Abbo et al., 2005; Allen et al., 2004; Van Looveren and Goossens, 2004; Berlau et al., 1999). Skin colonization of patients plays an important role in the subsequent contamination of the hands of hospital staff during contacts, thereby contributing to the spread of the organisms (Getchell-White et al., 1989). High colonization rates of the skin, throat, respiratory system or digestive tract of various degrees of importance have been documented in several outbreaks. However, clinical significance of skin and mucosal Acinetobacter carriage are difficult to draw if the organisms are not identified correctly to the species level. Besides that, Acinetobacters are also ubiquitous organisms in soil, water and sewage (Towner, 1996). It has been estimated that Acinetobacter may constitute as much as 0.001% of the total heterotrophic aerobic population of soil and water (Baumann, 1968). They have been found at densities exceeding 104 organisms per 100 ml in freshwater ecosystems and 106 organisms per 100 ml in raw sewage (LaCroix and Cabelli, 1982). They can be isolated from heavily polluted water, such as that found in wastewater treatment plants, but are found more frequently near the surface of fresh water and where fresh water flows into the sea (Droop and Jannasch, 1977).

Acinetobacters also are found in a variety of foodstuffs, including eviscerated chicken carcasses, various poultry and other meats, milk products and vegetables. It has been reported that Acinetobacters constitute up to 22.7% of the total microflora of chicken carcasses. It is also known that Acinetobacters are involved in the economically important spoilage of foods such as bacon, chicken, eggs and fish, even when stored under refrigerated conditions or following irradiation treatment (Towner, 1996). It is worth noting that there is a significant population difference between the Acinetobacters found in clinical and other environments. The vast majority of clinically significant isolates belong to the A. baumannii – A. calcoaceticus complex, whereas genomic species 7 (A. johnsonii),
8 (A. lwoffii) and 9 (not named) seem to predominate in foods and the environment. Other genomic species appear to comprise only minority components of the different populations investigated, but they may have evolved to acquire a selective advantage in as yet unrecognized specialized ecological niches.
1.2 Phenotypic and genotypic characteristics of *Acinetobacter* spp.

The original concept of the genus *Acinetobacter* (Bouvet and Jeanjean, 1989) included a heterogeneous collection of strict aerobes, nonmotile, non-fermentative, Gram-negative, catalase positive, and oxidase-negative saprophytes that could be distinguished from other non-fermentative bacteria by their lack of pigmentation (Ingram and Shewan, 1960). Extensive nutritional studies (Baumann *et al*., 1968) showed clearly that the oxidase-negative strains differed from the oxidase-positive strains, and in 1971, the Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria recommended that the genus *Acinetobacter* comprise only oxidase-negative strains (Lessel, 1971).

*Acinetobacters* are short, plump, Gram-negative rods, typically 1.0 to 1.5 mm by 1.5 to 2.5 mm in the logarithmic phase of growth but often becoming more coccoid in the stationary phase. *Acinetobacters* often appear as pairs or clusters (Figure 1.1). Gram staining as well as variations in cell size and arrangement can often be observed within a single pure culture. *Acinetobacter* spp. normally forms smooth, sometimes mucoid, pale yellow to greyish-white colonies on solid media. However, there are also studies that showed some environmental strains produced a diffusible brown pigment (Pagel and Seyfried, 1976). The colonies are comparable in size to those members of *Enterobacteriaceae*. Most strains are unable to reduce nitrate to nitrite in the conventional nitrate reduction assay. Some clinical isolates, particularly *A. haemolyticus*, may show hemolysis on sheep blood agar plates.

*Acinetobacters* can grow in a simple mineral medium containing a single carbon and energy source and they also can grow over a wide range of temperatures. Clinical isolates can grow at 37°C, but some environmental isolates prefer incubation temperatures of between 20 to 30°C. However, only *A. baumannii* are able to grow at a higher
temperature of 44°C which is the basis of differentiation between *A. baumannii* and *A. calcoaceticus* (Bouvet and Grimont, 1987).

There is no single biochemical test that enables ready differentiation of this genus from similar bacteria, but the nonfastidious nature and wide biochemical activities of the members of the genus makes them readily distinguishable from other bacteria at the genus level by the combination of nutritional tests applied to nonfastidious, nonfermentative organisms in general, including most commercially available diagnostic devices and systems. Phenotypic identification to the genomic species level is more problematic and time-consuming. A scheme of 22 phenotypic tests has been described that differentiates most of the genomic species known at the present time (Kampfer *et al*., 1993), but this scheme is laborious and time-consuming.

As far as commercial identification systems are concerned, the widely used API 20NE system, based largely on carbon source assimilation tests, contained only *A. baumannii*, *A. haemolyticus*, and *A. lwoffii* in the 1993 database release, together with *A. junii* and *A. johnsonii* as a combination, whereas the type species *A. calcoaceticus* and the other genomic species were not included at all. This system sometimes has problems with sensitivity and reproducibility (Kropec *et al*., 1993), and the differences between the genomic species are so slight that a reliable identification seems unrealistic. Indeed, two studies comparing the API 20NE system with species identification by DNA-DNA hybridization have demonstrated a poor correlation (Horrevorts *et al*., 1995; Weermink *et al*., 1995). However, promising results have been obtained with an automated Biolog system which involves the detection of oxidation with 95 different carbon sources (Dijkshoorn, 1996).
In general, genotypic identification of *Acinetobacter* spp. can be achieved using a genus-specific 16S rDNA-targeted oligonucleotide probe (Wagner *et al.*, 1994). However, most work on the development of molecular methods has been dedicated to developing methods for distinguishing the individual genomic species. The “gold standard” method is DNA-DNA hybridization (Tjernberg *et al.*, 1989b), but this technique is rather laborious and is normally used only in special situations in reference laboratories. Consequently, many research groups have concentrated on the development of alternative molecular methods for distinguishing individual genomic species. Unambiguous differences in rDNA sequences have been found in the highly variable regions of 16S rDNA molecules from at least 21 different genomic groups (Ibrahim *et al.*, 1997), although the limited number of strains examined means that these findings cannot be relied upon for absolute identification of genomic species at the present time. It also should be noted that the groupings based on 16S rDNA analysis did not completely correlate with those based on DNA-DNA homology data. This is in contrast with an alternative strategy in which
phylogenetic groupings were based on the nucleotide sequences of topoisomerase (gyrB) genes (Yamamoto and Harayama 1996).

As an alternative to direct sequence-based identification, a range of more rapid molecular fingerprinting methods have been developed for distinguishing individual genomic species, with varying degrees of success. These methods can be divided into those based on structural features, such as outer-membrane protein patterns (Ino and Nishimura, 1989), and those based on nucleic acid analysis. The most widely used techniques amongst the latter group include amplified fragment length polymorphism (AFLP) analysis (Janssen and Dijkshoorn, 1996), amplified rDNA restriction analysis, (ARDRA) (Vaneechoutte et al., 1995), ribotyping (Gerner-Smidt, 1992), tDNA spacer fingerprinting (Ehrenstein et al., 1996), 16S-23S spacer analysis (Dolzani et al., 1995), and 16S rDNA sequencing (Misbah et al., 2005).

Some of the methods used for species identification such as ribotyping and AFLP could also be used for strain characterization at the subspecies level. PCR-based methods have a lower level of discrimination and reproducibility but easier to perform were devised such as randomly amplified polymorphic DNA-PCR (RAPD-PCR) and repetitive extragenic palindromic (REP) PCR fingerprinting (Snelling et al., 1996). However, among the methods, Pulsed-field gel electrophoresis (PFGE) became the most commonly used method for epidemiological strain typing not only for *Acinetobacters* but for all the bacteria in general (Eckhardt et al., 2003). All these methods are comparative typing methods that require visual or computer-aided side-by-side comparison of molecular fingerprint patterns while multi locus sequence typing (MLST) is a library typing method that was found useful for the study of the population structure of multiple microorganisms (Ecker et al., 2006). MLST is a technique for characterizing isolates of bacterial species using the sequences of internal fragments of usually seven house-keeping genes.
Approximately 450-500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequencer. For each house-keeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST). Each isolate of a species is therefore unambiguously characterized by a series of seven integers which correspond to the alleles at the seven house-keeping loci. In MLST the number of nucleotide differences between alleles is ignored and sequences are given different allele numbers whether they differ at a single nucleotide site or at many sites. The rationale is that a single genetic event resulting in a new allele can occur by a point mutation or by a recombinational replacement that will often change multiple sites depending on the number of nucleotide differences between alleles would erroneously consider the latter allele to be more different to the original allele than the latter. Most bacterial species have sufficient variation within house-keeping genes to provide many alleles per locus, allowing billions of distinct allelic profiles to be distinguished using seven house-keeping loci (Bartual et al., 2005). With the use of these methods, the molecular epidemiology of Acinetobacter spp. can be studied with their mode of spread, the role of hospital personnel in their transmission and that of environmental surfaces. Spread from one patient to another in the same hospital, spread to another hospital in the same geographical region or spread even to more distantly located regions could also be demonstrated.
1.3 Molecular techniques used for genotypic characterization of *Acinetobacter* spp.

In this study, as two molecular techniques were used for genotypic characterization of *Acinetobacter* spp. strains, namely, Pulsed-field gel electrophoresis (PFGE) and Fluorescent *in-situ* hybridization (FISH), a short review of both of these techniques is described below. The PFGE technique was used to evaluate the distribution of *Acinetobacter* strains within the hospital settings of University Malaya Medical Centre (UMMC). On the other hand, FISH technique was used as a method to rapidly identify *Acinetobacters*.

1.3.1 Pulsed-Field Gel Electrophoresis (PFGE).

Pulsed-field gel electrophoresis was introduced in 1982 (Maule *et al.*, 1996) and was used as a molecular typing tool. The ability of PFGE to separate large DNA fragments has provided a useful tool to study microbial genomes. There are various types of PFGE systems such as orthogonal field alternation gel electrophoresis (OFAGE), transverse alternating field electrophoresis (TAFE), and contour-clamped homogeneous electric fields (CHEF), which differ in electrode geometry, homogeneity and method of re-orientation of the electric fields. All the systems actually share the same principle of DNA separation (Birren and Lai, 1993).

All PFGE systems have two electric fields that are applied at an angle greater than 90°. The voltage supplied by the power will force the DNA to change orientation periodically from one electric field configuration to the other. The migration rate of DNA molecules through an agarose gel is dependent on switch time, voltage (field strength), field angle and run time. Each time the field is switched, separation is achieved because the time required to change the direction is dependent on the size of the DNA molecules. Larger molecules take longer time to re-orient and therefore have less time to move during
each pulse. Thus, they migrate at a slower than smaller molecules and therefore as the DNA size increases, the switch time needs to be increased to resolve the molecules. As a result, this method allows the separation of DNA fragments with sizes of 10 kb to 10,000 kb (Suwanto, 1994).

PFGE has been widely used as a molecular typing technique for separating large DNA patterns generated after digestion with low-frequency-cleavage restriction endonucleases (Suwanto, 1994; Birren and Lai, 1993). PFGE involves embedding the organisms in agarose, lysing the organisms in-situ and digesting the chromosomal DNA with restriction endonucleases that cleave infrequently (Birren and Lai, 1993; Maslow et al., 1993). The genome size is equal to the sum of the size fragments produced by each rare cutting restriction enzyme. Although many molecular biological methods can be performed to estimate genome size, PFGE offers better results. PFGE banding can be analyzed directly and it gives the overall picture of the genomic profile which allows the construction of the physical map of a genome (Kramer and Jolly, 1989). The DNA profiles generated by PFGE are stable, reproducible and discriminatory. This approach has proven to be useful for the investigation of the molecular epidemiology of Acinetobacter spp. infection (Eckhardt et al., 2003).

Although DNA sequence-based methods are now emerging rapidly, PFGE is still the method of choice for epidemiological typing of many microorganisms. This method has been used for typing of Acinetobacter strains in numerous studies, usually with ApaI or SmaI as restriction enzyme (Seifert et al., 2005; Seifert and Gerner-Smidt, 1995). When applied on a set of strains of the Acinetobacter calcoaceticus-baumannii complex, PFGE with ApaI was found to be more discriminating than ribotyping (Seifert and Gerner-Smidt, 1995). In contrast, ribotyping was found to be not useful for taxonomic identification of the species in the complex. A comparative study of a selection of Acinetobacter baumannii
strains, performed by three laboratories, showed that PFGE profiles can be compared between laboratories if the procedure is rigorously standardized (Seifert et al., 2005). Thus, this standard procedure offers the opportunity to set up an international database for monitoring strains spreading regionally or globally.

In hospital epidemiology, it is common practice to consider strains with PFGE band differences of up to three bands as being probably part of the outbreak or closely related and differences of between four and six band as possibly part of the outbreak or possibly related during outbreaks (Tenover et al., 1995). However, to date, besides the standard method described by Tenover et al., 1995, there is no other systematic study that has yet been performed to assess the band variation specifically for Acinetobacters during outbreaks or endemic episodes.

1.3.2 Rapid identification of Acinetobacter spp. using Fluorescent in-situ Hybridization (FISH).

As far as bacterial identification is concerned, fluorescent in-situ hybridization (FISH) with fluorescently labeled oligonucleotide probes targeting the rRNA is a rapid and easy-to-perform method that has been used for the detection of other microorganisms except Acinetobacters (Wellinghausen et al., 2007; Jansen et al., 2000; Kempf et al., 2000).

Generally, fluorescent in-situ hybridization (FISH) is a molecular-cytogenetic investigation method and thus covers a gap between classical cytogenetic and molecular-genetic techniques. By the broad spectrum of application possibilities it leads to important new developments in basic and applied cytogenetics. It enables the labeling of whole chromosomes and defined chromosome regions and further localizes the gene. This technique allows DNA sequences to be detected on metaphase chromosomes, in interphase
nuclei, in a tissue section, or in blastomeres and gametes. In basic scientific research special fields of application comprise characterization of somatic cell hybrids, analyses of meiosis and of karyotype evolution. In clinical and tumor cytogenetic it helps to identify chromosome re-arrangements, marker chromosomes, chromosome mosaicism and specific tumor cell lines. Overall, FISH is a powerful technique that can be used in genetic counseling, medicine, and also species identification. FISH technique as in species identification has also been used in routine screening of positive blood cultures from clinical settings (Jansen et al., 2000).

FISH is an easy and rapid technique which takes less than 2 hours as compared to conventional methods which take about 72 hours. Generally, in this application, first, a probe is constructed. The probe has to be long enough to hybridize specifically to its target but not too large to impede the hybridization process, and it should be tagged directly with fluorophores, with targets for antibodies or with biotin. This can be done in various ways, for example nick translation and PCR using tagged nucleotides as shown in Figure 1.2.

Then, an interphase or metaphase chromosome preparation is produced. The chromosomes are firmly attached to a substrate, usually glass slide. Repetitive DNA sequences must be blocked by adding short fragments of DNA to the sample. The probe is then applied to the chromosome DNA and incubated for ~12 hours while hybridizing. Several wash steps remove all unhybridized or partially-hybridized probes. The results are then visualized and quantified using a microscope that is capable of exciting the dye and recording images.

If the fluorescent signal is weak, amplification of the signal may be necessary in order to exceed the detection threshold of the microscope. The signal strength depends on many factors which include probe labeling efficiency, the type of probe, and the type of dye affect the fluorescent signal. Fluorescently-tagged antibodies or streptavidin are bound
to the dye molecule. These secondary components are selected so that they have a strong signal.

To date, no invesigation has utilized this technique for bacterial identification of *Acinetobacters*. In the current study, this technique was used to specifically identify *Acinetobacters* down to $10^3$ CFU/ml (Wong *et al.*, 2007).
Figure 1.2: Schematic representation of Fluorescent in-situ hybridization (FISH) techniques.

(Adapted from: http://en.wikipedia.org/wiki/Image:FISH_%28Fluorescent_In_Situ_Hybridization%29.jpg)
1.4 Emergence of nosocomial infections by *Acinetobacter* spp.

Among the *Acinetobacter* genomic species, *Acinetobacter baumannii* is recognized as species most frequently isolated from patients. *Acinetobacters* have been isolated from various types of opportunistic infections, including septicemia, pneumonia, endocarditis, meningitis, skin and wound infection, and urinary tract infection (Joly-Guillou and Bergogne-Bérézin, 1992; Bergogne-Bérézin *et al*., 1987; French *et al*., 1980). The distribution by site of *Acinetobacter* infection does not differ from that of other nosocomial Gram-negative bacteria. In several surveys (Joly-Guillou *et al*., 1992; Glew *et al*., 1977), the main sites of *Acinetobacter* infection are the lower respiratory tract and the urinary tract. Often, *Acinetobacter* spp. emerged as important pathogens in the ICU setting (Ng *et al*., 1993; Siegman-Igra *et al*., 1993; Bergogne-Bérézin and Joly-Guillou, 1991), and this is probably due to the increasingly invasive diagnostic and therapeutic procedures used in hospital ICUs over the last two decade (Hartstein *et al*., 1988). The true frequency of nosocomial infection caused by *Acinetobacter* spp. is not easy to assess, partly because the isolation of these organisms from clinical specimens may not necessarily reflect a true infection but, could on the other hand result from colonization (Struelens *et al*., 1993).

In a United States survey (Talbot *et al*., 2006), it was reported that the occurrences of *Acinetobacter* spp. infections have escalated to levels of 6.9, 2.4, 2.1, and 1.6% as causes of health care-associated pneumonia, bloodstream infections, surgical-site infections, and urinary tract infections, respectively. Similarly, the SENTRY Antimicrobial Surveillance Program lists *Acinetobacter* spp. as the causative agent in 2.3 to 3.0% of health care-associated pneumonia and as the eighth most common pathogen (4.0%) isolated from ICU patients (Jones, 2003) worldwide. Thus, *Acinetobacter* spp. is emerging as an increasingly important multidrug resistant pathogen, spreading in hospitals, and causing severe adverse outcomes. Besides that, *Acinetobacter* spp. seems to be spreading...
from hospital to hospital, and it has established endemicity in various geographical areas through multiple hospital outbreaks (Go et al., 1994). It has become a leading nosocomial pathogen in many hospitals as compared to other non-fermenting Gram-negative bacilli.

In many cases, the dissemination of multidrug resistance in *Acinetobacter* spp. is due to patient-to-patient spread of resistant organisms (Turton et al., 2004). Other risk factors for the acquisition of *Acinetobacter* spp. include prolonged hospital stay, particularly in the ICUs, treatment with antibiotics, invasive procedures and devices, and severely ill patients or those who are immunocompromised. This explains in part the propensity of *Acinetobacter* spp. to cause extended outbreaks which are mainly located in ICUs.

In several outbreaks of nosocomial pulmonary infection caused by *Acinetobacter* spp. in ICUs (Bergogne-Bérézin and Joly-Guillou, 1991; Cefai et al., 1990; Vandenbroucke-Grauls et al., 1988; Stone and Das 1985), the role played by *Acinetobacter* spp. in ventilator-associated pneumonia appears to be increasing. Regardless of the bacteriological method used to define the cause of pneumonia precisely, there are studies reported that about 3 to 5% of nosocomial pneumonias are caused by *Acinetobacter* spp. (Craven et al., 1990). In addition, some researchers have demonstrated the increasing incidence of *Acinetobacter* spp. in nosocomial pneumonia for the subset of ICU patients requiring mechanical ventilation. Besides that, in studies which included only mechanically ventilated patients, at least one *Acinetobacter* spp. reported in 15% of pneumonia cases (Fagon et al., 1989). The similar findings were also reported in bacteriological studies which restricted to uncontaminated specimens obtained by bronchoscopic techniques (Torres et al., 1990). Although other studies have reported lower infection frequencies of 3 to 5% (Craven et al., 1990), these data suggest that nosocomial pneumonia caused by *Acinetobacter* spp. is emerging as one of the leading complications
of mechanical ventilation. However, a number of other factors have been identified as contributing factors of pneumonia or colonization of the lower respiratory tract by *Acinetobacter* spp. in ICU. These include advanced age, chronic lung disease, immunosuppression, surgery, use of antimicrobial agents, presence of invasive devices such as endotracheal and gastric tubes, and type of respiratory equipment (Lortholary *et al.*, 1995; Struelens *et al.*, 1993; Bergogne-Bérézin and Joly-Guillou, 1991). High mortality rates of 30 to 75% have been reported for nosocomial pneumonia caused by *Acinetobacter* spp., with the highest rates reported in ventilator-dependent patients (Bergogne-Bérézin and Joly-Guillou, 1991; Torres *et al.*, 1990; Fagon *et al.*, 1989). This indicates that the prognosis associated with this type of infection is considerably as worse as those associated with other nosocomial pathogens.

On the other hand, bacteremia also is an important nosocomial infection caused by the members of the genus *Acinetobacters*. Among this genus, *Acinetobacter baumannii* is the most common species causing significant bacteremia in most series of adult patients (Seifert *et al.*, 1993). This microorganism may be found either as a single pathogen or as part of polymicrobial bacteremia. Immunocompromised patients are the largest group of adult patients. In these patients, the source of bacteremia is often a respiratory tract infection, with the highest rate of nosocomial bacteremia occurring during the second week of hospitalization. In addition, malignant disease, trauma, and burns seem to be among the most common predisposing factors. A second important group of patients may consist of neonates. A study from Japan has described 19 neonates with *Acinetobacter* septicemia in the neonatal ICU over a period of 30 months. In this study, all cases were of late-onset type septicemia in infants hospitalized for long periods, with a mortality rate of 11% (Sakata *et al.*, 1989). The predisposing risk factors for septicemia were low birth weight, previous antibiotic therapy, mechanical ventilation, and the presence of neonatal convulsions.
Therefore, *Acinetobacter* spp. should be added to the list of organisms capable of causing severe nosocomial infection in neonatal ICUs. As far as risk factors for adults are concerned, surgical wound infections caused by *Acinetobacter* spp. have been described, and such wound infections may lead to bacteremia. There are also a number of reports in the literature describing *Acinetobacter* bacteremia in burn patients (Green and Milling, 1983; Graber et al., 1962). Several studies have reported that there is a correlation between vascular catheterization and *Acinetobacter* infection (Seifert et al., 1993; Rolston et al., 1985). Changing the catheter insertion site every 48 h and appropriate adherence to aseptic protocols may reduce the risk. A correlation between *Acinetobacter* bacteremia and the use of transducers for pressure monitoring has been performed (Beck-Sague et al., 1990), and it was suggested that prompt attention to sterilization techniques when handling equipment such as transducers may also reduce the infection rate. In general, the underlying disease seems to determine the prognosis of the patient. The prognosis of patients with malignant disease and burns is rather poor, but trauma patients have a better prognosis.

Another infection caused by *Acinetobacter* spp. is the secondary meningitis being the predominant form of *Acinetobacter* meningitis (Berk and McCabe, 1981). Until the year 1967, there were about 60 reports of incidents of *Acinetobacter* meningitis, most of which were community acquired. However, since 1979, the vast majority of cases have been nosocomial infections, with almost all caused probably by *Acinetobacter baumannii*. The mortality rates from different series were ranged from 20 to 27%. In most cases, majority patients have been adult men and had undergone lumbar punctures, myelography, ventriculography, or other neurosurgical procedures, although one patient had posttraumatic otorrhea without intervention (Siegman-Igra et al., 1993). A report of *Acinetobacter* meningitis associated with a ventriculoperitoneal shunt with concomitant tunnel infection in which *Acinetobacter baumannii* was isolated from cerebrospinal fluid
has been described (Seifert et al., 1995). Risk factors include the presence of a continuous connection between the ventricles and the external environment, a ventriculostomy, or a cerebrospinal fluid fistula. In addition, other important risk factor is the presence of an indwelling ventricular catheter for more than 5 days. Besides this factor, the heavy use of antimicrobial agents in the neurosurgical ICU also seems to be an important factor. An outbreak subsided spontaneously only when the selective pressure of antibiotics was reduced (Siegman-Igra et al., 1993). Outbreak of *Acinetobacter* meningitis was described in a group of children with leukemia (Kelkar et al., 1989) following the administration of intrathecal methotrexate. In this outbreak, out of the 20 children who received intrathecal methotrexate, 8 returned within 2 to 19 h of treatment with signs and symptoms of acute meningeal irritation. *Acinetobacter* spp. was isolated from the cerebrospinal fluid of five of these patients, as well as from the methotrexate solution. As a result of meningitis, three of the children died, and five recovered. In this case, the outbreak was caused by the use of inappropriately sterilized needles.

Other than respiratory infection, bacteremia, and secondary meningitis, urinary tract infection is also being an important nosocomial infection, however, this infection caused only infrequently by *Acinetobacter* spp. Urinary tract infection occurs most commonly in elderly debilitated patients, in patients confined to ICUs, and in patients with permanent indwelling urinary catheters. Majority patients about 80% were men (Pedraza et al., 1993), perhaps reflecting the higher prevalence of indwelling urinary catheters in this population as a result of prostatic enlargement. However, it should be noted that not every isolation of *Acinetobacter* spp. from the urinary tract of patients with an indwelling urinary catheter can be correlated with actual infection (Hoffmann et al., 1982).

Besides these infections, very few rare cases of native-valve infective endocarditis caused by *Acinetobacter* spp. have also been reported (Gradon et al., 1992). In this case,
dental procedures and open heart surgery have been identified as possible inciting events. *Acinetobacter* infective endocarditis does not differ clinically from infective endocarditis caused by other microorganisms. However, there are wide variations in the presentation and clinical course of the disease. In addition, *Acinetobacter* spp. can also cause peritonitis in patients undergoing continuous ambulatory peritoneal dialysis. It is difficult to be certain that all such cases are nosocomial, but technical failure and diabetes mellitus are also often underlying risk factors. The mean duration of risk factors before the onset of peritonitis were ranged from 2 to 13 months. In this case, the most common manifestations were abdominal pain or cloudy dialysate, but only a minority of patients had fever. Most of the patients respond to antibiotic therapy without the need to interrupt continuous ambulatory peritoneal dialysis (Lye et al., 1991; Valdez et al., 1991; Galvao et al., 1989). *Acinetobacter* cholangitis and septic complications following percutaneous transhepatic cholangiogram and percutaneous biliary drainage have been reported among elderly patients with obstructive jaundice caused by malignant disease or choledocholithiasis. It was reported in one study that 13.5% of patients undergoing transhepatic cholangiography or biliary drainage had developed infection with the most common isolates being *Enterobacter cloacae* and *Acinetobacter* spp. (Sacks-Berg et al., 1992). Other rare case reports include typhlitis after autologous bone marrow transplantation (Nagler et al., 1992) and osteomyelitis and extremity infections following injury (Dietz et al., 1988; Martin et al., 1988). In some cases, eye infections following trauma have also been reported (Melki and Sramek, 1992; Mark and Gaynon, 1983). Besides that, penetrating keratoplasty (Zabel et al., 1989) and the fitting of contact lenses (Barre and Cook, 1984) have also caused ophthalmic infections with *Acinetobacter* spp.
1.5 Treatment of Acinetobacter infections.

Numerous reports in the medical and scientific literature have documented the high rates of antibiotic resistance found in Acinetobacter spp. (Struelens et al., 1993; Buisson et al., 1990; Leonov et al., 1990; Bergogne-Bérézin and Joly-Guillou, 1985; Larson, 1984; French et al., 1980). Frequent multiple antibiotic resistance exhibited by nosocomial Acinetobacters and the resulting therapeutic problems involved in treating patients with nosocomial infections in ICUs is becoming a serious problem worldwide. Until the early 1970s, nosocomial Acinetobacter infections could be treated successfully with gentamicin, minocycline, nalidixic acid, ampicillin, or carbenicillin, either as single agents or in antibiotic combinations, but increasing rates of resistance began to be noticed between 1971 and 1974. Since 1975, successive surveys have shown increasing resistance in clinical isolates of Acinetobacter spp. (Godineau-Gauthey et al., 1988; Joly-Guillou and Bergogne-Bérézin, 1985; Obana et al., 1985; Garcia et al., 1983). High proportions of strains have become resistant to older antibiotics; indeed, many Acinetobacters are now resistant to clinically achievable levels of most commonly used antibacterial drugs, including aminopenicillins, ureidopenicillins, narrow-spectrum (cephalothin) and expanded-spectrum (cefamandole) cephalosporins (Joly-Guillou and Bergogne-Bérézin, 1985; Morohoshi and Saito, 1977), cephamycins such as cefoxitin (Garcia et al., 1983), most aminoglycosides-aminocyclitolts (Joly-Guillou and Bergogne-Bérézin, 1985; Goldstein et al., 1983; Devaud et al., 1982; Dowding, 1979), chloramphenicol, and tetracyclines. For some relatively new antibiotics, such as broad-spectrum cephalosporins (cefotaxime, ceftazidime), imipenem, tobramycin, amikacin, and fluoroquinolones, partial susceptibility remains, but the MICs of these antibiotics for Acinetobacter isolates have increased substantially in the last decade. Imipenem remains the most active drug used and until recently, was shown to be 100% sensitive to strains (Seifert et al., 1993; Amor et al.,
1993; Vila et al., 1993; Muller-Serieys et al., 1989). In some reports, the only active drugs were imipenem and the polymyxins. Unfortunately, since then the most recent extensive analyses of hospital outbreaks have documented the spread of imipenem-resistant strains (Go et al., 1994; Tankovic et al., 1994). This is a particularly worrying development which threatens the continued successful treatment of *Acinetobacter* infections. Most resistance to imipenem has been observed in strains identified as *Acinetobacter baumannii*, while the MIC of carbapenems for other *Acinetobacter* strains has remained below 0.3 mg/liter, but the widespread emergence and/or spread of resistance to imipenem is likely to pose a serious threat in the near future. Differences in antibiotic susceptibility have been observed between countries, probably as a result of environmental factors and different patterns of antimicrobial usage. Thus, most studies report 50 to 80% of isolates to be not susceptible to gentamicin and tobramycin, while aminoglycosides no longer seem to be active at clinically achievable levels against *Acinetobacter baumannii* isolates from Germany (Seifert et al., 1993). Similarly, in France most *Acinetobacter* isolates, which were originally susceptible to fluoroquinolones, became resistant (75 to 80%) to pefloxacin and other fluoroquinolones within 5 years of the introduction of these antibiotics. Species other than *Acinetobacter baumannii* isolated from the hospital environment such as *Acinetobacter iwoffii*, *Acinetobacter johnsonii*, and *Acinetobacter junii* are involved less frequently in nosocomial infection and are generally more susceptible to antibiotics (Gerner-Smidt, 1987; Traub and Spohr, 1989). Strains of *Acinetobacter iwoffii* are more susceptible to β-lactams than *Acinetobacter baumannii*. *Acinetobacter haemolyticus* isolates are normally not susceptible to aminoglycosides and rifampin, but rifampin which has a mean MIC for *Acinetobacter baumannii* at 2 to 4 mg/liter has been used effectively
in synergic combination with imipenem in ICUs in France (Bergogne-Bérézin and Joly-Guillou, 1985).

However, as an alternative therapeutic option, of all the antibiotics, sulbactam is now often used for the treatment of MDR *Acinetobacter baumannii*, usually as ampicillin/sulbactam. For isolates with moderate resistance to imipenem, this is still the most effective therapy, while for high-level resistance; colistin is preferable (Montero *et al.*, 2004). Finally, tigecycline is a new agent with promising activity against *Acinetobacter baumannii* (Pachon-Ibanez *et al.*, 2004). Tigecycline is the first glycylcycline to be launched and is one of the very few new antimicrobials with activity against Gram-negative bacteria. It evades acquired efflux and target-mediated resistance to classical tetracyclines, but not chromosomal efflux in Proteae (Ruzin *et al.*, 2005) and Pseudomonas (Dean *et al.*, 2003). Tigecycline has shown equivalence to imipenem/cilastatin in intra-abdominal infection and to vancomycin plus aztreonam in skin and skin structure infection (Olivia *et al.*, 2005; Fomin *et al.*, 2005; Sacchidanad *et al.*, 2005; Breedt *et al.*, 2005). Tigecycline may prove particularly useful for treatment of surgical wound infections, where both gut organisms and MRSA are likely pathogens. It is also likely to find a role in the treatment of infections due to multiresistant pathogens, including *Acinetobacter* spp. and ESBL producers, as well as MRSA and Enterococci (Fritsche *et al.*, 2004; Milatovic *et al.*, 2003).
1.6 Emergence of beta-lactam antibiotics.

β-lactam antibiotics are useful therapeutic agents. All the antibiotics contain a β-lactam ring as shown in Figure 1.3 (Koneman et al., 1997; Mims et al., 1993). The β-lactam family of antibiotics consists of different groups of compounds such as penicillins, cephalosporins, cephameicins, monobactams, and carbapenems (Table 1.2).

β-lactam antibiotics act by inhibiting peptidoglycan synthesis in eubacterial cell walls. The target of these antibiotics is the transpeptidation reaction involved in the cross-linking step of peptidoglycan biosynthesis (Koletar, 1995). This reaction is unique to bacteria thus the β-lactam antibiotics are highly specific against bacteria and are useful therapeutic agents.

**Figure 1.3: Basic structure of beta-lactam antibiotic.**

The first β-lactam, benzylpenicillin was discovered by Alexander Fleming in 1929. Before World War II, penicillin production was limited and extremely expensive. During World War II, additional antibiotics were discovered and patterns of susceptibility against
various organisms were established. In 1943, Waksman discovered streptomycin and soon after that, Dubos discovered gramicidin and tyrocidin (Koneman et al., 1997). A year later, Dugger’s research resulted in the discovery of chlortetracycline (Koneman et al., 1997). The introduction of penicillin was miraculous in treating bacterial infections. The need for antimicrobial susceptibility testing became evident soon after antibiotics became commercially available. In very early reports, all isolates of *S. aureus* tested were susceptible to penicillin (North and Christie, 1945; Sprink et al., 1944).

However, in the year 1942, Ramelkamp and Maxon described increased resistance of *S. aureus* isolates to penicillin (Ramelkamp and Maxon, 1942). The mechanism of dissemination and resistance was a result of clonal spread of strains containing plasmids carrying genes for the production and regulation of an inducible Class A β-lactamase that could inactivate penicillin G. Penicillin induced *S. aureus* to produce large amounts of β-lactamase. Much of the enzyme was excreted extracellularly, providing collective resistance to a population of bacteria. This resistance was overcome by the introduction of methicillin, a semisynthetic penicillin which is poorly hydrolyzed by *S. aureus* β-lactamase.

Then, the first semisynthetic penicillin with activity against Gram-negative bacilli was introduced shortly after the introduction of methicillin and ampicillin. Soon species those were intrinsically susceptible to ampicillin, developed resistance to β-lactam antibiotics. The first strain of *E. coli* that was resistant to ampicillin was isolated in Athens in 1963 and was identified as producing TEM-1 β-lactamase (Datta and Kontomichalou, 1965). Since then, more than 30 different plasmid mediated β-lactamases has been identified among the *Enterobacteriaceae* and *Pseudomonas* spp. Among the *Enterobacteriaceae*, TEM-1 and SHV-1 occurred most frequently, whereas PSE-1 was
predominant in *Pseudomonas* spp. (Medeiros, 1989; Medeiros and Jacoby, 1986; Medeiros, 1984). Soon after, in the mid-1960s, the first-generation cephalosporins were released for clinical use (Medeiros, 1997). Cephalosporins are derivatives of the fermentation products of *Cephalosporium acremonium*.

**Table 1.2: The β-lactam family.**

<table>
<thead>
<tr>
<th>β-lactam Group</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural penicillins</td>
<td>Penicillin G, penicillin V</td>
</tr>
<tr>
<td>Penicillinase-resistant penicillins (PRP)</td>
<td>Nafcillin, methicillin</td>
</tr>
<tr>
<td>PRP: isoxazolyl penicillins</td>
<td>Oxacillin, cloxacillin, dicloxacillin</td>
</tr>
<tr>
<td>Amino-penicillins</td>
<td>Ampicillin, amoxicillin</td>
</tr>
<tr>
<td>Carboxy-penicillins</td>
<td>Carbenicillin, ticarcillin</td>
</tr>
<tr>
<td>Ureidopenicillins</td>
<td>Piperacillin, azlocillin, mezlocillin</td>
</tr>
<tr>
<td>First-generation cephalosporins</td>
<td>Cephalothin, cefazolin, cephradine</td>
</tr>
<tr>
<td>Second-generation cephalosporins</td>
<td>Cefamandole, cefuroxime, cefonicid, cefaclor</td>
</tr>
<tr>
<td>Cephamycins</td>
<td>Cefoxitin, cefotetan, cefmetazole</td>
</tr>
<tr>
<td>Third-and-fourth generation cephalosporins</td>
<td>Ceftriaxone, cefotaxime, ceftizoxime, cefoperazone, cefpirome, cefpiramide, ceftazidime, cefepime</td>
</tr>
<tr>
<td>Monobactams</td>
<td>Aztreonam</td>
</tr>
<tr>
<td>Carbapenems</td>
<td>Imipenem, meropenem</td>
</tr>
<tr>
<td>Beta-lactamase inhibitors</td>
<td>Clavulanate acid, sulbactam, tazobactam</td>
</tr>
</tbody>
</table>

The first-generation cephalosporins were generally more β-lactam-resistant and permeated the outer membrane of Gram-negative bacilli more rapidly than the penicillins,
making them more effective antibiotics (Medeiros, 1997). However, clinical isolates soon appeared with diminished permeability. A TEM-1-producing Salmonella isolate which had lost OmpC through mutation as well as repression of OmpF porin synthesis, was found to be resistant to cephalosporins (Medeiros et al., 1987).

During this period too, in the 1970s nosocomial infections due to Gram-negative bacilli had become more prevalent, while those caused by S. aureus declined (Medeiros, 1997). Strains of K. pneumoniae that harboured plasmids containing genes that encoded for TEM-1 as well as multiple antibiotic-resistance genes became endemic in many hospitals. Species that were rarely isolated in the previous era such as S. marcescens and Acinetobacter spp. began to cause outbreaks in hospitals worldwide (Retailliau et al., 1979; Medeiros and O’Brien, 1968).

In October 1978 cefoxitin was approved for clinical use in United States. It was the first derivative of a new class of β-lactam antibiotic (cephamycins) produced by a filamentous Gram-positive soil bacterium, Streptomyces clavuligerus. At that time cefoxitin was highly resistant to hydrolysis by all known plasmid-mediated β-lactamases. However, it was readily inactivated by the chromosomal Class C β-lactamases from Enterobacter, Serratia, Citrobacter, Morganella and Pseudomonas spp. (Medeiros, 1997). Later, the first oxyiminocephalosporins, cefuroxime was synthesized by adding an additional methoxyimino moiety to the R group attached to the acetamido bond of the cephalosporins molecule. The compound was resistant to plasmid-mediated β-lactamases and more stable than cefoxitin to the β-lactamases produce by Enterobacter spp, K. oxytoca, C. freundii, and Providencia stuartii. Further modification of the R group produced cefotaxime which was even more stable to β-lactamases, inhibiting most strains of Morganella morganii and S. marcescens as well as many cefuroxime-resistant strains of
the above mentioned species (Medeiros, 1997). Further improvements via the addition of a novel side chain to the six-membered ring resulted in ceftriaxone, which has a similar antibacterial spectrum but a more prolonged half-life. A large bicyclic moiety added also to the six-member ring produced cefepime, which binds less readily to Class C β-lactamases of *Enterobacteriaceae* (Sanders, 1993). Ceftazidime has a methylethoxyimino group, a larger branched substituent bearing a carboxylate, and has greater activity against *P. aeruginosa* (Medeiros, 1997). All these antibiotics have enjoyed widespread clinical use since their introduction in the late 1970s and early 1980s.

Monobactams which are intrinsically produced by *Pseudomonas acidophila* are novel monocyclic antibiotics that have a β-lactam ring but lack the thiazolidine ring of penicillins (Imada *et al*., 1981). They have little activity against Gram-negative bacilli and none against Gram-positive bacteria or anaerobes (Medeiros, 1997). However, a completely synthetic monobactam, aztreonam, has good antipseudomonal activity and is active against many β-lactamase producing Gram-negative bacilli.

Carbapenems are another novel class of β-lactam antibiotics which are produced by *Streptomyces cattleya*. At the time of introduction, none of the known Class A or Class C β-lactams could inactivate imipenem efficiently. Only a few relatively rare bacterial pathogens that produced metallo-enzymes (Class B) were known to hydrolyze imipenem rapidly (Medeiros, 1997). However, with more widespread clinical use of this agent, an increasing variety of carbapenem-hydrolyzing enzymes have been identified among strains of *Acinetobacter* spp., *P. aeruginosa*, *Serratia marcescens*, *Klebsiella pneumoniae* and other members of the *Enterobacteriaceae*.

The first inhibitor of β-lactamase which was available for clinical use in 1984 was clavulanic acid. It is packaged in combination with ampicillin and is a natural product of
*S. clavuligerus*. The second group of inhibitors, the penicillanic acid sulfones, sulbactam and tazobactam are semisynthetic derivatives of penicillanic acid (Medeiros, 1997). These inhibitors bind, acylate the enzyme, and inactivate the active-site of serine in the Class A β-lactamases thus preventing the β-lactamases from hydrolyzing the penicillins in the drug combination (Medeiros, 1997). Production of large amounts of Class C enzyme confers clinically relevant resistance to cephamycins, oxyiminocephalosporins, monobactams, clavulanic acid, and penicillinic acid sulfones. Carbapenems are the only exception, probably because the β-lactamase hydrolyzes these drugs at an extremely slow rate and the drug can permeate very rapidly into the periplasmic space (Raimondi *et al.*, 1991; Yang and Livermore, 1988).

Over the last thirty years, manipulation of the side chains of the penicillins and cephalosporin nuclei has produced a wide range of powerful antibiotics. However, these novel antibiotics are still susceptible to inactivation by the vast and rapidly evolving array of β-lactamases produced by Gram-negative bacteria (Moosdeen, 1997). Thousands of antibiotics have been discovered but only a few of them have the right combination of properties which confers high activity against the invading organism, low toxicity in mammals, and physical and metabolic stability that justifies their use in humans. These antibiotics are either obtained from a direct microbial source or by chemical modification of known antibiotics. Bacterial resistance to the action of β-lactams will continue to change. Thus, it will be crucial to ensure that proper surveillance of antibiotic use is ongoing and that the data acquired will be used to help establish appropriate guidelines and policies for the use of antibiotics, particularly the β-lactams.
1.7 Antibiotic resistance mechanisms in *Acinetobacter* spp.

As with other Gram-negative organisms, most resistance to β-lactams in *Acinetobacter* spp. is associated with the production of β-lactamases which include the widely distributed TEM-1 and TEM-2 enzymes (Joly-Guillou *et al.*, 1988; Goldstein *et al.*, 1983; Devaud *et al.*, 1982; Philippon *et al.*, 1980). A report by Joly-Guillou *et al.*, 1988 showed that analysis of 76 ticarcillin-resistant (MIC >256 mg/liter) *Acinetobacter* strains for their β-lactamase content found penicillinase activity in only 41% of the resistant strains. Most of these strains produced an enzyme with a pI of 5.4 which correspond to that of TEM-1 like enzyme whereas a few had an enzyme with a pI of 6.3 which correspond to that of the β-lactamase CARB-5. Some β-lactamase activity was also identified with a pI above 8.0 that were presumed to be chromosomally encoded cephalosporinases because of their high pI. A separate study by Vila *et al.* (1993) has identified cephalosporinase activity in 98% of the clinical isolates of *Acinetobacter baumannii* studied and suggested that cephalosporinases are the predominant β-lactamases in this species. Four such enzymes designated as ACE-1 to ACE-4 have been studied in detail by Hood and Amyes (1991). All four enzymes were identified as cephalosporinases, although some possessed a small activity against penicillins and none had detectable hydrolyzing activity against aztreonam or the broad-spectrum cephalosporins, ceftazidime or cefotaxime. These enzymes showed their maximum activity against cephaloridine and, except for ACE-4, showed good activity against cephradine. In addition, enzyme ACE-1 showed the broadest spectrum of activity with some hydrolysis of cefuroxime. Therefore, the contribution of these chromosomal β-lactamases appears to be important in the expression of β-lactam resistance. Besides that, these β-lactamases may also act as dual mechanisms which are mediated by a reduction in permeability and altered penicillin-
binding proteins that probably already confer some inherent resistance (Obara and Nakae, 1991; Sato and Nakae, 1991). The acquisition of plasmid-encoded penicillinases does not seem to have importance in the long-term β-lactam resistance of this genus. The most worrying development is the identification of a novel β-lactamase, designated ARI-1 from an imipenem-resistant strain of Acinetobacter baumannii which was isolated from a blood culture at the Royal Infirmary, Edinburgh, in the 1985 (Paton et al., 1993). This enzyme hydrolyzes imipenem and azlocillin but not cefuroxime, ceftazidime, or cefotaxime. Direct conjugative transfer of the ARI-1 gene from its original Acinetobacter baumannii host to an Acinetobacter junii recipient has been demonstrated by Scaife et al. (1995) and they showed that the same plasmid was visualized in the donor and recipient strains. These last observations suggest strongly that ARI-1 is a plasmid-encoded carbapenemase, a development that may have extremely serious long-term consequences such as acquisition of carbapenemases from one to another strain.

In many cases, carbapenem have become the drug of choice for treatment of infections due to multidrug-resistant Acinetobacter spp. (Bergogne-Bérézin and Towner, 1996). Unfortunately, the prevalence of carbapenem-resistant isolates appears to be increasing. The early reports described Acinetobacter spp. with β-lactamase-independent carbapenem resistance (Clark, 1996; Gehrlein et al., 1991), but the most recent reports have described β-lactamase-mediated resistance (Poirel and Nordmann, 2002; Bou et al., 2000). There are several factors leading to carbapenem resistance in Acinetobacter spp. which include acquisition of β-lactamas, the ability of other β-lactamases to hydrolyze carbapenems, presence of mobile genetic elements, reduced expression of outer membrane proteins, penicillin-binding proteins and most importantly presence of carbapenem
hydrolyzing β-lactamases (Quale et al., 2003; Fernandez-Cuenca et al., 2003; Bou et al., 2000).

Based on molecular studies, two types of carbapenem-hydrolyzing enzymes have been described: serine enzymes possessing a serine moiety at the active site, and metallo-β-lactamases (MBLs), requiring divalent cations, usually zinc, as metal cofactors for enzyme activity (Buynak et al., 2004; Bush, 2001; Bush, 1999; Bush et al., 1995). The serine carbapenemases are invariably derivatives of Class A or Class D enzymes and usually mediate carbapenem resistance in Acinetobacter spp. and also other Gram-negative bacteria. The enzymes characterized from Class A enzymes include NmcA, Sme1-3, IMI-1, KPC1-3, and GES-2 and these genes are only found in other Gram-negative bacteria such as Enterobacter cloae, Serratia marcescens, Klebsiella pneumoniae, and Pseudomonas aeruginosa (Poirel et al., 2001; Yigit et al., 2001; Queenan et al., 2000; Rasmussen et al., 1996; Nordmann et al., 1993; Yang et al., 1990). However, with regard to the activity of these enzymes for carbapenems, they do not always mediate high-level resistance and not all are inhibited by clavulanic acid (Nordmann and Poirel, 2002). Many variants of the SHV, VEB, PER and CTX-M enzymes are found occasionally in this organism, including many that are extended-spectrum β-lactamases (ESBLs) with potent activity against third-generation cephalosporins. In contrast, the oxacillinases have been characterized from Acinetobacter baumannii only and include OXA 23 to 27 (Afzal-Shah et al., 2001; Bou et al., 2000), OXA-40 (Héririer et al., 2003), and OXA-48 (Poirel et al., 2004). These enzymes have weak carbapenemase activity but however, they are able to confer resistance to imipenem and meropenem and are only partially inhibited by clavulanic acid. The Class A and Class D carbapenemases are encoded by genes that have been produced by the bacterium and can be chromosomally encoded. These
carbapenemases sometimes associate with integrons or are carried on plasmids (Nordmann and Poirel, 2002). MBLs, like all β-lactamases, can be divided into those that are normally chromosomally mediated and those that are encoded by transferable genes. The early studies on chromosomally mediated MBLs mainly centered around other Gram-negative bacteria such as *Bacillus cereus* (Lim *et al*., 1988), and *Stenotrophomonas maltophilia* (Walsh *et al*., 1994). However, primarily due to genomic sequencing, increasingly more chromosomally mediated genes are being discovered but are often found in non-clinically associated bacteria (Naas *et al*., 2003; Saavedra *et al*., 2003; Mammeri *et al*., 2002; Rossolini *et al*., 2001; Simm *et al*., 2001). Over the last decade there have been several articles summarizing the levels of MBLs in the bacterial community (Livermore, 2002; Nordmann and Poirel, 2002; Bush, 2001; Livermore and Woodford, 2000; Bush, 1999; Bush, 1998; Payne, 1993). However, in the past 3 to 4 years many new transferable types of MBLs have been studied and appear to have rapidly spread. This problem is becoming a serious issue and this could simulate the global spread of extended-spectrum β-lactamases (ESBLs).

Aminoglycosides such as gentamicin, tobramycin, netilmicin, and amikacin are used widely for the treatment of *Acinetobacter* infections, and increasing numbers of highly resistant strains have been reported since the late 1970s. All four types of aminoglycoside-modifying enzymes (AAC, ANT, AAD, APH) have been identified within clinical *Acinetobacter* strains (Table 1.3), but geographic variations in the incidence of particular genes has been observed. For an example, the gene for AAC(3)-Ia was found frequently in *Acinetobacter* strains from Belgium (36 of 45 strains) but was observed less frequently in strains from the United States (3 of 17 strains) and not at all in strains from Argentina (Shaw *et al*., 1993; Shaw *et al*., 1991). In addition, some strains have been
observed to contain more than one aminoglycoside resistance gene, with as many as six different resistance genes being identified in some isolates. It has been suggested that the novel gene \( aac(6')-Ig \), identified only in \textit{Acinetobacter haemolyticus} and was responsible for amikacin resistance and may also be utilized to identify this species (Lambert \textit{et al.}, 1993). Few studies have investigated the genetic nature of aminoglycoside resistance in \textit{Acinetobacter} spp., and reported that the aminoglycoside resistance genes are found in both plasmid and transposons (Elisha and Steyn, 1991; Lambert \textit{et al.}, 1990; Goldstein \textit{et al.}, 1983; Devaud \textit{et al.}, 1982; Gomez-Lus \textit{et al.}, 1980; Murray and Moellering, 1980).

\textbf{Table 1.3: Aminoglycoside-modifying enzymes identified in \textit{Acinetobacter} spp.}

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Reference(s)</th>
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<tr>
<td>1) Acetylating:</td>
<td></td>
</tr>
<tr>
<td>AAC(69)</td>
<td>Lambert \textit{et al.}, 1993</td>
</tr>
<tr>
<td>AAC(29)I</td>
<td>Dowding, 1979</td>
</tr>
<tr>
<td>AAC(3)I</td>
<td>Vila \textit{et al.}, 1993</td>
</tr>
<tr>
<td>AAC(3)II</td>
<td>Murray and Moellering, 1980</td>
</tr>
<tr>
<td>AAC(3)Va</td>
<td>Shaw \textit{et al.}, 1993</td>
</tr>
<tr>
<td>AAC(3)IV</td>
<td>Shaw \textit{et al.}, 1993</td>
</tr>
<tr>
<td>2) Adenylating:</td>
<td></td>
</tr>
<tr>
<td>ANT(30)I</td>
<td>Shannon \textit{et al.}, 1978</td>
</tr>
<tr>
<td>AAD(30)(9)a</td>
<td>Vila \textit{et al.}, 1993</td>
</tr>
<tr>
<td>ANT(20)I</td>
<td>Murray and Moellering, 1980</td>
</tr>
<tr>
<td>AAD(20)a</td>
<td>Shaw \textit{et al.}, 1993</td>
</tr>
<tr>
<td>2) Phosphorylating:</td>
<td></td>
</tr>
<tr>
<td>APH(39)I</td>
<td>Shaw \textit{et al.}, 1993</td>
</tr>
<tr>
<td>APH(39)II</td>
<td>Murray and Moellering, 1979</td>
</tr>
<tr>
<td>APH(39)III</td>
<td>Murray and Moellering, 1979</td>
</tr>
<tr>
<td>APH(39)VI</td>
<td>Vila \textit{et al.}, 1993</td>
</tr>
<tr>
<td>APH(30)I</td>
<td>Elisha and Steyn, 1989</td>
</tr>
</tbody>
</table>

\(a\) Enzymatic activity detectable only in vitro.
(Adapted from Bergogne and Towner, 1996 with a modification.)

The emergence of \textit{Acinetobacter} spp. as important hospital pathogens has occurred at the same time as increased use of fluoroquinolones for the treatment of serious infection. The development of fluoroquinolones resistance is often quite difficult to demonstrate in...
the laboratory, and it has been extrapolated to suggest that resistance will be rare in the clinical situation. This is true for bacteria such as *Escherichia coli* but does not seem to be the case for nonfermentative Gram-negative bacteria such as *Acinetobacter* spp. Although the precise mechanism is virtually unknown in this organism, it is clear that *Acinetobacter* spp. can readily develop fluoroquinolone resistance. Resistance to fluoroquinolones in other bacterial genera has often been attributed to changes in the structure of the DNA gyrase subunits, usually by *gyrA* mutations. Vila et al. (1994) has demonstrated PCR amplification to amplify DNA of the active-site region of the *gyrA* gene from 13 clinical isolates of *Acinetobacter baumannii* with a range of ciprofloxacin MICs from 0.25 to 64 mg/liter. As a result from sequencing of the PCR product, it was found that the susceptible bacteria had 87 nucleotide differences, correlating with 13 amino acid differences, compared with the same 290-bp fragment from *Escherichia coli*. The residues Gly-81, Ser-83, Ala-84, and Gln-106 had led to fluoroquinolone resistance in *Escherichia coli* and were all of them were conserved in the susceptible strains of *Acinetobacter baumannii*. All nine isolates of *Acinetobacter baumannii* with ciprofloxacin MICs of > 2 mg/liter showed a substitution of Ser-83 to leucine and Ala-84 to proline. Four strains with an MIC of 1 mg/liter did not show any change at Ser-83, but one exhibited a change from Gly-81 to valine. The results correlated with those from *Escherichia coli* in that substitution of Ser-83 contributed to ciprofloxacin resistance in *Acinetobacter baumannii*. Besides that, the *gyrB* mutations have caused changes in the β-subunit and occurred less frequently in other bacteria and also rarely result in such high levels of resistance. So far, no studies have examined these genes in *Acinetobacter* spp. *Acinetobacter* strains are less permeable to antibacterial agents as compared to other Gram-negative organisms, and fluoroquinolone resistance can also be conferred by outer
membrane changes that result in decreased permeability. Selection of resistance by fluroquinolones can result in cross-resistance to β-lactams in *Escherichia coli* and *Pseudomonas aeruginosa* (Neu, 1988). This suggests that resistance results from alterations in the outer membrane, leading to decreased permeability. Quibell *et al.* (1993) has demonstrated a study on the development of fluoroquinolone resistance in *Pseudomonas aeruginosa* and suggested that outer membrane protein changes are responsible for the development of resistance genes contributing to fluoroquinolone resistance and this is also likely to be true for *Acinetobacter* spp.

Although it is known that various antibiotic resistance genes carried on plasmids of different incompatibility groups can be transferred into *Acinetobacter* spp. from *Escherichia coli* (Chopade *et al.*, 1985), however, there have been very few studies of antibiotic resistance mechanisms in clinical isolates of *Acinetobacter* spp. High-level trimethoprim resistance (MIC > 1,000 mg/liter) has been reported (Muller-Serieys *et al.*, 1989; Chirnside *et al.*, 1985; Goldstein *et al.*, 1983), and the genes encoding such resistance was often associated with multiple other resistance genes in transposon structures on large conjugative plasmids. Similarly, the chloramphenicol acetyltransferase I (CAT1) gene has been associated with both chromosomal and plasmid DNA in a clinical *Acinetobacter* isolate. It was suggested that the CAT1 gene might be transposon encoded and had improved its survival potential by locating in both replicons (Elisha and Steyn, 1991).
1.8  **Enzymatic mediated mechanisms of resistance in *Acinetobacter* spp.**

Beta-lactamases are the most common and most important mechanism of resistance to β-lactam antibiotics where they are capable of hydrolyzing the four members of β-lactam antibiotics including penicillins, cephalosporins, monobactam and carbapenems. These β-lactamases may be plasmid- or chromosomally-mediated (Livermore, 1996). Up to 2001 some 340 discrete β-lactamases have been identified (Bush, 2001) and they are divided into four groups in the scheme developed by Bush *et al.* (1995). An earlier scheme proposed by Ambler *et al.* (1991) is also frequently used to classify β-lactamases. These two schemes are shown in Table 1.4.

**Table 1.4: Classification of beta-lactamases.**

<table>
<thead>
<tr>
<th>Ambler Class</th>
<th>Bush Group</th>
<th>Description</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1</td>
<td>Often chromosomal enzymes in Gram-negatives but some are plasmid-mediated. Not inhibited by clavulanic acid.</td>
<td>AmpC, CEP-1, CMY</td>
</tr>
<tr>
<td>A</td>
<td>2a</td>
<td>Staphylococcal and enterococcal penicillinases.</td>
<td>MJ-2, NPS-1</td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>Broad spectrum β-lactamases including TEM-1 and SHV-1, mainly occurring in Gram-negatives.</td>
<td>TEM-1, SHV-1</td>
</tr>
<tr>
<td></td>
<td>2be</td>
<td>Extended-spectrum β-lactamases.</td>
<td>SHV, TEM</td>
</tr>
<tr>
<td></td>
<td>2br</td>
<td>Inhibitor-resistant TEM (IRT) β-lactamases.</td>
<td>TEM-41</td>
</tr>
<tr>
<td></td>
<td>2c</td>
<td>Carbenicillin-hydrolyzing enzymes.</td>
<td>PSE-1, CARB-3</td>
</tr>
<tr>
<td></td>
<td>2d</td>
<td>Cloxacillin (oxacillin) hydrolyzing enzymes.</td>
<td>OXA-1, PSE-2</td>
</tr>
<tr>
<td></td>
<td>2e</td>
<td>Cephalosporinases inhibited by clavulanic acid.</td>
<td>FEC-1, L2</td>
</tr>
<tr>
<td></td>
<td>2f</td>
<td>Carbapenem-hydrolyzing enzyme inhibited by clavulanic acid.</td>
<td>OXA-18, CARB</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>Metallo-enzymes that hydrolyze carbapenems and other β-lactams except monobactams. Not inhibited by clavulanic acid.</td>
<td>IMP-1, VIM-1</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>Miscellaneous enzymes that do not fit into other groups including oxacillinas</td>
<td>OXA-2, PSE-2</td>
</tr>
</tbody>
</table>

(Adapted from Bush 1989 with a modification.)
The level of resistance conferred by a β-lactamase depends on its quantity as well as its catalytic properties (Livermore, 1996). Plasmid-mediated β-lactamases are constitutive in Gram-negative bacteria, but their amount varies with gene copy number (Livermore et al., 1986). For inducible β-lactamase, the enzyme quantity is even more critical. A β-lactam that is labile may remain active in inducible strains, but a loss of activity can occur as a result of mutations (Sanders and Sanders, 1992; Livermore and Yang, 1987). Hence, it is important to know the types of enzymes produced by various pathogens as this has an impact on the selection of antimicrobial agents.

*Acinetobacter* is a genus that appears to have a propensity to develop antibiotic resistance extremely rapid, perhaps as a consequence of its long-term evolutionary exposure to antibiotic-producing organisms in a soil environment. This is in contrast to more “traditional” clinical bacteria such as *Enterobacter* spp. and *Salmonella* spp., which seem to require more time to acquire highly effective resistance mechanisms in response to the introduction of modern radical therapeutic strategies. It is thus possible that *Acinetobacter* spp. are able to respond rapidly when challenged with antibiotics and when coupled with widespread use of these antibiotics in the hospital environment, they have become successful nosocomial pathogens.

There are some preliminary studies have shown evidence that some functionally related genes can be located at several different positions on the chromosome of *Acinetobacters* (Gralton et al., 1997; Towner, 1978). β-lactamase production is one of the main mechanisms of resistance to β-lactams in *Acinetobacter* spp. These β-lactamases can be chromosomally-mediated enzymes like AmpC β-lactamases (AmpC) which belongs to the Class C β-lactamases (Bou and Martinez-Beltran, 2000; Perilli et al., 1996). However, this constitutively expressed enzyme does not share strong similarity with AmpC
cephalosporinases of *Enterobacteriaceae* family as this particular enzyme belongs to the Class A β-lactamases (Barlow and Hall, 2002). Furthermore, phylogenetic analysis suggests that this cephalosporinase should be placed in a unique subgroup among the Class C β-lactamases (Hujer *et al*., 2005). To date, there has been no evidence to indicate that the chromosomal cephalosporinase is inducible (Hujer *et al*., 2005).

In addition to the Class C cephalosporinase discussed, other β-lactamases have also been reported in *A. baumannii* that are chromosomally mediated. These include the TEM-1 type (Bou *et al*., 2000; Vila *et al*., 1993), SHV type (Huang *et al*., 2004; Bergogne-Bérézin and Towner, 1996), CTX-M type (Nagano *et al*., 2004), PER-1 (Yong *et al*., 2003; Poirel *et al*., 1999; Vahaboglu *et al*., 1997), and VEB-1 (Carbonne *et al*., 2005; Poirel *et al*., 2003) β-lactamases. Although they are important, it is difficult to assess their impact on resistance in the presence of the AmpC cephalosporinase.

On the other hand, there are also several studies have reported that more than 80% of *Acinetobacter* isolates carry multiple indigenous plasmids of variable molecular size (Seifert *et al*., 1994; Gerner-Smidt *et al*., 1989). However, problems in isolating plasmid DNA from *Acinetobacter* spp. have been reported often because of difficulties in lysing the cell wall of these organisms. Most indigenous plasmids from *Acinetobacters* seem to be relatively small (<23 kb), and therefore probably lack conjugative functions. As with many other groups of organisms, interest has focused particularly on plasmids associated with resistance to antibiotics.

Although many clinical isolates of *Acinetobacter* show widespread and increasing resistance to a whole range of antibiotics, few studies have demonstrated plasmid-mediated transfer of resistance genes such enzymes like TEM-1 or CARB-5 penicillinases (Vila, 1998; Paul *et al*., 1989) which belong to Class A β-lactamases and ARI-1 gene which
encodes Class D $\beta$-lactamases (Paton et al., 1993). This may partly reflect a lack of conjugative functions on indigenous plasmids, but also may reflect the absence of a suitable test system for detecting such transfer. For historical reasons, attempts to transfer plasmids from clinical isolates of any Gram-negative species have tended to use *Escherichia coli* K12 as a recipient strain. Complex and varied transfer frequencies of standard plasmids belonging to different incompatibility groups have been observed between *E. coli* K12 and *Acinetobacter* strain EBF 65/65, and a number of these plasmids required an additional mobilizing plasmid for re-transfer to occur (Chopade et al., 1985). Accordingly, it is not surprising that most reported cases of indigenous transmissible antibiotic resistance from *Acinetobacter* have been associated with plasmids belonging to broad host-range incompatibility groups (Towner, 1991a).

Apart from antibiotic resistance, genes encoding resistance to heavy metals (Kholodii et al., 1993) and important metabolic steps in the degradation of organic compounds and environmental pollutants, such as polychlorinated biphenyls (PCBs), have been shown to be carried on plasmids in *Acinetobacter* (Fujii et al., 1997; Towner, 1991a). Studies to date indicate clearly that though there is a pool of plasmid-mediated genetic information that is confined largely to *Acinetobacter*, a group of plasmids can cross the boundaries between *Acinetobacter* and other distinct genetic pools. A range of cloning and shuttle vectors for *in-vitro* genetic manipulation experiments in *Acinetobacter* have been described (Minas et al., 1993; Gutnick et al., 1991; Hunger et al., 1990; Singer et al., 1986; Ditta et al., 1985). In such cases, transposons probably play an important role in ensuring that particular novel genes can become established in a new gene pool, even if the plasmid vectors that transferred them are unstable. There have been several reports of chromosomally located transposons carrying multiple antibiotic resistance genes in clinical
isolates of *Acinetobacter* (Towner, 1991a). In general, such transposons closely resemble those found in other Gram-negative bacteria. Transposons also have been used in conjunction with suicide plasmid vectors to introduce mutations to the *Acinetobacter* chromosome (Leahy *et al*., 1993; Towner, 1991a).

Apart from transposons, there is another type of mobile DNA elements that can transfer antibiotic resistance genes in bacteria, also known as integrons. However, integrons are different from transposons in two important characteristics, whereby; i) transposons have repeat sequences at their ends, but the regions surrounding the antibiotic resistance genes in the integrons were not repeats, and ii) the integrons contained a site-specific integrase gene of the same family as those found in the bacteria but lacked many gene products associated with the transposons. Integrons are conserved genetic elements which encode a site-specific recombination system that enables the insertion, deletion and rearrangement of discrete genetic cassettes within the integron structure (Stokes and Hall, 1989). Most, but not all, cassettes identified to date have been associated with antibiotic resistance, and large numbers of clinical isolates of *Acinetobacter* have been shown to carry integrons incorporated into their chromosome (Gallego and Towner, 2001; Seward and Towner, 1999; Gonzalez *et al*., 1998). It is clear that clinical isolates of *Acinetobacter* seem to share resistance mechanisms with many other genera, and it has been suggested that integron structures make an important contribution to the dissemination of antibiotic resistance genes in the clinical setting.

To date, there are more than 9 classes of integrons, with the Class I integrons being the most documented and well characterized (Gu *et al*., 2007). Class 1 integrons consist of three different segments. The 5’ conserved segment (5’CS) which contain an *intI* gene encoding an integrase and an *attI* recombination site, the 3’ conserved segment (3’CS) which contains a combination of the three genes: *qacE* (antiseptic resistance gene); the *sulI*...
(sulfonamide resistance gene); and orf5 (an open reading frame of unknown function), and a variable region of resistance gene cassettes situated between the 5’ and 3’ conserved segments (Rowe-Magnus and Mazel, 1999; Hall and Collis, 1998; Paulsen et al., 1993). The movements of cassettes are catalyzed by the integrase, which can excise or integrate cassettes by site-specific recombination between two specific sequences, either attI and attC or two attC sites. Cassette mobility results in the dissemination of resistance genes, and more than 50 cassettes have been described for gram-negative bacteria (Hall and Collis, 1998). This genetic flexibility allows numerous cassette rearrangements under antibiotic selective pressure, and study of these various assortments can lead to a better understanding of integron evolution.

So far, in *Acinetobacters*, many OXA-type β-lactamases which include OXA 23 to 27 and also some of the variants were found as part of integrons (Navia et al., 2002; Poirel et al., 2002; Poirel et al., 2001; Vila et al., 1997). Besides that, there were also reports of antibiotic resistance in *Acinetobacter* spp, particularly to aminoglycosides which has been associated increasingly with the presence of integrons (Seward and Towner, 1999; Young et al., 1995). In addition, IMP and VIM type of metallo-β-lactamases which encode the Class B β-lactamases were also found to be located in the integrons (D’Agata, 2004; Nordmann and Poirel, 2002). However, the cassette content in this organism has not been fully characterized yet (Seward and Towner, 1999; Gonzalez et al., 1998).
1.9 Non-enzymatic mediated mechanisms of resistance in *Acinetobacter* spp.

Generally, there are two types of non-enzymatic mechanisms of resistance which are involved in *Acinetobacters* such as the efflux pumps and also outer membrane proteins. The efflux systems are widely found in microorganisms and confer resistance to various compounds, including antibiotics, by extrusion of the drug. The ATP-dependent multidrug transporters use ATP as a source of energy, whereas the secondary multidrug transporters are sensitive to agents that dissipate the proton motive force, suggesting that they mediate the efflux of the toxic compounds from the cell in a coupled exchange with protons (Bambeke et al., 2000). These secondary multidrug transporters can be subdivided into distinct families: the major facilitator (MF) superfamily, the small multidrug resistance (SMR) superfamily, the multidrug and toxic compound extrusion (MATE) superfamily, and the resistance-nodulation-cell division (RND) family (Putmann et al., 2000). Most of the multidrug transporters belonging to the RND family interact with a membrane fusion protein (MFP) and an outer membrane protein (OMP) to allow drug transport across both the inner and the outer membranes of Gram-negative bacteria which can be organized as multicomponent systems (Tseng et al., 1999). These multicomponent efflux pumps are specific to Gram-negative bacteria, since their particular organization allows extrusion of antibiotics directly into the extracellular medium as shown in Figure 1.4.

In *Acinetobacters*, the chromosomally encoded pump is a tripartite efflux machinery that belongs to the RND-type superfamily (Saier, 1994). The AdeABC efflux pump (RND-type superfamily) consists of *adeA* (membrane fusion), *adeB* (multidrug transporter), and *adeC* (outer membrane) genes. These three genes are contiguous and adjacent by two-component regulatory systems; *adeR*, and *adeS*, which are transcribed in the opposite direction as shown in Figure 1.5 (Marchand et al., 2004). The two-component systems are signal transduction pathways in bacteria that respond to environmental
conditions (Koretke et al., 2000). They consist of a sensor kinase and its cognate response regulator. Signal transduction by the histidine protein kinase domain of the sensor and the response regulator domain of the transcriptional activator involve the reversible phosphorylation of each domain and the transfer of phosphoryl groups between these domains. The sensor monitors certain environmental conditions and, accordingly, modulates the active state of the response regulator, which controls gene expression. Two-component systems mediate adaptive responses to a broad range of environmental stimuli (Koretke et al., 2000). Besides the AdeABC efflux pumps, the AdeDE efflux pump has been reported to confer resistance to amikacin, ceftazidime, chloramphenicol, ciprofloxacin, erythromycin, meropenem, rifampin, and tetracycline in *Acinetobacter* genomic DNA group 3 (Chu et al., 2006; Chau et al., 2004). Recently, it has been reported that AdeIJK efflux pump has contributed resistance to β-lactams, chloramphenicol, tetracycline, erythromycin, lincosamides, fluoroquinolones, fusidic acid, novobiocin, rifampin, trimethoprim, acridine, safranin, pyronine, and sodium dodecyl sulfate (Damier-Piolle et al., 2008). In addition, the author has also reported that the AdeABC and AdeIJK efflux systems have contributed in a more than additive fashion to tigecycline resistance.
Figure 1.4: Topology of multicomponent (left) and monocomponent (right) efflux pumps as they can be found in the MFS, RND, and ABC superfamilies and in the MFS, RND, ABC, and MATE superfamilies, respectively. Arrows show directions of antibiotic transport.

(Adapted from Bambeke et al., 2003).

Figure 1.5: Schematic representation of ade gene cluster.

These adeA, adeB, and adeC genes are contiguous (arrow to the right) and adjacent by two-component regulatory systems; adeR, and adeS, which are transcribed in the opposite direction (arrow to the left).
The other non-enzymatic mechanisms of resistance are the outer membrane proteins. Generally, the outer membrane of a Gram-negative bacterium is a protective structure that shields the bacterium from external hazards such as detergents and foreign enzymes. However, it also needs to be permeable to essential nutrients. Small nutrients (<about 600 Daltons) can diffuse through numerous pores in the membrane structure. These channels are formed by the porins, which are some of the most abundant proteins in Gram-negative bacteria. A porin is a trimer of three identical polypeptide chains that forms a single beta barrel with a channel down the centre through which nutrients diffuse. A long extracellular loop containing one alpha helix restricts the size of the channel; its relatively small size limits the size of the nutrients that can enter the cell in this way. This is a passive diffusion process, so there is no energy utilized.

The outer membrane of Gram-negative bacteria is an unusual, asymmetric bilayer that creates selective permeability that permits nutrients and vitamins to enter the cell, but as mentioned before, it also excludes many toxic molecules, like detergents and antibiotics. These selective permeability properties are critical to the survival of bacteria, including all of the pathogenic Gram-negative bacteria that cause diseases in animals and humans. Probably, there are certain substrate-specific transport systems that may present in *Acinetobacter* spp. outer membrane to permit the uptake of essential nutrients. Figure 1.6 shows the uptake of drug molecules through the porins of Gram-negative bacteria in general. The low outer membrane permeability might confer high-level and broad-spectrum antibiotics resistance on microorganisms.

Sato and Nakae (1991) have determined that the 45.5 and 47 kDa minor outer membrane proteins have a channel-forming function while Nitzan *et al.* (2002) and Jyothiri *et al.* (1999) established that this was the function of the 37 and 43 kDa major outer membrane proteins. However, these proteins were the only porins identified and were
demonstrated as belonging to the Omp-A like family of proteins (Gribun et al., 2003). The loss of 22, 33, 25, and 29 kDa protein bands seen on outer membrane protein extracts of resistant clinical isolates by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) suggested a possible role for these outer membrane proteins in carbapenem resistance (Limansky et al., 2002; Bou et al., 2000; Clark, 1996), although no structural and functional studies were carried out for these proteins.

In the year 2005, Siroy et al., has investigated the function of the 25/29 kDa protein band of A. baumannii, called CarO, which allowed the influx of carbapenem antibiotics by a predicted transmembrane β-barrel. In their study, the authors provided evidence that only CarO was able to form channels in lipid bilayers with no binding site for imipenem, thus, suggesting non-specific monomeric channel function rather than a specific function. This similar finding was also suggested previously by Mussi et al. (2005).

In regard to the findings on outer membrane proteins as part of mechanisms of antimicrobial resistance in Acinetobacter spp., it has been reported that nutrient uptake especially iron by the microorganisms is essential for survival and multiplication in human hosts (Caleb et al., 2003; Teresa et al., 2001). However, as defense mechanisms, availability of iron for the microorganisms is limited in the human host as most of it is complexed to transferrin, ferritin, heme, hemoglobin, and hemosiderin (Teresa et al., 2001). To establish an infection, pathogenic bacteria must compete with and obtain iron from the host’s iron binding proteins (Teresa et al., 2001). There are a number of diverse mechanisms for bacteria to obtain and utilize iron from the host (Teresa et al., 2001). One of these methods is by secretion of low molecular weight and high affinity iron chelators called siderophores and their specific cell surface receptor, termed Iron Regulated Outer Membrane Proteins (IROMPs). These receptors are expressed under iron restricted
conditions (Caleb et al., 2003). Goel and Kapil (2001) have reported that monoclonal antibodies raised against the IROMPs of *Acinetobacter baumannii* are bactericidal and kill the bacteria when grown in iron deficit medium. They suggested that these antibodies may have a role as protective antibodies in the course of infection inside a human host.

**Figure 1.6: Uptake of drug molecules through Gram-negative bacteria (right).**

Since multiple mechanisms are responsible for carbapenem resistance in *Acinetobacter* spp., this study was designed to elucidate the role of these mechanisms which include involvement of antibiotic resistance genes, mobile genetic elements, outer membrane proteins, and efflux pumps in the development of multidrug resistance.
1.10 Objectives:

1) To determine the antibiotic susceptibility patterns of Acinetobacter spp. isolated at the University Malaya Medical Centre (UMMC).

2) To develop and assess the Fluorescent in-situ Hybridization (FISH) technique to rapidly identify Acinetobacter spp.

3) To confirm the identification of Acinetobacter spp. at species level using Amplified Ribosomal DNA Restriction Analysis (ARDRA).

4) To type and characterize carbapenem sensitive and resistant strains of Acinetobacter spp. using PFGE.

5) To study the mechanisms to β-lactams and carbapenem resistance in Acinetobacter spp.:
   a) Enzymatic mechanisms of resistance:
      i) Chromosomally-mediated resistance.
      ii) Plasmid-mediated resistance.
      iii) Transposon and integron-mediated resistance.
   b) Non-enzymatic mechanisms of resistance:
      i) Efflux mechanisms.
      ii) Outer Membrane Permeability.
Chapter 2

Materials and Methods
2.1 Bacterial strains.

Forty-nine strains that were identified as *Acinetobacter* spp. were obtained from the Diagnostic Bacteriology Laboratory, University Malaya Medical Centre (UMMC) between August 2003 to March 2004. The strains were obtained from invasive and non-invasive sites which include blood, tracheal secretion, sputum, throat swab, peritoneal fluid, wound, bronchial lavage, and urine. Besides that, these strains were also selected based on their resistance to carbapenems, imipenem and meropenem. Screening for resistance to carbapenems was done by disc diffusion susceptibility test using CLSI (2005) guidelines. The identity of the isolates was confirmed using standard laboratory methods which include Gram-stain, colony morphology, non-lactose fermenter, and reaction to the oxidase test. The identity of the isolates was reconfirmed using API20NE kit (bioMerieux, France).

A single colony from a pure culture of *Acinetobacter* spp. grown on Mueller-Hinton (MH) agar was resuspended in 1 ml Brain Heart Infusion (BHI) broth (Sigma, US) containing 20% glycerol in sterile glass, screw-capped vials and stored at -70°C until further use. Strains that were used routinely, were streaked onto nutrient agar (NA) slopes incorporated with 4 µg/ml of imipenem in 5 ml capacity Bijou bottles and incubated at 37°C overnight before being stored at 4°C. Prior to use, the culture from NA slopes were streaked on MH agar and carefully examined for purity and colonial characteristics.
2.1.1 Conventional methods of identification and characterization of the bacterial isolates.

2.1.1.1 Gram stain.

3-4 colonies of a pure culture were smeared thinly onto a glass slide and air-dried prior to being fixed over a gentle flame. Crystal violet stain was added over the fixed culture and left to stand for 10 to 60 seconds. The stain was poured off and the excess stain was gently rinsed off with water. Then, iodine solution was added onto the smear, enough to cover the fixed culture. The slide was let to stand for 10 to 60 seconds before iodine solution was poured off and rinsed with running tap water. A few drops of acetone were added to the slide and rinsed off with water after 5 seconds. The slide was then counterstained with diluted Carbol Fuchsin solution for 40 to 60 seconds before rinsing off with water. Finally, the slide was air-dried prior to being examined under a microscope.

2.1.1.2 Motility test.

A small drop of liquid culture of *Acinetobacter* spp. grown in nutrient broth was placed onto a slide and covered with a clean cover slip. The motility test was examined using a microscope.

2.1.1.3 Methyl Red – Voges-Proskauer test (MRVP).

*Acinetobacter* spp. was inoculated into 3ml of peptone water and incubated overnight at 37°C. Two drops of methyl red solution were added and the culture was examined. A red colour indicated a positive reaction while a yellow colour indicated negative reaction. After completion of MR test, 0.6 ml of α - naphthol solution and 0.2 ml of 40% KOH aqueous solution was added and the colour changes was examined after 15 minutes. A positive reaction was indicated by a definitive red colour.
2.1.1.4 **Indole test.**

About 1 ml of xylol was added to a 48 hour culture of *Acinetobacter* spp. in peptone water and 0.5 ml of Ehrlich’s reagent was run down the side of the tube. The appearance of a pink or red colour in the solvent indicated the presence of indole.

2.1.1.5 **Triple Sugar Iron test (TSI).**

A wire loop of *Acinetobacter* spp. culture was inoculated by stabbing into the Triple sugar iron gel and streaking over the surface of a slope of the agar which was incubated overnight to examine, if any, the colour changes to green.

2.1.1.6 **Simmon’s Citrate Agar test.**

A wire loop of *Acinetobacter* spp. culture was inoculated by stabbing into the Simmon’s citrate gel and streaked over the surface of a slope of the agar and was incubated overnight to examine the colour changes. A blue colour and streak of growth indicates that citrate was utilized (positive test) and original green colour indicates that citrate was not utilized (negative test).

2.1.1.7 **Oxidase test.**

2-3 drops of the oxidase reagent was placed on a piece of filter paper in a Petri dish and a wire loop of *Acinetobacter* spp., was smeared across the impregnated paper. A positive reaction is indicated by the appearance of a dark purple colour on the paper within 10 seconds.

2.1.1.8 **Bacterial identification using API20NE kit.**

API20NE is a standardized system for the identification of non-fastidious, non-enteric Gram-negative rods, combining 8 conventional tests which include potassium nitrate (NO₃), L-tryptophane (TRP), D-glucose (GLU), L-arginine (ADH), urea (URE), esculin ferric citrate (ESC), gelatin (GEL), and 4-nitrophenyl-βD-galactopyranoside.
(PNPG), and also the 12 assimilation tests which include D-glucose (GLU), L-arabinose (ARA), D-mannose (MNE), D-mannitol (MAN), N-acetyl-glucosamine (NAG), D-maltose (MAL), potassium gluconate (GNT), capric acid (CAP), adipic acid (ADI), malic acid (MLT), trisodium citrate (CIT), and phenylacetic acid (PAC). Following the tests, results were analyzed using the database incorporated in the APILAB software (bioMerieux, France). These tests were performed according to the manufacturer’s instructions.
2.1.2 Antimicrobial Susceptibility Test.

2.1.2.1 Disc Diffusion Test.

The disc diffusion test as described by Kirby-Bauer has been widely used in clinical laboratories worldwide since 1966, when this method was first described (Hindler and Jorgensen, 1995). The disc diffusion test provides a simple, rapid and inexpensive method of determining the antibiotic susceptibility profiles of clinical isolates. This test involves placing an antibiotic-impregnated disc on an agar surface which has been lawned with a pure bacterial culture. This test depends on the formation of a gradient of antimicrobial concentrations as the antimicrobial agent diffuses radially into the agar. The drug concentration decreases with increasing distances from the disc. At the point where the concentration of drug at a defined distance from the disc is unable to inhibit the growth of the test organism, a zone of inhibition is formed. This test allows several antimicrobial agents to be tested on a single plate.

Disc diffusion testing was carried out on 49 strains using Clinical Laboratory Standard Institute (CLSI) guidelines, 2005. Mueller-Hinton (MH) agar was used as the test medium as recommended by CLSI. A single pure colony from an overnight culture was inoculated into 3 ml of tryptone water (Sigma, USA) and vortexed to mix well. The inoculum size was standardized by comparing the turbidity of the bacterial suspension to that of a 0.5 McFarland Standard to give a bacterial concentration of approximately $10^8$ CFU/ml. A sterile cotton-tipped swab was dipped into the standardized suspension and was used to lawn the bacterial suspension onto the entire surface of a 90 mm MH agar plate. The plate was rotated 60° and lawned in the same manner. The plate was then rotated another 60° and swabbed again. The inoculum was allowed to dry before the
appropriate antibiotic discs were placed on the agar. Not more than six antibiotics discs were placed on the plate to avoid overlapping of zones of inhibition.

The plate was then incubated at 37°C overnight in ambient air. The diameter of the zone of growth inhibition of the bacteria produced by the activity of the antimicrobials was measured and the susceptibility of the isolate to a particular antibiotic was interpreted as susceptible, intermediate or resistant using the CLSI guidelines (Table 2.1). *A. baumannii* ATCC 15308 was used as quality control strain as recommended by the CLSI guidelines in each batch of susceptibility testing.

The antibiotic discs were stored under defined conditions in order to ensure that the drugs maintained their potency. For long-term storage, discs were stored at -20°C or below in a frost-free freezer. Antibiotic discs in-use were stored in a refrigerator at 2 to 8°C for a maximum of one week in a tightly-sealed container with a desiccant.

The antibiotics studied were imipenem, ceftazidime, piperacillin, aztreonam, amoxicillin, amikacin, and gentamicin which were obtained from Oxoid Company, UK, and cefoperazone, ciprofloxacin, and meropenem which were obtained from BBL, Becton Dickinson, USA.

**Table 2.1: Zone diameter interpretive standard for Acinetobacter spp. (CLSI, 2005).**

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Disc Content (µg)</th>
<th>Zone Diameter, (nearest whole mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Resistant</td>
</tr>
<tr>
<td>Imipenem</td>
<td>10 µg</td>
<td>≤ 13</td>
</tr>
<tr>
<td>Meropenem</td>
<td>10 µg</td>
<td>≤ 13</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>100 µg</td>
<td>≤ 17</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>30 µg</td>
<td>≤ 15</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>30 µg</td>
<td>≤ 11</td>
</tr>
<tr>
<td>Amikacin</td>
<td>30 µg</td>
<td>≤ 14</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 µg</td>
<td>≤ 12</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>75 µg</td>
<td>≤ 15</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>30 µg</td>
<td>≤ 14</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>5 µg</td>
<td>≤ 15</td>
</tr>
</tbody>
</table>
2.1.2.2 Minimum Inhibition Concentration (MIC).

MIC determination is a method used in antimicrobial susceptibility testing to determine the minimum concentration of an antibiotic that is required to inhibit the growth of an organism after a suitable period of incubation. Normally, serial 2-fold dilutions of the antibiotics are tested under defined conditions against a standard inoculum of the test organism. MIC values can be determined either by an agar dilution method, broth dilution method or by a gradient diffusion method (E-test). In this study, the agar dilution method was used to determine the MIC of the 49 strains of Acinetobacter spp. This test was carried out according to the Clinical Laboratory Standard Institute (CLSI, 2005) guidelines. The control strain used was A. baumannii ATCC 15308.

In order to prepare stock solutions and working concentrations of the antimicrobial agents used, the potency of the antibiotic powders and the solvents used to dissolve the antibiotic has to be known (Table 2.2). It is important to know the potency because the actual concentration of the antibiotic in the powder may differ between drug production lots and most antibiotics contain salts that are inactive. The potency of the antibiotic refers to the actual weight of pure antibiotic per mg of the total antibiotic powder. The weight of the antibiotic that is required to prepare a working concentration can be calculated using the following formula:

\[ W = \frac{V \times C}{P} \]

\[ W = \text{Weight of antibiotic powder in mg} \]
\[ V = \text{Volume of diluent needed in ml} \]
\[ C = \text{Concentration of antimicrobial required in } \mu\text{g/ml} \]
\[ P = \text{Assay potency of the agents provided by manufacturer in } \mu\text{g/mg} \]
Table 2.2: Antibiotics evaluated for *in-vitro* activity against *Acinetobacter* spp. strains.

<table>
<thead>
<tr>
<th>Antibiotic powders for <em>in-vitro</em> use</th>
<th>Source</th>
<th>Potency (µg/mg)</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td>Merck Sharp &amp; Dohme (I.A.) Corp.</td>
<td>500</td>
<td>Phosphate buffer 0.01M, pH 7.2</td>
</tr>
<tr>
<td>Meropenem</td>
<td>Zeneca Limited</td>
<td>500</td>
<td>Water</td>
</tr>
<tr>
<td>Amikacin</td>
<td>Sigma Chemical Co.</td>
<td>1000</td>
<td>Water</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>Sigma Chemical Co.</td>
<td>900</td>
<td>Water</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>Sigma Chemical Co.</td>
<td>1000</td>
<td>Saturated NaHCO₃ + water</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Sigma Chemical Co.</td>
<td>600</td>
<td>Water</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>Sigma Chemical Co.</td>
<td>1000</td>
<td>Water</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>E.R. Squibb &amp; Sons, Inc.</td>
<td>940</td>
<td>Saturated NaHCO₃</td>
</tr>
</tbody>
</table>

Each antimicrobial agent was weighed out according to its potency and dissolved in the appropriate solvent. A stock solution of 12.8 mg/ml was prepared in a total volume of 3 ml whereby the antibiotic powder or solution was dissolved in a small volume of the appropriate solvent and was topped up to 3 ml with sterile distilled water. The solution was then filter sterilized by using a disposable membrane (Cameo 25E, Nitrocellulose) with 0.2 µm pore size. Doubling dilution of the stock solution was made and is illustrated in Table 2.3. The remaining stock solution was dispensed as 2 ml aliquots in sterilized glass vials and stored in −20°C to be used later. The period that the antimicrobial agents could be stored depended on the stability of the antibiotics.

Then, Mueller-Hinton (MH) agar plates were prepared by dissolving MH agar powder in distilled water and sterilized by autoclaving at 121°C with a pressure of 1.25 kg/cm². The agar was then allowed to equilibrate in a water bath to a temperature of 45–50°C. Then, 24 ml of the MH agar was poured into 90 mm sterile petri dishes
containing 1 ml of the appropriate concentration of the antibiotic to give the final concentration required. The plate was swirled gently to ensure that the antibiotic was distributed evenly in the agar. The plates were left to solidify at room temperature before being stored at 4°C in a sealed plastic bag until used which was within 5 days of preparation. Control plates without any antibiotics were also prepared.

Table 2.3: Preparation of antibiotic concentration.

Scheme for preparing dilution of antimicrobial agents to be used in agar dilution susceptibility test.

<table>
<thead>
<tr>
<th>Step</th>
<th>Initial concentration (µg/ml)</th>
<th>Source</th>
<th>Vol. of water added (ml)</th>
<th>Intermediate concentration (µg/ml)</th>
<th>Final concentration agar (24 ml) + antibiotic (1 ml) (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12800</td>
<td>Stock</td>
<td>-</td>
<td>12800</td>
<td>512</td>
</tr>
<tr>
<td>2</td>
<td>12800</td>
<td>Step 1</td>
<td>1 ml</td>
<td>6400</td>
<td>256</td>
</tr>
<tr>
<td>3</td>
<td>6400</td>
<td>Step 2</td>
<td>1 ml</td>
<td>3200</td>
<td>128</td>
</tr>
<tr>
<td>4</td>
<td>3200</td>
<td>Step 3</td>
<td>1 ml</td>
<td>1600</td>
<td>64</td>
</tr>
<tr>
<td>5</td>
<td>1600</td>
<td>Step 4</td>
<td>1 ml</td>
<td>800</td>
<td>32</td>
</tr>
<tr>
<td>6</td>
<td>800</td>
<td>Step 5</td>
<td>1 ml</td>
<td>400</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
<td>400</td>
<td>Step 6</td>
<td>1 ml</td>
<td>200</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>200</td>
<td>Step 7</td>
<td>1 ml</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>100</td>
<td>Step 8</td>
<td>1 ml</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>Step 9</td>
<td>1 ml</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>25</td>
<td>Step 10</td>
<td>1 ml</td>
<td>12.5</td>
<td>0.5</td>
</tr>
<tr>
<td>12</td>
<td>12.5</td>
<td>Step 11</td>
<td>1 ml</td>
<td>6.25</td>
<td>0.25</td>
</tr>
<tr>
<td>13</td>
<td>6.25</td>
<td>Step 12</td>
<td>1 ml</td>
<td>3.125</td>
<td>0.125</td>
</tr>
<tr>
<td>14</td>
<td>3.125</td>
<td>Step 13</td>
<td>1 ml</td>
<td>1.5625</td>
<td>0.0625</td>
</tr>
</tbody>
</table>

The inoculum of *Acinetobacter* spp. was prepared from an overnight culture in Luria-Bertani broth (LB) which was subsequently diluted in sterile distilled water (dH₂O). The inoculum was then standardized to give a concentration of 10⁸ CFU/ml by comparing the OD of the suspension to that of a 0.5 McFarland standard using a spectrophotometer at a light wavelength of 600 nm. The standardized inoculum was further diluted with dH₂O to
10 fold to obtain a concentration of $10^7$ CFU/ml. The culture was spot inoculated on the plates using an automatic multi-point inoculator (AM80 Automatic Inoculator, Denley-Tech, Switzerland) with 3 mm pins that enable the inoculation of 21 isolates per plate, at a time.

The standardized inoculum was inoculated within 15 minutes of preparation. This was to ensure that the concentration of the inoculum is within $10^7$ CFU/ml prior to inoculation. Agar plates that had been prepared earlier were allowed to equilibrate to room temperature in a laminar flow hood (NUAIRE, Biological Safety Cabinets) for approximately 30 minutes with their lids ajar to hasten drying of the agar surface prior to incubation. The inoculated plates were allowed to stand at room temperature until the spots dried. The agar plates were then inverted and incubated at 37°C overnight. The MIC for each antimicrobial agent was then recorded. In each batch, one control plate known as the ‘before plate’ was inoculated first before inoculating the antibiotic containing plates and another plate known as ‘after plate’ was inoculated after inoculating all the antibiotic containing plates. This was to ensure that there was no contamination or significant antimicrobial carry-over during the inoculation. *A. baumannii* ATCC 15308 was included in each assay to ensure that the antibiotic concentrations were correctly prepared. This also ensured that other variables such as the inoculum size, incubation, temperature, and media were appropriately controlled.
2.2 Rapid bacterial identification using Fluorescent *in-situ* Hybridization (FISH).

Oligonucleotide probes were designed based on the 16S rRNA gene of the *Acinetobacter* spp. and *Pseudomonas* spp. and then labeled with TAMRA and 6-FAM respectively, at the 5’ end as shown in Table 2.4. In addition, a universal eubacterial probe (Amann *et al.*, 1990) was used as a positive control. 10-15 µl of aliquots from the positive blood culture samples were applied onto glass slides, air-dried and fixed with 4% formaldehyde in 96% ethanol. The slides were then dried and covered with 50µl hybridization buffer [0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.1% SDS] containing labeled probes (10 ng/ml) prior to incubation at 50°C for 40 minutes. The slides were then washed and air dried prior to visualization using a specialized Carl-Zeiss microscope. The probes were tested individually against various gram negative bacteria (*Vibrio cholera, Stenotrophomonas maltophilia, Burkholderia cepacia, Chromobacterium violacesceum, Escherichia coli, Klebsiella pneumoniae*) to test their specificity. *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 15308 were included as positive controls. The sensitivity of the *Acinetobacter* spp. and *Pseudomonas* spp. probes respectively were determined using a pure bacterial culture that was spiked into uninoculated blood culture bottles (Bactec Plus/Paeds Plus culture vial aerobic/anaerobic; Becton Dickinson, Heidelberg, Germany). Briefly, the colonies from pure bacterial cultures incubated overnight on Mueller-Hinton (MH) agar were suspended in 0.85% NaCl and the turbidity adjusted to an OD$_{625}$ equivalent to 0.5 McFarland standards, which is approximately $10^8$ CFU/ml. Further 10-fold dilutions of the suspension were made up to $10^1$ CFU/ml. Following this, 5 ml of healthy volunteer blood were inoculated into aerobic blood culture media and subsequently spiked with 100 µl of diluted culture suspension
which ranged from $10^8$ to $10^3$ CFU. The final concentration of the bacteria in the blood culture bottles ranged from $10^6$ to $10^1$ CFU allowing for the volume for the culture media in the blood culture bottles. The blood culture bottles were then incubated overnight at 37°C prior to FISH technique. Simultaneously, from each dilution factor, 10$\mu$l of aliquots from each bottle was subcultured onto agar plates right before and after incubation period. In addition, one blood culture bottle was also incubated without any organisms and was subcultured before and after overnight incubation and this was used as negative control.

Table 2.4: Probe Sequences.

<table>
<thead>
<tr>
<th>Microorganism (Probe name)</th>
<th>Gene</th>
<th>Gene Accession Number</th>
<th>Nucleotide sequence</th>
<th>Probe size</th>
<th>Nucleotide position</th>
<th>Fluorescent tag (5’-end)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter spp.</td>
<td>16S rRNA</td>
<td>Z93435</td>
<td>GCTTGCTACCGGACCTAGCGGC</td>
<td>22 bp</td>
<td>62 – 83</td>
<td>TAMRA</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>16S rRNA</td>
<td>AF448034</td>
<td>ATGAAGGAGCTTGCTCCTGGATTGACG</td>
<td>26 bp</td>
<td>14 – 39</td>
<td>6-FAM</td>
</tr>
</tbody>
</table>
2.3 Identification of *Acinetobacter* spp. at species level using Amplified Ribosomal DNA Restriction Analysis (ARDRA).

A 1500 bp fragment of the 16S rRNA gene is amplified using PCR and digested with different restriction enzymes to give patterns that are specific for each genospecies. The procedure was performed as described by Jawad *et al.*, 1998 with a modification. Briefly, 1500 bp fragment of the 16S rRNA gene was amplified using the universal primers forward 5’-GGCTCAGATTGAACGCTGGCGGC-3’ and reverse 5’-TACCTTGTTACGACTTCACCCCA-3’. The PCR reaction mixture of a final volume of 50 µl contained 20 pM of each primer, 200 µM deoxynucleotide triphosphate (dNTP), 1X reaction buffer, 1.5 mM MgCl₂, 2.5 U Taq DNA Polymerase (Fermentas, Lithuania, USA) and approximately 1 µl of template DNA. The thermocycle protocol used was: an initial denaturation step at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 45s, annealing at 58°C for 45s, elongation at 72°C for 3 min, followed by a final extension step at 72°C for 7 min. The restriction enzymes *Alu*I, *Cfo*I, *Mbo*I, *Rsa*I and *Msp*I (Gibco BRL, Paisley, UK) were used in separate reactions to digest the amplimers, according to the manufacturer’s instructions. Restriction fragments were resolved in 2% agarose gel and the profiles were compared with those reported by Jawad *et al.*, 1998 and Vaneechoutte *et al.*, 1995. The genospecies identification of all the 49 *Acinetobacter* strains was confirmed using the ARDRA technique.
2.4 Molecular typing of *Acinetobacter* spp. isolates using Pulsed-field Gel Electrophoresis (PFGE).

Molecular typing of *Acinetobacter* spp. isolates was performed using Pulsed-Field Gel Electrophoresis (PFGE) according to the modified method of Grundman *et al.* (1995). Briefly, a single colony of the organism obtained from an overnight pure culture of *Acinetobacter* spp. on MH agar was inoculated into 3 ml of Luria-Bertani Broth (LB) containing 4 g/ml of imipenem, prior to overnight incubation at 37°C with aeration. One milliliter of this culture was centrifuged (Centrifuge 5810, Eppendorf, Germany) at 4°C at 8,000 rpm for 5 minutes. The resulting pellet was resuspended in 1 ml of cold Sodium Buffer, SB (see Appendix) and the cells were harvested by centrifugation at 8,000 rpm for 5 minutes. The cells were mixed with 0.5 ml warm SB buffer and 0.5 ml molten 1.6% low-melting temperature agarose (FMC, Rockland, USA). This mixture was then pipetted into a plug mould and allowed to solidify. Each plug was placed in 2 ml of lysis solution containing 500 µg/ml lysozyme and 2 µg/ml RNase. The plugs were incubated overnight at 37°C with gentle agitation. After incubation, the lysis solution was replaced with EDTA-Sarcosine-Proteinase K (ESP) containing 1 mg/ml Proteinase K and the plugs were incubated overnight at 50°C. The plugs were then washed 5 times with Tris-EDTA (TE) at 1-hour intervals before being stored in fresh TE at 4°C until further use.

Each plug was cut into a 2 mm slice which was pre-incubated with 80 µl of digestion buffer mixtures for 30 minutes at 4°C. The digestion buffer was then discarded and replaced with fresh buffer containing 20 units of *ApaI* (Promega, Madison, USA). Restriction enzyme digestion was carried out at 4°C for at least five hours prior to an overnight incubation at 37°C. After digestion, the plug slice was washed in 1 ml TE buffer for 30 minutes at 37°C before being loaded into 1.2% agarose gel (Pronadisa, Hispanlab,
Spain). Electrophoresis was carried out using the CHEF III PFGE machine (Bio-Rad Laboratories, California, USA) at 200V with pulse time 5s – 35s for 26 hours at 13°C in 0.5X TBE buffer. The PFGE Lambda Marker (New England Bio-Lab, Massachusetts, USA) was used as a molecular size marker.

After the electrophoresis was completed, the gel was stained with 10 µg/ml ethidium bromide (Sigma, USA) before being viewed under UV transillumination. Images of ethidium bromide-stained gels were captured using a gel documentation system, (Alpha InnoTech System).

PFGE DNA fingerprints were carefully analyzed to determine a common pattern among the isolates. Based on the fragment-for-fragment (pairwise) comparisons, the categories of relatedness of other patterns were assigned in reference to the common pattern. The interpretive criteria as recommended by Tenover et al. (1995) were used to ascertain the relatedness of each strain. Strains which had indistinguishable DNA patterns were considered genetically related. Strains were considered closely related if the PFGE patterns were changed by a single genetic event such as a point mutation, insertion, or deletion that resulted in two to three band differences with the common pattern. Other strains with patterns that differed by ≥4 up to 6 bands to the common patterns were considered possibly related. Strains with patterns that differed by more than seven bands were considered unrelated. The criteria of interpretation are shown in Table 2.5.
Table 2.5: Criteria for interpreting PFGE patterns by Tenover et al. (1995).

<table>
<thead>
<tr>
<th>Categories of relatedness</th>
<th>No. of band differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indistinguishable</td>
<td>0</td>
</tr>
<tr>
<td>Closely related</td>
<td>2 – 3</td>
</tr>
<tr>
<td>Possibly related</td>
<td>4 – 6</td>
</tr>
<tr>
<td>Different</td>
<td>≥ 7</td>
</tr>
</tbody>
</table>

The PFGE photograph was uploaded onto a computer to be analyzed using the GelCompar II software (Biosystematica, United Kingdom) which compares the mobilities and the number of DNA fragments by pairwise comparison of all the strains on the gel. The degree of similarity among the DNA patterns generated was scored by the Dice coefficient or the coefficient of similarity \( F \) to give the proportion value of the shared DNA fragments among the strains. The following formula was used to calculate the \( F \) value in this program.

\[
F = \frac{2n_{xy}}{n_x + n_y}
\]

\( n_x = \) the total number of DNA fragments from strain \( x \)
\( n_y = \) the total number of DNA fragments from strain \( y \)
\( n_{xy} = \) the number of DNA fragments shared between the two strains

Two identical DNA fragment length patterns were indicated by the maximum \( F \) value \( (F = 1) \). The overall similarities based on the \( F \) values of the strains were summarized in a dendogram in which the similarities were shown in percentages.
2.5 Characterization of beta-β-lactamase enzymes using isoelectric focusing (IEF).

Isoelectric focusing has been used to study the β-lactamases produced by Gram-negative bacteria. This method separates the proteins which appear as sharp bands at their isoelectric point (pI) in an electrophoretically produced pH gradient resulting from the presence of ampholites contained in the gel. Specially synthesized polyamino and polycarboxy groups act as ampholites and these compounds have a high buffering capacity at their pI. Each of them will migrate and focus at its pI in an electric field, thus, forming a constant pH zone where they focus. A high degree of resolution is obtained by this method because when a protein is introduced into a pH gradient during the IEF procedure, it will be repelled electrostatically by one of the electrodes according to its charge in the electric field and will migrate to an equilibrium position in the pH gradient at which it has no overall charge thereby halting the migration (Matthew et al., 1975). Depending on the pI value, the β-lactamases will separate and can be visualized as bands after specific staining.

Nitrocefin (Oxoid, UK) is a chromogenic substrate used to detect the β-lactamases produced by various bacteria. The intact substrate molecule is yellow and will turn pink when it is hydrolysed by the β-lactamases present in the gel, so that focused bands with β-lactamase activity appear pink on a yellow background (Matthew et al., 1975).

In this study, beta-lacatamase extracts were prepared as described by Bonfiglio et al. (1998). A single colony from a pure overnight culture on MH agar was inoculated into 50 ml LB broth and was grown overnight at 37°C with continuous shaking until the late logarithmic phase is achieved giving an optical density at 675 nm of 0.7 to 1.0. The bacterial cells were harvested by centrifugation at 4,000 rpm at 4°C for 20 minutes in 50 ml falcon tube using a refrigerated centrifuge (Centrifuge 5810,
Eppendorf, Germany). The supernatant was removed and the cell pellet was washed twice with ice-cold 50 mM sodium phosphate buffer. The cell pellet was then resuspended thoroughly in 1 ml of phosphate buffer and frozen at –20°C.

The pellet was thawed at room temperature the next day and sonicated 5 times for duration of 30s with intermediate 30s intervals for each cycle. The cell debris and unbroken cells were removed by centrifugation at 12,000 rpm for 10 minutes at 4°C. The supernatant was collected in a fresh eppendorf tube and centrifuged again at 12,000 rpm for 30 minutes at 4°C. The supernatant containing crude β-lactamases was kept in a fresh eppendorf tube and frozen at –20°C until further use.

The isoelectric focusing was carried out using the PhastSystem (Pharmacia, Sweden). This system separates proteins based on the principle that each protein has their own pI. Thus, when commercially prepared polyacrylamide gels known as PhastGels IEF (Pharmacia, Sweden) which are incorporated with carrier ampholites are used, the β-lactamase enzymes focus to a point based on the pH gradient on the gel. A gel with carrier ampholites that produces a pH gradient of 3–9 was chosen as the pI values of the β-lactamases of interest were in this range. There are three stages in the IEF procedure which include pre-focusing, sample application and focusing.

At the pre-focusing step, the pH gradient is formed and the sample applicators are loaded onto the sample applicator arm at either the anode or cathode side. For the determination of the pI of an unknown sample, it is suggested to apply the sample at either position. Each sample was loaded into the well of the sample applicator through capillary action, whereby each well could absorb up to a maximum of 4 µl of sample.

The sample was applied to the gel at 15 Vh. The voltage was kept quite low at this step (200V) to avoid streaking patterns caused by contamination or poorly soluble proteins.
At the beginning of the focusing step, the applicators were raised and the proteins migrate to their isoelectric point. Separation ended once the volthours reached 410 Vh. The optimized parameters for IEF using the PhastGel IEF 3–9 of PhastSystem (Pharmacia, Sweden) is shown below:

<table>
<thead>
<tr>
<th>Step</th>
<th>Voltage (V)</th>
<th>Current (mA)</th>
<th>Power (W)</th>
<th>Temperature (°C)</th>
<th>Time (Vh)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>2000</td>
<td>2.5</td>
<td>3.5</td>
<td>15</td>
<td>75</td>
</tr>
<tr>
<td>Step 2</td>
<td>200</td>
<td>2.5</td>
<td>3.5</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Step 3</td>
<td>2000</td>
<td>2.5</td>
<td>3.5</td>
<td>15</td>
<td>410</td>
</tr>
</tbody>
</table>

An IEF Standards Broad Range pI 4.45-9.6 marker (Bio-Rad, USA) was run together with samples. The marker is a mixture of nine natural proteins with isoelectric points ranging from 4.45 to 9.6. Five (4.45-4.75, 7.0, 7.1, 7.5, 9.6) of the twelve proteins are prestained to provide continuous monitoring of the focusing process and also to ensure that the IEF separation was complete. Another gel was run with only the marker and stained with Coomassie Blue to observe pI bands of the rest of the standard. This gel served as the standard to determine the pI values of the β-lactamases. The gel was stained with nitrocefin solution (0.05 mg/ml) by flooding the gel with the solution and leaving it at room temperature for 1 to 2 minutes to allow the bands to appear. As soon as pink or red bands appeared, the pI values were estimated and recorded. This had to be done quickly otherwise the bands would diffuse out giving less sharp bands and inaccurate pI values.
2.6 Preliminary screening test for the presence of metallo-β-lactamases.

2.6.1 Double disc-synergy test.

The method of Arakawa et al. (2000) was followed to perform double disc-synergy test for screening of metallo-β-lactamases. Briefly, an overnight culture of the test strain was suspended to the turbidity of a 0.5 McFarland standard and used to lawn a MH agar. After the culture was dry, a 10-µg imipenem disc (BBL, Cockeysville, MD) and a blank filter paper disc were placed within a centre-to-centre distance of 15mm. Subsequently, 5 µl of 0.5 M EDTA was applied to the blank disc. After overnight incubation, the presence of an enlarged zone of inhibition was interpreted as EDTA-synergy test positive. In addition, a ceftazidime disc and a 2-mercaptoethanol impregnated filter paper disc were used to increase the sensitivity of the test, as some strains were resistant to low levels of imipenem only. Positive controls of IMP-1 and VIM-2 producing Acinetobacter spp. and Pseudomonas aeruginosa were used in this experiment. These strains were kindly provided by Prof. Emeritus Yunsop Chong, and Prof. Kyungwon Lee from Department of Clinical Pathology and Research Institute of Bacterial Resistance, Yonsei University College of Medicine, Seoul, Korea.

2.6.2 Modified Hodge test.

The Modified Hodge test of Lee et al. (2001) was followed. Briefly, the surface of a MH agar plate was lawn even with an overnight culture suspension of Escherichia coli ATCC 25922, which was adjusted to one-tenth turbidity of the 0.5 McFarland standards. After allowing the lawn to dry, an imipenem disc was placed in the centre of the plate and imipenem-resistant isolates from the overnight culture plates were streaked heavily from the edge of the disc to the periphery of the plate. The presence of a distorted inhibition zone after overnight incubation was interpreted as Modified Hodge test positive.
The same positive controls, IMP-1 and VIM-2 producing *Acinetobacter* spp. and *Pseudomonas aeruginosa* that were used in double disc-synergy test above were also included in this experiment.

2.6.3 **Fractionation of metallo-β-lactamases for kinetic study.**

Isolates which carried the putative metallo-β-lactamases, were used in the kinetic studies to analyze the rate of antibiotics being hydrolyzed by this enzyme. Log-phase cells were harvested from a 10 litre broth culture, washed and resuspended in phosphate buffer. Cells were disrupted by sonication and the cell debris removed by ultracentrifugation at 10,000 rpm for 20 mins. The resulting supernatant containing the metallo-β-lactamases were purified using the Rotofor system (Biorad, Singapore) which enabled the collection of fractions based on isoelectric point (pI) values. The β-lactamase activity was determined spectrophotometrically by measuring the hydrolysis of an oxacillin, imipenem and meropenem as substrates. As described by Bush *et al.* (1989), metallo-β-lactamases are only inhibited by EDTA and not by clavulanic acid. Therefore, in this study, enzymes were inhibited with EDTA for 10 min at 37°C before the addition of substrate. Relative $V_{max}$ rates were determined from the Hanes plots generated.
2.7 Detection of antibiotic resistance genes in *Acinetobacter* spp. using polymerase chain reaction (PCR).

In order to confirm the detection of β-lactamases, particularly, metallo-β-lactamases from the preliminary findings from this study, polymerase chain reaction (PCR) was carried out. PCR is an *in-vitro* technique which allows the amplification of a specific deoxyribonucleic acid (DNA) region that lies between two regions of known DNA sequences and this technique was invented by Kary Mullis in 1985 (Saiki *et al*., 1985). In this study, PCR amplification was used for the detection of IMP- and VIM- gene (metallo-β-lactamases), OXA-gene (oxacillinases, another type of carbapenemases), and AmpC-gene (non-carbarbapenemases).

### 2.7.1 PCR amplification of *bla*<sub>IMP</sub> and *bla*<sub>VIM</sub> genes encoding metallo-β-lactamases.

Primers specific for *bla*<sub>IMP</sub> and *bla*<sub>VIM</sub> genes were used to detect the presence of the metallo-β-lactamase genes encoding carbapenem resistance in *Acinetobacter* spp. Amplification of *bla*<sub>IMP</sub> gene was initially performed according to the method of Yum *et al.* (2002) using primers specific to *bla*<sub>IMP</sub>-1 5’-GATGGTATGTTGCTCTTGT-3’ (forward) and 5’-TTAATTTGCGGACTTAGGC-3’ (reverse). This set of primers gave rise to an amplicon size of 448 bp. However, these primers could not amplify the entire coding region of *bla*<sub>IMP</sub> since the primers were only specific to *bla*<sub>IMP</sub>-1 gene. Another set of primers IMP-F, 5’-CCCTCTAGAACATAGTTCG-3’ and IMP-R, 5’-GTTGCAACGTTCTGGTTACATAT-3’) flanking the entire coding region *bla*<sub>IMP</sub> was designed. This set of primers (IMP-F and IMP-R) would give an amplicon size of 795 bp. In order to reconfirm and complete the sequencing of the coding region of *bla*<sub>IMP</sub>, internal reverse primer 5’-AACTGGAAGCTTAGGC-3’ was designed downstream from the 5’-coding region. The parameters for amplification of *bla*<sub>IMP</sub> are shown in Table 2.6.
Amplification of bla\textsubscript{VIM} gene was performed according to Poirel \textit{et al.} (2000) and the primers used were 5’-ACCTTCAGACTTTGAGTTAAG-3’ (forward) and 5’-CACTCAACGACTAAGCG-3’ (reverse). These primers were expected to give an amplicon with a size of 801 bp.

Template DNA was prepared by boiling the supernatant of a bacterial suspension in sterile distilled water for 10 minutes. 50 µl of reaction mixture containing 20 pmol (each) primer, 200 µM (each) deoxynucleotide triphosphate (dNTPs), 1X reaction buffer, 1.5 mM MgCl\textsubscript{2}, 2.5U Taq Polymerase (Fermentas, Lithuania, USA), and 1 µl of template DNA was used. In preparing the PCR reaction mixture, a PCR master mix containing all the components except the DNA template was first prepared. The master mix was then dispensed equally into each reaction tube. Appropriate volume of template DNA was added to each tube to give a final volume of 50 µl and mixed well. The mixtures were subjected to PCR amplification immediately using a Thermal Cycler (BioRad, Hercules, USA). The parameters for bla\textsubscript{IMP} and bla\textsubscript{VIM} amplification are shown in Table 2.6.

A bla\textsubscript{IMP} and a bla\textsubscript{VIM} positive control strains were provided by Prof. Yunsop Chong and Prof. Kyungwon Lee from Yonsei University College Medicine, Korea. A tube without DNA was included to serve as negative control during the amplification of bla\textsubscript{IMP} and bla\textsubscript{VIM} respectively.

The PCR products were run on a 2% agarose gel (Pronadisa, Hispanlab, Spain) for one and half hours prior to staining with ethidium bromide and viewed under UV light (Vilber Lourmat, France). The gel was then photographed using the Alpha Inno Tech System. A 100 bp DNA ladder (Promega, Wisconsin, USA) was used to estimate the size of the PCR products. Only the amplicon of the appropriate size was sent to confirm the identity of the amplified product.
Table 2.6: Parameters for amplification of bla\textsubscript{IMP} and bla\textsubscript{VIM}.

<table>
<thead>
<tr>
<th>Step</th>
<th>bla\textsubscript{IMP}</th>
<th>bla\textsubscript{VIM}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation, 94°C</td>
<td>5 minutes</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Denaturation, 94°C</td>
<td>30 seconds</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>58°C, 30 seconds</td>
<td>56°C, 30 seconds</td>
</tr>
<tr>
<td>DNA extension, 72°C</td>
<td>2 minutes</td>
<td>45 seconds</td>
</tr>
<tr>
<td>Final extension, 72°C</td>
<td>7 minutes</td>
<td>7 minutes</td>
</tr>
<tr>
<td>Cycles</td>
<td>25 cycles</td>
<td>25 cycles</td>
</tr>
</tbody>
</table>

2.7.2 PCR amplification of bla\textsubscript{OXA-23} gene (Class D β-lactamases).

The OXA gene encodes for another type of carbapenemases and therefore PCR was performed to detect this gene in Acinetobacter spp. Primers specific for bla\textsubscript{OXA-23} were used to detect the presence of the bla\textsubscript{OXA-23} gene encoding Class D β-lactamases in Acinetobacter spp. as described by Alfaz \textit{et al.} (2001). Amplification of bla\textsubscript{OXA-23} gene was performed using primer pairs 5’-GATGTGTCATAGTATTCGTCG-3’ (forward) and 5’-TCACAACAACTAAAAGCACTG-3’ (reverse). This set of primers gave rise to an amplicon of 1058 bp.

The PCR reaction mixture in a final volume of 50 µl contained 20 pmol (each) primer, 200 µM (each) deoxynucleotide triphosphate (dNTPs), 1X reaction buffer, 1.5 mM MgCl\textsubscript{2}, 2.5U Taq DNA Polymerase (Fermentas, Lithuania, USA). This master mix was then dispensed equally into each tube and vortexed. Approximately, 1 µl of template DNA was added. The tubes were then subjected for PCR amplification using Thermal Cycler (BioRad, Hercules, USA). The parameters for bla\textsubscript{OXA-23} amplification are shown in Table 2.7.

The PCR products were run on a 2% agarose gel (Pronadisa, Hispanlab, Spain) for one and half hours prior to staining using ethidium bromide and viewed under UV light (Vilber Lourmat, France). The gel was then photographed using Alpha Inno Tech System.
A 100 bp DNA ladder (Promega, Wisconsin, USA) was used to estimate the size of the PCR products. Only the amplicon of the appropriate size was sent for further confirmation by sequencing.

### Table 2.7: Parameters for amplification of $bla_{\text{OXA-23}}$

<table>
<thead>
<tr>
<th>Step</th>
<th>$bla_{\text{OXA-23}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation, 94°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Denaturation, 94°C</td>
<td>25 seconds</td>
</tr>
<tr>
<td>Annealing, 52°C</td>
<td>40 seconds</td>
</tr>
<tr>
<td>DNA extension, 72°C</td>
<td>50 seconds</td>
</tr>
<tr>
<td>Final extension, 72°C</td>
<td>6 minutes</td>
</tr>
<tr>
<td>Cycles</td>
<td>30</td>
</tr>
</tbody>
</table>

#### 2.7.3 PCR amplification of $bla_{\text{AmpC}}$ gene (Class C β-lactamases).

Besides detection of carbapenemase genes (IMP, VIM, and OXA), other β-lactamases such as cephalosporinases (AmpC) was also detected using PCR amplification. Primers specific to $bla_{\text{AmpC}}$ were used to detect the presence of the $bla_{\text{AmpC}}$ gene encoding Class C β-lactamases in *Acinetobacter* spp as described by Bou and Martinez (2000). Amplification of $bla_{\text{AmpC}}$ gene was performed using primer pairs 5’-ACTTACTTCAACTCGCAGACG-3’ (forward) and 5’-TAAACACCACATATGTGCCG-3’ (reverse) which gave rise to an amplicon of 663 bp.

The PCR reaction mixture in a final volume of 50 µl contained 20 pmol (each) primer, 200 µM (each) deoxynucleotide triphosphate (dNTPs), 1X reaction buffer, 1.5 mM MgCl$_2$, 2.5U *Taq* DNA Polymerase (Fermentas, Lithuania, USA). The master mix was then dispensed equally into each tube and vortexed. Approximately 1 µl of template DNA was added. The tubes were then subjected for PCR amplification using Thermal Cycler
(BioRad, Hercules, USA). The parameters for $bla_{\text{AMPC}}$ amplification are shown in Table 2.8.

The PCR products were run on a 2\% agarose gel (Pronadisa, Hispanlab, Spain) for one and half hours prior to staining using ethidium bromide and viewed under UV light (Vilber Lourmat, France). The gel was then photographed using Alpha Inno Tech System. A 100 bp DNA ladder (Promega, Wisconsin, USA) was used to estimate the size of the PCR products. Only the amplicon of the appropriate size was sent for further confirmation by sequencing.

**Table 2.8: Parameters for amplification of $bla_{\text{AMPC}}$.**

<table>
<thead>
<tr>
<th>Step</th>
<th>$bla_{\text{AMPC}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation, 94°C</td>
<td>4 minutes</td>
</tr>
<tr>
<td>Denaturation, 94°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Annealing, 50°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>DNA extension, 72°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Final extension, 72°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Cycles</td>
<td>30</td>
</tr>
</tbody>
</table>
2.8 Determination of metallo-β-lactamase gene location.

2.8.1 Plasmid analysis of *Acinetobacter calcoaceticus*.

Plasmids were isolated according to the modified protocol of Sambrook *et al.* (1989). Briefly, 5 ml of an overnight culture in Luria-Bertani (LB) broth was centrifuged at 14,000 rpm for 15 minutes. The supernatant was discarded and the cell pellet was resuspended in 150 µl of Solution I (see Appendix) to lyse the cells. This was followed by alkaline lysis, where 200 µl of Solution II (see Appendix) was added. The mixture was then neutralized with 200 µl of potassium acetate solution (Solution III, see Appendix). Most of the chromosomal DNA and bacterial protein will form a complex with potassium and removed by centrifugation at 14,000 rpm for 5 minutes. The supernatant was transferred to a new eppendorf tube containing 700 µl isopropanol and recentrifuged again. The pellet was washed with 500 µl of 70% ethanol and the supernatant was decanted after another centrifugation. The pellet was resuspended in 80 µl of TE and 5 µl of RNase was added and the tube was incubated at 37°C for 30 minutes. After the incubation, 100 µl of 3M NaOAc, 50 µl TE-saturated phenol, and 50 µl CHISAM (phenol:chloroform-isoamyl alcohol); (24:24:1 v/v) were added and vortexed to mix. After centrifugation at 14,000 rpm for 10 minutes, the upper layer of the supernatant was transferred to a fresh eppendorf tube containing 100 µl isopropanol and recentrifuged again. The pellet was then washed with 500 µl 70% ethanol centrifuged at 14,000 rpm for 5 minutes. The pellet was resuspended in 30 µl of Mili Q water. The DNA was run on 0.8% agarose gel prepared in TBE buffer and stained with ethidium bromide in order to view the plasmids. The mobility of four reference plasmids carried in a reference strain, *E. coli* 39R861 were used to estimate the molecular weights of the plasmids isolated.
2.8.2 Characterization of integrons in *Acinetobacter* spp.

Primers specific for *IntI* as described previously by Honang *et al.* (2003) were used to detect the presence of the Class I integrase genes encoding a mobile genetic element that carries the antibiotic resistance gene cassettes in *Acinetobacter* spp. Amplification of *Int* gene was initially performed using primer pairs specific to *IntI* 5’-AGATTTGATCGCGTCAGGA-3’ (forward) and 5’-TATTGTGTCTGGCTGCGGA -3’ (reverse) which gave rise to an amplicon of 569 bp. As, these primers cannot amplify the entire coding region of integron, a new set of primers CSF, 5’-GCCAACCATGCACCATG-3’ (forward) and CSR, 5’-ATGCACACTAGAGCTCA-3’ (reverse) flanking the entire coding region of inetegron were used. This set of primers (CSF and CSR) gave an amplicon size of 2924 bp and targets the conserved region in all Gram-negative organisms. The parameters for PCR amplification of these genes are shown in Table 2.9.

Total genomic DNA extracts of *Acinetobacter* spp. isolates were used as template in PCR experiments to detect the presence of class 1 integrase genes and the entire coding region of the integrons. Each PCR was performed in a final 50 µl volume containing 1.5mM MgCl$_2$, 600 µM deoxynucleoside triphosphate, 20 pmol of each primers, 2.5U *Taq* DNA polymerase and 5 µl of DNA extract.

The PCR products were run on a 2% agarose gel (Pronadisa, Hispanlab, Spain) for one and half hours prior to staining using ethidium bromide and viewed under UV light (Vilber Lourmat, France). The gel was then photographed using Alpha Inno Tech System. A 100 bp and 1 kb DNA ladder (Promega, Wisconsin, USA) was used respectively to estimate the size of the PCR products. Only the amplicon of the appropriate size was sent for further confirmation by sequencing.
2.8.3 Typing of Class 1 integron using restriction enzymes analysis.

PCR products using CSF and CSR primer pairs were used for digestion of 2924 bp of the *Acinetobacter* spp. isolates by enzyme *Alu*I. Restriction digest was carried out in a total volume of 50 µl containing 20 µl of PCR product, 5 µl of 10X restriction buffer, 24 µl of sterile water, and 10U of *Alu*I (1 µl). The reaction mixture was then incubated at 37°C for at least 2 hours for complete digestion.

The digestion products were run a 2% agarose gel (Pronadisa, Hispanlab, Spain) for one and half hours viewed under UV light (Vilber Lourmat, France). The gel was then photographed using Alpha Inno Tech System. A 1 kb DNA ladder (Promega, Wisconsin, USA) was used to estimate the digestion patterns and analyzed.

### Table 2.9: Parameters for amplification of integrons.

<table>
<thead>
<tr>
<th>Step</th>
<th><em>IntI</em></th>
<th>CSF and CSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation, 94°C</td>
<td>5 minutes</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Denaturation, 94°C</td>
<td>30 seconds</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>56°C, 30 seconds</td>
<td>58°C, 30 seconds</td>
</tr>
<tr>
<td>DNA extension, 72°C</td>
<td>45 seconds</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Final extension, 72°C</td>
<td>7 minutes</td>
<td>7 minutes</td>
</tr>
<tr>
<td>Cycles</td>
<td>25 cycles</td>
<td>25 cycles</td>
</tr>
</tbody>
</table>
2.8.4 Confirmation of location of metallo-β-lactamase gene in *Acinetobacter* spp. using Southern Blot Hybridization.

Following electrophoresis and staining of DNA, the agarose gel was cut to size by removing the wells at the top of the gel (to prevent buffer transfer through the wells) and any blank or unrelated lanes that do not need to be transferred. The cut agarose was put under UV treat for 5 minutes on the transilluminator. The gel was soaked in base solution for 45 minutes, followed by the neutralizing solution for 90 minutes. The gel may be stored in the neutralizing solution for many hours. Two Whatman 3 mM filters were cut to the same size as the gel, and another to act as a wick, running under the gel and extending into troughs containing the transfer solution. Then, a nitrocellulose or nylon membrane was cut using the 3 mM filters as a template to the size of the gel. Nylon membranes are preferred due to their strength upon repeated handling. The nylon membranes are handled by their edges with tweezers. The wick was pre-wet in 20X SSPE and its ends were position in a reservoir that contains about 500 ml of 20X SSPE. The gel was turned upside down on the wick so that the smooth side will face the nylon membrane. The bubbles trapped between the gel, membrane and filter paper were avoided. Then, the membrane was pre-wet in distilled water. The membrane was placed on top of the gel so that its edge is aligned with the cut wells. This membrane was covered with the two Whatman 3 mM filters that were pre-wet in 20X SSPE. Then, a few inches of paper towels and a weight (about 1 kg) were placed on top of this. The genomic DNA was allowed to transfer overnight as shown in Figure 2.1. Before removing the membrane after the transfer, the position of the wells was marked with a pencil. The membrane was soaked in 5X SSPE for 30 minutes and to remove excess moisture, a Whatman 3 mM filter paper was used. The membrane was placed in Saran wrap, and UV treated for 2 to 5 minutes with the DNA side facing the UV
light. The membrane kept in Saran wrap can be stored at 4°C until further use. The membrane was then hybridized with a labeled probe.

PCR products of \( \text{bla}_{\text{IMP}} \) (IMPF and IMPR primers) and Class 1 integron (CSF and CSR primers) were used as probes in two different experiments. Generally, in PCR tube, about 10–100 ng of template DNA, 5 µl of 10X Random Primer Mix were mixed together to a final volume of 31 µl with nuclease-free water. Then, the reaction mixture tube was heat to 97°C in Thermal Cycler (BioRad, Hercules, USA) or boiled for 3 minutes. The tube was removed and chilled quickly on ice. The tube was then centrifuged briefly at room temperature to collect droplets formed by condensation and the tube was placed on ice prior labeling. The following reagents were added to the tube containing template DNA to a final volume of 50 µl:

\[
\begin{align*}
5 \mu l & \quad 10X \text{Klenow Reaction Buffer} \\
5 \mu l & \quad 10X \text{Klenow Labeling Mix} \\
8 \mu l & \quad \text{ddH}_2\text{O} \\
1 \mu l & \quad \text{Klenow Enzyme} \\
50 \mu l & \quad \text{Total volume}
\end{align*}
\]

The reaction mixture tube was then incubated at 37°C for 30 minutes in a water bath. After incubation, the tube was then removed from the water bath and the reaction was stopped by adding 2 µl of 0.5 M EDTA (pH 8.0). The labeled probe was then purified and quantified in order to continue with Southern blot hybridization. The probe is stable and can be stored at -20°C up to six months.
Figure 2.1: Diagram of DNA blotting to a membrane.

Adapted from:
2.9 Non-Enzymatic Mechanisms.

Besides involvement of antibiotic resistance genes in *Acinetobacter baumannii/calcoaceticus*, which include metallo-β-lactamases, oxacillinases, and mobile genetic elements, other possible mechanisms of resistance such as efflux pumps and outer membrane proteins were also included in this study.

2.9.1 Efflux pumps and detection of *adeA, adeB, adeC, adeR, and adeS* genes using PCR amplification.

The main genes involved in efflux pump system of *Acinetobacter baumannii/calcoaceticus* are *adeA, adeB, adeC, adeR, and adeS*. In order to detect these genes, primers of the *adeA, adeB, adeC, adeR, and adeS* were designed and subjected to PCR amplification. The forward and reverse primer sequences of each gene are listed in Table 2.10. *SmaI* restriction sites were added to each forward and reverse primer sequences of each gene for ligation purposes. The presence of each gene was performed using *Pfu* DNA polymerase according to the manufacturers’ recommendations (Fermentas Inc., Lithuania). PCR mixes contained 1 µl of heat-extracted template DNA, 1 µl (20 pmol) of each primer and PCR Premix containing 2.5U of *Taq* DNA polymerase in a final volume of 50 µl. The thermocycle protocol used was: an initial denaturation step at 94°C for 5 mins, followed by 25 cycles of denaturation at 94°C for 30s, annealing at 56°C for 30s, elongation at 72°C for 45s, followed by a final extension step at 72°C for 7 mins.

The PCR products were run on a 2% agarose gel (Pronadisa, Hispanlab, Spain) for one and half hours prior to staining using ethidium bromide and viewed under UV light (Vilber Lourmat, France). The gel was then photographed using Alpha Inno Tech System. A 100 bp DNA ladder (Promega, Wisconsin, USA) was used to estimate the size of the
PCR products. The amplicon of the appropriate size was sent for further confirmation by sequencing.

In addition to this study, cloning work was performed to inactivate each of these genes to study their contribution in carbapenem resistance particularly in this organism. The rationale of this study was to identify the molecular mechanism that confers the particularly carbapenem resistance of *Acinetobacter baumannii* strains. To do this, the three-component efflux system (*adeA, adeB, adeC*) that included an RND multidrug transporter will be disrupted by insertional inactivation to show their involvement in antibiotic resistance. The method of Heritier *et al.* (2005) was utilized with some modification. Briefly, plasmid pAT801 which confer resistance to ampicillin was used as a shuttle vector. This vector is able to replicate in *A. baumannii* and *E. coli* and it consists of part of pWH1266 and pUC18 vectors. Inactivation of *bla*TEM-1 resistance gene of pAT801 was performed as described previously by Heritier *et al.* (2005), giving rise to plasmid pAT801-RA conferring resistance to rifampin. An internal fragment to the *adeA, adeB, adeC, adeR*, and *adeS* gene was amplified using primer pairs as listed in Table 2.10. Each PCR product was cloned into the *SmaI* digested pAT801-RA vector individually and electro-transformed into another strain harboring this gene in order to disrupt the sequences. The transformants were selected on ticarcillin (50 µg/ml) and rifampin (25 µg/ml) containing plates. The successful disruption of *adeA, adeB, adeC, adeR*, and *adeS* genes individually was screened by M13 primers and primers specific to the inserts. The derivative mutants were then subjected to antimicrobial susceptibility testing using various antibiotics including imipenem, meropenem, ceftazidime, cefotaxime, gentamicin, amikacin, ciprofloxacin and amoxicillin.
Table 2.10: Primer sequences of the *adeABC*, and *adeRS* genes.

<table>
<thead>
<tr>
<th>Resistance Genes</th>
<th>Forward sequence</th>
<th>Reverse Sequence</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>adeA</em></td>
<td>AAAATTTTCTCTAGCCGATGTCGCTCAA</td>
<td>AAAATTTTATACCTGAGGCTCGCCACTG</td>
<td>510</td>
</tr>
<tr>
<td><em>adeB</em></td>
<td>AAAATTTTACCGCGCTAACTTAGGTATGC</td>
<td>AAAATTTTTCTGAACTTGCACCTGAGGCA</td>
<td>931</td>
</tr>
<tr>
<td><em>adeC</em></td>
<td>AAAATTTTGCGGCAGGTTAGCCCATCG</td>
<td>AAAATTTTGCAGGAACAGGATGACCTGCT</td>
<td>435</td>
</tr>
<tr>
<td><em>adeR</em></td>
<td>AAAATTTTGAATCTACGATATTGGCGACAT</td>
<td>AAAATTTTACGGTTTGCTCTAGTGACATC</td>
<td>560</td>
</tr>
<tr>
<td><em>adeS</em></td>
<td>AAAATTTTGATTGGCATGCGCCTCGCAA</td>
<td>AAAATTTTGCCTGCATGTGAATAGCGTA</td>
<td>580</td>
</tr>
</tbody>
</table>

Legend: * = CCCGGG (SmaI restriction site added to the primer sequences)
2.9.2 **Outer membrane protein (OMP).**

Besides the involvement of efflux pumps in *Acinetobacter baumannii/calcoaceticus*, the role of outer membrane protein (OMP) as another type of non-enzymatic mechanism of resistance was also included in this study. Generally, Gram-negative bacteria are surrounded by the OMP as a permeability barrier. Often, most of hydrophilic solutes cross the OMP through water-filled channels of porins. Porin channels are totally non-specific and therefore, it is expected that other small hydrophilic agents such as β-lactam antibiotics may utilize the porin pathway. Besides that, porin channels can also be very narrow and act as effective barriers for the penetration of large or hydrophobic compounds. The OMP barrier thus creates various degrees of intrinsic resistance to antimicrobial agents in bacteria. Furthermore, the level of resistance can be increased by genetic or physiological alterations that lower the permeability of this membrane. Therefore the rationale of this study was to look at the function of OMP as an important barrier in *Acinetobacter baumannii/calcoaceticus*.

2.9.2.1 **OMP extraction and analysis using SDS-PAGE.**

Outer membrane protein was prepared based on modified method of Matsuyama et al. (1984). A single pure colony of overnight culture on Mueller Hinton (MH) agar was inoculated into 5 ml of Luria Broth (LB) containing 4 µg/ml imipenem and incubated with shaking (150 rpm) for approximately 5 hours (late log phase) at 37°C. Then the culture was inoculated into 500 ml of fresh LB broth. The culture was allowed to grow at 37°C with aeration (150 rpm) until late log phase at optical density of (OD$_{650}$) of 0.4. The culture was transferred to 250 ml Nalgene tubes and spun down at 4, 000 rpm for 20 minutes at 4°C in a refrigerated centrifuge. The cell pellets were pooled and washed with 10 mM phosphate buffer, pH 7.2. The cells were then resuspended in 20 ml of the same buffer before being subjected to cell disruption by sonication for 30s at 30KHz output with 30s intervals. This was repeated 6 times to ensure
complete destruction of the cells. Cell debris was removed by centrifugation at 4,000 rpm for 40 minutes at 4°C. The supernatant was transferred to an ultratube and the outer membrane fraction was pelleted by ultra centrifugation at 100,000X g for 35 minutes at 4°C. The pellet was then resuspended in 20 ml of 10 mM phosphate buffer, pH 7.2 containing 2% (v/v) Triton X-100. The suspension was incubated at 37°C for 20 minutes. The Triton X-100 insoluble fraction was recovered by ultra centrifugation at 100,000X g for 35 minutes at 4°C. The pellet was then washed once with 10 ml phosphate buffer, pH 7.2 and recovered by centrifugation at 100,000X g for 35 minutes at 4°C. The protein pellet was resuspended in 100 µl of Laemmli buffer and stored at -20°C until further use. Prior to SDS-PAGE analysis, the concentration of protein was determined using method described by Bradford (1976). Each protein sample was then separated on a Dual Vertical Mini-gel Unit (C.B.S. Scientific Co., California). The gel was prepared using the following components:

**Table 2.11: 12 % Separating gel.**

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide / Bis-acrylamide</td>
<td>9.6 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8.8</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>200 µl</td>
</tr>
<tr>
<td>ddH2O</td>
<td>5.1 ml</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulfate (freshly prepared)</td>
<td>100 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

**Table 2.12: 4 % Stacking gel.**

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide / Bis-acrylamide</td>
<td>2.6 ml</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH 6.8</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>200 µl</td>
</tr>
<tr>
<td>ddH2O</td>
<td>12.2 ml</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulfate (freshly prepared)</td>
<td>100 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 µl</td>
</tr>
</tbody>
</table>
All the reagents were mixed together in a beaker before adding in ammonium persulfate and TEMED to initialize polymerization. The separating gel solution was swirled gently and pipetted into the gel caster using 1.0 mm spacer to a level of about 4.0 mm from the top. N-butanol was gently layered on the top of the gel solution to even the surface of the gel. After polymerization, the n-butanol was removed and the gel was flushed with deionized water. The top layer was dried with filter paper before pouring in the stacking gel solution. Immediately, a 10-toothed comb was inserted into the stacking gel.

After the stacking gel had polymerized, the comb was gently removed from the gel. A total of 25 µg of protein of each sample was denatured by boiling for 10 minutes and cooled immediately prior to loading. This is to ascertain that the differences in protein profiles were not ascribed to loading variants. A protein marker (New England Biolabs) was run together with the samples. One well served as a negative control which contained only sample dilution buffer and loading dye. This was to ensure that any bands that appeared were not as a result of experimental errors or contamination in the dilution buffer or loading dye. Electrophoresis was performed at a constant voltage of 100V in the stacking gel. The voltage was increased to 120V once the dye had reached the separating gel. The electrophoresis was terminated before the front dye reached the bottom edge of the gel. The apparatus was disassembled and the gel was carefully removed and stained with Coomassie Brilliant Blue for an hour followed by destaining step with destaining solution overnight.

2.9.2.2 Potential target for antimicrobial therapy.

To this end polyclonal antibodies were raised against a specific outer membrane protein called Iron-regulated Outer Membrane Protein (IROMP). Following a report by Goel and Kapil (2001) who showed that the monoclonal antibodies raised against IROMPs
specifically kill the bacteria grown in iron deficit medium, I decided to carry out a study to investigate IROMPs as possible target for antimicrobial therapy.

### 2.9.2.2.1 Extraction of IROMPs.

IROMPs were first characterized according to the method of Goel and Kapil (2001) with a modification. Briefly, *Acinetobacter baumannii/calcoaceticus* were grown in iron depleted chemically defined medium (CDMFe⁻) and in iron rich (CDMFe⁺) medium. Medium without iron (CDMFe⁻) contained less than 0.04 mM Fe³⁺, and for medium with iron (CDMFe⁺), FeSO₄ was added to give a concentration of 60 mM. Overnight cultures in CDMFe⁻ and CDMFe⁺ were allowed to grow at 37°C with aeration (150 rpm) until late log phase at optical density of (OD₆₅₀) of 0.4. The cultures were then subjected to OMPs extraction according to the modified method described by Matsuyama *et al.* (1984) as mentioned earlier in section 2.8.2.1. IROMPs extracted from the SDS-PAGE gel were used as antigen to immunize rabbits.

### 2.9.2.2.2 Preparation of polyclonal sera.

To raise polyclonal antibodies in rabbit, first dose of antigen (300 µg/ml) was given intraperitoneally mixed with 250 µl of complete Freund’s adjuvant followed by three doses (300 µg/ml each) in Freund’s incomplete Adjuvant at intervals of one week. A final booster was given in normal saline, intraperitoneally, just four days before sacrifice. The experiments reported herein were conducted according to the principles in the Guidelines for the Care and Use of Laboratory Animals. Then, ELISA was carried out to test the reactivity of the polyclonal antibodies from rabbit sera with the antigens (10 µg/ml). Briefly, the microtitter plates (96 wells) were coated overnight at 4°C with antigens diluted in phosphate buffer saline (PBS). The plates were washed four times by dispensing 250 µl PBS-T (PBS + 0.05% Tween) to each well. Then, the plates were blocked with 1% BSA in PBS for 1 h at 37°C, and washed four times with
PBS-T. Rabbit sera of 100 µl with a 2-fold serial dilution, containing polyclonal antibody against IROMPs were added to the wells. The plates were incubated for 1 h at room temperature and washed four times with PBS-T. Goat anti-rabbit IgG horseradish peroxidase (Incstar Corporation, Boulevard) diluted at 1:2,000 was added to the wells. After 1 h of incubation at room temperature, the plates were washed four times with PBS-T and the last wash was with PBS only before the addition of the 2,2’-azinobis(3-ethylbenzthiazoline sulfonic acid) (ABTS) substrate (Kirkegaard & Perry). Optical densities at 410 nm (OD\textsubscript{410}) were measured after 15 min of incubation at room temperature.

2.9.2.2.3 Bactericidal activity.

Total viable count was performed to demonstrate bactericidal activity of polyclonal antibodies raised against IROMPs according to the modified method described by Poolman \textit{et al.} (1985). First, a standard growth curve was plotted and used as control. Briefly, 1 ml of known amount of \textit{Acinetobacter baumannii/calcoaceticus} culture approximately 10\textsuperscript{8} CFU/ml was inoculated into CDMFe\textsuperscript{−} medium and CDMFe\textsuperscript{+} medium. The cultures were vortexed well and incubated at 37°C with shaking at 250 rpm. Every half an hour, 10 µl of the culture suspensions were diluted in 90 µl of Luria broth in a test tube and followed by a 10-fold serial dilution until to 10\textsuperscript{1} CFU/ml. Each serially diluted culture suspensions about 100 µl each was spread onto Mueller-Hinton agar plates. The plates were incubated at 37°C overnight. Total viable count was done between 18 and 24 h. This procedure was repeated every half hour until the bacterial growth reached late log phase. Following this, total viable count method was performed on polyclonal antibodies to obtain bactericidal activity. In this experiment, polyclonal antibodies with 2 different dilution factors were used to obtain different concentration of antisera; 1:5 and 1:10. The appropriate concentrations of polyclonal antibodies were added to
the *Acinetobacter baumannii/calcoaceticus* culture medium at early stationary phase. Finally, the growth curve graphs were plotted and the data obtained were analyzed.
Chapter 3

Results
3.1 Bacterial strains.

A total of 49 strains were obtained from patients who were hospitalized in University Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia from August 2003 until March 2004. The strains were isolated from various body sites of infections from patients whom were hospitalized mostly in intensive care unit (ICU), surgical and general wards as listed in Table 3.1. All the *Acinetobacter* spp. isolates were sporadic cases of bacterial infections and presumably community-acquired. Majority of the isolates were from tracheal secretion (45%), wound swab (12%), bronchoalveolar lavage (11%), sputum (8%), blood (6%), peritoneal fluid (4%), followed by urine (2%), and other body sites such as epidermal catheter, abrasion over back, suture line swab, foot pressure sore swab, pus swab, and double lumen tips (12%). Of which 89.8% of the strains were from non-invasive sites while 10.2% were from invasive sites. However, for simplicity of analysis, the sites of isolation were re-assigned as upper respiratory tract (tracheal secretion, nasopharyngeal secretion), lower respiratory tract (sputum, bronchoalveolar lavage), peritoneal fluid, blood, wound, urine, and others.

Table 3.1: Strain number, patient RN number, ward, source and date of isolation of 49 *Acinetobacter* spp. isolates.

<table>
<thead>
<tr>
<th>NO</th>
<th>PATIENT RN NO.</th>
<th>COLLECTED</th>
<th>WARD</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>1106728</td>
<td>29/8/2003</td>
<td>ICU</td>
<td>Tracheal aspiration</td>
</tr>
<tr>
<td>1</td>
<td>1147833</td>
<td>3/12/2003</td>
<td>8U</td>
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</tr>
<tr>
<td>49</td>
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<td>Sputum</td>
</tr>
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<td>Sputum</td>
</tr>
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</tr>
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<td>Tracheal secretion</td>
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<tr>
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<td>WARD</td>
<td>SOURCE</td>
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<td>--------------</td>
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<td>ICU</td>
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<td>BAL</td>
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<td>8U</td>
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<td>Peritoneal fluid</td>
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<td>Sputum</td>
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<td>124</td>
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<td>13U</td>
<td>Tracheal aspiration</td>
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</table>
3.1.1 Identification using standard laboratory methods.

The identification of the organisms was performed using standard laboratory methods which include sugar tests and Gram stain. The results of standard identification of Acinetobacter spp. as comparison to other family members of Enterobacteriaceae and Non-Enterobacteriaceae are shown in Table 3.2. The members of the genus Acinetobacters are Gram-negative coccobacilli that are strictly aerobic, non-fermentative, oxidase-negative, catalase-positive, and non-motile bacteria. They appear as white mucoid colonies on Mueller Hinton (MH) agar as shown in Figure 3.1. Besides that, Gram stain of Acinetobacter spp. under light microscope with 100X magnification showed that they appear as plump, and short rods as shown in Figure 3.2. They utilize carbon and ammonium as its only source of energy and the results of the sugar tests carried out to confirm this test are shown in Figure 3.3 to Figure 3.5.

In addition, API20NE test was carried out as confirmatory test for the identification of this organism. This test is also to differentiate between Acinetobacter spp. with other Non-Enterobacteriaceae. The API20NE test showed that all the 49 strains were identified as Acinetobacter baumannii/calcoaceticus complex as shown in Fig 3.6.

An additional test to differentiate between Acinetobacter baumannii and Acinetobacter calcoaceticus was performed by growing the organisms at 44°C (Bou and Martinez, 2000). Only Acinetobacter baumannii can grow at 44°C. As a result, among the 39 imipenem-resistant strains, 36 were Acinetobacter baumannii and only 3 were Acinetobacter calcoaceticus.
Table 3.2: Comparison of biochemical test among family members of *Enterobacteriaceae* and *Non-Enterobacteriaceae*.

<table>
<thead>
<tr>
<th>Species</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>Slant</th>
<th>E</th>
<th>H₂S</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
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<td><em>Acinetobacter</em> spp.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Alkaline</td>
<td>Alkaline</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td><em>P. aeruginosa</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Alkaline</td>
<td>Alkaline</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>Acid</td>
<td>Acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>Acid</td>
<td>Acid</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>C. freundii</em></td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Enterobacter</em> spp.</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>Acid</td>
<td>Acid</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>M. morganii</em></td>
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<td>+</td>
<td>+</td>
<td>-</td>
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<td>Alkaline</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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</table>

Abbreviation:

- A – Motility
- B – Indole
- C – Methyl Red
- D – Voges-Proskauer
- E – TSI
- F – Citrate
- G – Oxidase
- H – Catalase
- I – Lactose
Figure 3.1: Morphology of *Acinetobacter* spp.

Some of *Acinetobacter* spp. isolates appear as white mucoid colonies.

Figure 3.2: Gram stain of *Acinetobacter* spp.

Gram stain shows that they are Gram-negative coccobacilli or rod shaped.
Figure 3.3: Citrate test of *Acinetobacter* spp.

- Positive test.
- Citrate utilized as its only source of carbon; and ammonium as its only source of nitrogen.

Figure 3.4: MRVP test of *Acinetobacter* spp.

- Negative test.
- No fermentation of glucose.

Figure 3.5: TSI test of *Acinetobacter* spp.

- Negative test.
- No fermentation of glucose, lactose or sucrose; peptone utilized.
All 49 *Acinetobacter* spp. isolates were identified as *A. baumannii/calcoaceticus* complex.

### 3.1.2 Antibiotic susceptibility.

A total of 49 pre-selected strains from the disc diffusion results were tested for MICs levels against the following antibiotics: Imipenem (IMP), Meropenem (MEM), Ceftazidime (CAZ), Cefotaxime (CTX), Aztreonam (ATM), Ciprofloxacin (CIP), Ticarcillin/clavulanate (T/CLA), Gentamicin (CN), Amikacin (AK), and Amoxicillin (AMX). Based on the susceptibility results, the strains were categorized into 4 different groups as shown in Table 3.3. This table also shows the range of MICs values of isolates in each group and the breakpoints for interpretive criteria as recommended by the CLSI (2005) guidelines. Since only carbapenem resistant strains of *Acinetobacter baumannii/calcoaceticus* were the main focus of this study, therefore, only Group A and Group D strains were fully utilized throughout all the experiments. The MICs values of IMP for Group A ranged from 32–64 µg/ml values whereas for Group D it ranged from 16–64 µg/ml.
Table 3.3: Different categories of strains based on the susceptibility to IMP and CAZ.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of isolates (n=49)</th>
<th>MICs IMP (µg/ml)</th>
<th>MICs CAZ (µg/ml)</th>
<th>Breakpoints IMP (µg/ml) S - R</th>
<th>Breakpoints CAZ (µg/ml) S - R</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>32 – 64</td>
<td>2 – 8</td>
<td>≤4 – ≥16</td>
<td>≤8 – ≥32</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>0.5 – 2.0</td>
<td>256 – &gt;512</td>
<td>≤4 – ≥16</td>
<td>≤8 – ≥32</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>0.125 – 4.0</td>
<td>1 – 8</td>
<td>≤4 – ≥16</td>
<td>≤8 – ≥32</td>
</tr>
<tr>
<td>D</td>
<td>35</td>
<td>16 – 64</td>
<td>16 – &gt;512</td>
<td>≤4 – ≥16</td>
<td>≤8 – ≥32</td>
</tr>
</tbody>
</table>

Legend:

S – Sensitive  R – Resistant

Group A : IMP resistant; CAZ sensitive (n=4)
Group B : IMP sensitive; CAZ resistant (n=4)
Group C : IMP sensitive; CAZ sensitive (n=6)
Group D : IMP resistant; CAZ resistant (n=35)
3.2 Fluorescent in-situ hybridization and its application in clinical samples.

Fluorescent in-situ hybridization is a technique that uses fluorescently labeled probes to detect microorganisms rapidly. In this study, two genus specific probes, namely, Acinetobacter spp. and Pseudomonas spp. were designed to detect members of Non-Enterobacteriaceae. In order to test the specificity of the Acinetobacter spp. probe that was designed, this probe was hybridized against various types of organisms. The probe was highly specific and hybridized to the target genus only and not to other bacterial genera (Figure 3.7). All the bacterial strains tested could be detected using the universal eubacterial probe. The rapid identification of Acinetobacter spp. and the high specificity of the probe showed that the Fluorescent in-situ hybridization is a powerful technique that can be use to identify microorganisms very easily. This is because the whole process for the bacterial identification using this technique only needs less than 2 hours as compared to the conventional identification which is laborious and requires 72 hours in total.

To test the sensitivity of the Acinetobacter spp. probe, blood spiking was done with a 10 fold serial dilution factor from an approximately $10^8$ CFU/ml of blood culture to $10^1$ CFU/ml. The results obtained showed that the probe was very sensitive. Microscopic sensitivity testing with serially diluted bacterial suspension revealed a limit of detection by FISH at $10^3$ CFU/ml (Figure 3.8).

Fluorescent in-situ hybridization technique was applied onto clinical samples, especially direct blood culture samples which were collected from the University Malaya Medical Centre (UMMC), which were smear positive for Acinetobacter spp. and were tested against the Acinetobacter spp. probe. As a result, 3 representative blood culture positive samples could successfully be detected using Acinetobacter spp. probe (Figure 3.9). This shows that the FISH technique could be very useful for bacterial identification in routine diagnostic laboratory. This
also would be an advantage for the clinical laboratories as FISH is an easy and simple method to use in that it detected all *Acinetobacter* spp. studied and did not react with any of the bacteria.

This part of the study was published in Indian Journal of Medical Microbiology, 2007 and entitled “Fluorescent *in-situ* Hybridization Assay for the detection of *Non-Enterobactericeae* in blood culture samples (Wong et al., 2007).
Figure 3.7: Specificity testing of *Acinetobacter* spp. probe using different types of organisms.

NB. EUB – eubacterial probe; *A* spp. – *Acinetobacter* species-specific probe; *S. maltophilia* – *Stenotrophomonas maltophilia*; *V. cholera* – *Vibrio cholera*; *C. violesceum* – *Chromobacterium violaceum*; *B. cepacia* – *Bukholderia cepacia*; *P. aeruginosa* – *Pseudomonas aeruginosa*.
Figure 3.8: Sensitivity testing of *Acinetobacter* spp. probe using blood spiking method.

Keyword:

Negative before / after = negative sample without any organism and picture taken before / after overnight incubation.

After $10^8 - 10^7$ = bacteria at concentration of $10^8 - 10^7$ CFU/ml and picture taken after overnight incubation.
Figure 3.9: Application of *Acinetobacter* spp. probe using Fluorescent *in-situ* hybridization in clinical samples.

BC : Blood culture
3.3 Species-level identification of *Acinetobacter* spp. using Amplified Ribosomal DNA Restriction Analysis (ARDRA).

In this study, however, the identification of *Acinetobacter* spp. using FISH has a limitation as it only allows bacterial identification at genus level. Therefore, in order to identify all the 49 *Acinetobacter* spp. isolates at species level, Amplified Ribosomal DNA Restriction Analysis (ARDRA) was carried out using five restriction enzymes (*Alu*I, *Cfo*I, *Mbo*I, *Rsa*I and *Msp*I). As a result, the combination of the patterns obtained after individual restriction analysis with *Cfo*I, *Alu*I, *Mbo*I, *Rsa*I, and *Msp*I has enabled to differentiate strains within the *A. calcoaceticus-A. baumannii* complex. For example, as shown in Figure 3.10, if RE pattern 1 of *Mbo*I observed then there are possibilities of species *A. calcoaceticus*, *A. baumannii*, or genomospecies 13 present. Then with the RE patterns obtained using *Alu*I, *A. calcoaceticus* was confirmed as it has unique pattern 2 whereas *A. baumannii* and genomospecies 13 share pattern 1. To find out whether *A. calcoaceticus* or genomospecies 13 present, then RE patterns using *Alu*I were used to differentiate and it was confirmed that *A. baumannii* present with unique pattern 1 and pattern 2 belongs to *A. calcoaceticus*. Therefore, these strains could be easily identified as separate groups and the RE patterns were concurrent with those reported by Jawad *et al.*, 1998 and Vaneechoutte *et al.*, 1995. In this study, it was confirmed that among the 39 carbapenem resistant strains of *Acinetobacter* spp., 3 were identified as *Acinetobacter calcoaceticus* and the remaining 36 were identified as *Acinetobacter baumannii*. 
Figure 3.10: RE Digestion of 16S rDNA using *Alu* I, *Cfo* I, and *Mbo* I enzymes for species level identification.

**LANE 1**: 100 bp
**LANE 2**: Strain 6
**LANE 3**: Strain 12
**LANE 4**: Strain 13
**LANE 5**: Strain 14
**LANE 6**: Strain 9
**LANE 7**: Strain 26
**LANE 8**: Strain 90
**LANE 9**: 100 bp
**LANE 10**: Strain 6
**LANE 11**: Strain 12
**LANE 12**: Strain 13
**LANE 13**: Strain 14
**LANE 14**: Strain 9
**LANE 15**: Strain 26
**LANE 16**: Strain 90
**LANE 17**: 100 bp
**LANE 18**: Strain 6
**LANE 19**: Strain 12
**LANE 20**: Strain 13
**LANE 21**: Strain 14
**LANE 22**: Strain 15
**LANE 23**: Strain 9
**LANE 24**: Strain 26
**LANE 25**: Strain 90
**LANE 26**: 100 bp
**LANE 27**: *A. baumannii* ATCC 15308
**LANE 28**: *A. baumannii* ATCC 15308
**LANE 29**: *A. baumannii* ATCC 15308
**LANE 30**: 100 bp
3.4 Molecular typing of *Acinetobacter baumannii/calcoaceticus* by PFGE.

Molecular typing of *Acinetobacter baumannii/calcoaceticus* isolates by Pulsed-Field Gel Electrophoresis (PFGE) generated DNA fragments which could be useful to distinguish the strains (Figure 3.10). There were all together 16 PFGE patterns assigned as A to P. In addition, the genetic relatedness between these strains was analyzed using the Dice coefficient and is depicted by a dendogram analysis using GelComparII Software (Figure 3.11). The dendogram was constructed by clustering the strains using the unweighted pair group method and demonstrated the overall genetic relatedness of *Acinetobacter baumannii/calcoaceticus* strains. The results showed that among 39 imipenem-resistant strains, 23 strains (59%) had 2 major identical PFGE patterns which were divided into 2 major genotypes assigned as genotype B, and C. Among these 2 major groups, genotype B was the dominant group found in 12 strains (31%), followed by genotype C found in 11 strains (28%). However, the remaining 16 strains (41%) had unique PFGE patterns. Isolates with genotype B and C were isolates mostly found in patients whom were admitted in intensive care unit (ICU) and surgical ward within the period of 3 months. The major sites of isolation in these patients were mainly from upper respiratory tract such as tracheal secretion, and nasopharyngeal secretion.
Figure 3.11: PFGE band patterns of *Acinetobacter baumannii/calcoaceticus* isolates.

<table>
<thead>
<tr>
<th>LANE</th>
<th>Genotype</th>
<th>Date of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>K</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td></td>
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<tr>
<td>4</td>
<td>G</td>
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<tr>
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<td>L</td>
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<td></td>
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</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LANE 1: *A. baumannii* (15308)
LANE 2: 26 (21/12)
LANE 3: 26 (21/12)
LANE 4: 63 (5/1)
LANE 5: 14 (8/12)
LANE 6: 15 (9/12)
LANE 7: 29 (24/12)
LANE 8: 30 (22/12)
LANE 9: 90 (26/1)
LANE 10: λ marker

(Ward P1, Ward ICU)

(Date of isolation shown in bracket)
Figure 3.12: Dendrogram analysis depicting the clonal relationship of the carbapenem resistant strains of *Acinetobacter baumannii/calcoaceticus* using GelCompar II Software.
3.5  **Isoelectric Focusing.**

Broad range pI markers were used to determine the isoelectric point (pI) values of the β-lactamases produced by all *Acinetobacter baumannii/calcoaceticus* isolates. A control strain producing characterized β-lactamases with known pI values was unfortunately not available to facilitate more accurate determination of the pI. Thus, the isoelectric point values of the β-lactamases from all the strains were estimated by comparing the pIs obtained with broad range pI markers with pI values that ranged from 4.45 to 9.6. IEF was carried out on PhastGel containing ampholites that gave a pI gradient of 3 to 9. The pI profiles obtained were ranged from 6.5 to >9.0. The putative enzymes of respected pI values were listed in Table 3.4.

IEF analysis showed that the 2 carbapenem hydrolyzing strains of *A. calcoaceticus*, S26 and S90 expressed several similar β-lactamases focusing at pI values of 6.65, 7.5, 8.9, and >9.0 (Figure 3.12). The pI value of 6.65 likely corresponded to that of OXA-23 (Donald *et al.*, 2000; Paton *et al.*, 1993), pI value of 7.5 corresponded to that of OXA-4 (Huovinen, 1988), pI value of 8.9 corresponded to that of metallo-β-lactamases (in this study), whereas pI value of >9.0 most likely corresponded to that of an AmpC-type cephalosporinase (Hornstein *et al.*, 1997). However, S90 had an additional band of pI 7.0.
Table 3.4: pI profiles and the putative enzymes in *Acinetobacter baumannii/calcoaceticus* isolates.

<table>
<thead>
<tr>
<th>STRAIN NO.</th>
<th>WARD</th>
<th>MIC</th>
<th>PUTATIVE ENZYMES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pI VALUES</td>
<td>GROUP A</td>
</tr>
<tr>
<td>1</td>
<td>ICU</td>
<td>32 64 4</td>
<td>6.65 OXA</td>
</tr>
<tr>
<td>11</td>
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<td>49</td>
<td>13U</td>
<td>64 64 8</td>
<td>6.65 OXA</td>
</tr>
<tr>
<td>99</td>
<td>13U</td>
<td>64 64 8</td>
<td>6.65 OXA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GROUP B</td>
</tr>
<tr>
<td>9</td>
<td>6TE</td>
<td>1 2 256</td>
<td>Nil Nil</td>
</tr>
<tr>
<td>21</td>
<td>ICU</td>
<td>0.5 1 512</td>
<td>9.6 OXA</td>
</tr>
<tr>
<td>93</td>
<td>ICU</td>
<td>1 4 &gt;512</td>
<td>Nil Nil</td>
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<tr>
<td>102</td>
<td>7U</td>
<td>2 4 512</td>
<td>7.1, 9.6 OXA, AmpC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GROUP C</td>
</tr>
<tr>
<td>3</td>
<td>6TD</td>
<td>0.125 0.5 1</td>
<td>Nil Nil</td>
</tr>
<tr>
<td>4</td>
<td>7U</td>
<td>0.25 0.5 4</td>
<td>Nil Nil</td>
</tr>
<tr>
<td>8</td>
<td>P2</td>
<td>0.25 0.5 2</td>
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</tr>
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<td>ICU</td>
<td>4 32 8</td>
<td>Nil Nil</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>GROUP D</td>
</tr>
<tr>
<td>6</td>
<td>6U</td>
<td>32 64 &gt;512</td>
<td>6.5, 6.65, 7.0, 8.2, 9.6 OXA, AmpC</td>
</tr>
<tr>
<td>12</td>
<td>CICU</td>
<td>16 32 512</td>
<td>6.5, 6.65, 7.0 OXA</td>
</tr>
<tr>
<td>13</td>
<td>8D</td>
<td>64 64 512</td>
<td>6.65, 8.2, 9.6 OXA, AmpC</td>
</tr>
<tr>
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<td>ICU</td>
<td>32 64 512</td>
<td>6.65, 8.2 OXA</td>
</tr>
<tr>
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<td>32 64 &gt;512</td>
<td>6.5, 6.65, 7.0, 8.2, 9.6 OXA, AmpC</td>
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<tr>
<td>22</td>
<td>CICU</td>
<td>32 64 &gt;512</td>
<td>6.65, 9.6 OXA, AmpC</td>
</tr>
<tr>
<td>26</td>
<td>P1</td>
<td>32 128 256</td>
<td>6.65, 7.5, 8.9, 9.6 IMP, OXA, AmpC</td>
</tr>
</tbody>
</table>

* Values in red indicates resistant strains, values in blue indicate sensitive strains.
Table 3.4: Continued.

<table>
<thead>
<tr>
<th>STRAIN NO.</th>
<th>WARD</th>
<th>MIC</th>
<th>PUTATIVE ENZYMES</th>
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<tbody>
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<td>IMP</td>
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<td>CAZ</td>
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<td>GROUP D</td>
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<td></td>
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<tr>
<td>29</td>
<td>ICU</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>30</td>
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<td>32</td>
<td>64</td>
</tr>
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<td>33</td>
<td>7U</td>
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<td>128</td>
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<td>35</td>
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<td>16</td>
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</tr>
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<td>45</td>
<td>6U</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>46</td>
<td>ICU</td>
<td>64</td>
<td>128</td>
</tr>
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<td>7U</td>
<td>32</td>
<td>128</td>
</tr>
<tr>
<td>52</td>
<td>7U</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>53</td>
<td>8U</td>
<td>32</td>
<td>128</td>
</tr>
<tr>
<td>54</td>
<td>P5</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>55</td>
<td>6U</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>57</td>
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<td>128</td>
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<tr>
<td>59</td>
<td>7U</td>
<td>32</td>
<td>128</td>
</tr>
<tr>
<td>61</td>
<td>ICU</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>62</td>
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<td>64</td>
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<td>P1</td>
<td>16</td>
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</tr>
<tr>
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<tr>
<td>124</td>
<td>13U</td>
<td>32</td>
<td>64</td>
</tr>
</tbody>
</table>

* Values in red indicates resistant strains, values in blue indicates sensitive strains.

Legend :

- **Blue** = Sensitive strains
- **Red** = Resistant strains
Figure 3.1: Isoelectric focusing of two *A. calcoaceticus*, metallo-β-lactamase producing strains. Gel was stained with nitrocefin.

Lane 1: Strain 26; pI value of 6.65, 7.5, 8.9 & >9.0,
Lane 2: pI marker,
Lane 3: Strain 90; pI value of 6.65, 7.0, 7.5, 8.9 & >9.0,
Lane 4: pI marker.

* pI values of 6.65, 7.5, and >9.0 not clearly visualized on the gel.
3.6 Screening for metallo-β-lactamases.

3.6.1 Double disc-synergy and Modified Hodge tests.

All the carbapenem resistant strains of \textit{A. baumannii} and \textit{A. calcoaceticus} were tested for the presence of metallo-β-lactamases using methods described by Arakawa \textit{et al.} (2000) and Lee \textit{et al.} (2001). In these experiments, 2 thiol compounds, EDTA and 2-mercaptoethanesulfonic acid were used because these agents able to inhibit only metallo-β-lactamases. Thus, the methods described by Arakawa \textit{et al.} (2000) and Lee \textit{et al.} (2001) were used as preliminary testing methods for screening metallo-β-lactamases. Out of the 39 carbapenem resistant strains screened, only two strains, both \textit{A. calcoaceticus}, S26 and S90 were positive for the presence of metallo-β-lactamases, whereby these enzymes were inhibited by EDTA or 2-mercaptoethanesulfonic acid (Figure 3.13 and 3.14) and 6, all \textit{A. baumannii} were equivocal at 30°C. The distortion patterns shown by both positive strains were similar to those of the IMP-1 and VIM-2 producing \textit{Acinetobacter} spp. and \textit{Pseudomonas aeruginosa} (positive control strains). These strains were kindly provided by Prof. Emeritus Yunsop Chong, and Prof. Kyungwon Lee from Department of Clinical Pathology and Research Institute of Bacterial Resistance, Yonsei University College of Medicine, Seoul, Korea. In addition to this study, both \textit{A. calcoaceticus} strains which were double disc-synergy test-positive showed a distorted inhibition zone around the IMP disc (Figure 3.15 and 3.16) by the Modified Hodge test, indicating that both strains are carbapenem-hydrolyzing strains.
Figure 3.14 and 3.15: EDTA- and 2MPA- double disc-synergy test positive.

![Augmentation of the inhibition zone](image1)

The presence of an augmentation of zone of inhibition shows double disc-synergy test positive.

Figure 3.16 and 3.17: Modified Hodge test.

![Distortion](image2)

The distorted inhibition zone showed positive test of carbapenem-hydrolyzing strains of S26 and S90, whereas, strain S62, S63, and S81 are carbapenem-non-hydrolyzing strains with no effect on the zone.
3.6.2 Metallo-β-lactamase purification and kinetics.

Metallo-β-lactamases, IMP-4 enzyme produced by S26 and S90 were purified using Rotorfor system (Biorad, Singapore). S26 had 2 enzymes purified at pI of 8.99 and 12.75 (Figure 3.17), whereas S90 had 3 enzymes purified at pI of 7.62, 8.80, and 12.93 (Figure 3.18). Since the IEF analysis suggested that pI 8.9 corresponded to that of \( \text{bla}_{\text{IMP-4}} \) enzyme, therefore fractions collected at pI 8.99 and pI 8.80 (S26 and S90, respectively) were used for further analysis. The purified IMP-4 enzyme from strains S26 and S90 had a wide range of activity against several antibiotics including carbapenems. However, this enzyme showed highest catalytic efficiency for nitrocefin as compared to other antibiotics (Table 3.5). The relative \( V_{\text{max}} \) rates (maximum rates of hydrolysis) were obtained for imipenem (0.82–0.97 mmol/L/h), meropenem (0.97–1.07 mmol/L/h), and oxacillin (1.11–1.24 mmol/L/h) (Table 3.6). On the basis of maximum rate of hydrolysis (\( V_{\text{max}} \)) values, both the metallo-β-lactamases strains, S26 and S90 tend to show higher hydrolysis rates to oxacillin than for imipenem and meropenem (Table 3.6). This suggests that IMP-4 enzyme has stronger hydrolysis activity against oxacillin as compared to carbapenems. However, this enzyme hydrolyzes meropenem faster than imipenem and resulted in higher MICs of meropenem in these two strains as previously shown in Table 3.4. Metallo-β-lactamases such as IMP-4 are the β-lactamases that require a metal ion for enzymatic activity and apparently they are not inhibitable by clavulanic acid but they are able to be inhibited by metal ion chelators such as EDTA. This inhibition assay was used to distinguish the presence of metallo-β-lactamases. In this study, the two strains, S26 and S90 showed 50% inhibitory effect when EDTA was incubated with the IMP-4 enzyme, indicating high affinity of this enzyme. This also suggests that the enzyme had the characteristics of metallo-β-lactamases since it was inhibited by EDTA. In addition, the AmpC enzyme produced by S90 was not inhibited by EDTA, excludes the possibility of a metalloenzyme.
Figure 3.18: Purified fractions of β-lactamases from S26 versus OD and pH.

Figure 3.19: Purified fractions of β-lactamases from S90 versus OD and pH.
Table 3.5: Kinetic parameters of the enzymes of S26 and S90.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>IMP-4 (S26)</th>
<th>IMP-4 (S90)</th>
<th>AmpC (S90)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kcat (s⁻¹)</td>
<td>Km (µM)</td>
<td>kcat/Km (mM⁻¹s⁻¹)</td>
<td>Kcat (s⁻¹)</td>
</tr>
<tr>
<td>IMP</td>
<td>40</td>
<td>125.76</td>
<td>318</td>
</tr>
<tr>
<td>MEM</td>
<td>40</td>
<td>125.76</td>
<td>318</td>
</tr>
<tr>
<td>OXA</td>
<td>40</td>
<td>125.76</td>
<td>318</td>
</tr>
<tr>
<td>CAZ</td>
<td>40</td>
<td>125.76</td>
<td>318</td>
</tr>
<tr>
<td>NIT</td>
<td>60</td>
<td>125.76</td>
<td>477</td>
</tr>
<tr>
<td>AMX</td>
<td>40</td>
<td>125.76</td>
<td>318</td>
</tr>
<tr>
<td>CN</td>
<td>40</td>
<td>125.76</td>
<td>318</td>
</tr>
</tbody>
</table>

**Keyword:**

kcat = molecules hydrolyzed per second;  
Km = binding affinity;  
kcat/Km = molecules hydrolyzed per second per molecule of enzyme
Table 3.6: Relative rate of hydrolysis to imipenem, meropenem, oxacillin, ceftazidime, nitrocefin, amoxicillin, and gentamicin for S26 and S90.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Original host</th>
<th>Relative rate of hydrolysis (Vmax)</th>
<th>IC$_{50}$ for inhibition (µM)</th>
<th>pI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IMP</td>
<td>MEM</td>
<td>OXA</td>
<td>CAZ</td>
</tr>
<tr>
<td>IMP-4</td>
<td>Strain 26</td>
<td>0.97</td>
<td>1.07</td>
<td>1.24</td>
<td>0.67</td>
</tr>
<tr>
<td>IMP-4</td>
<td>Strain 90</td>
<td>0.82</td>
<td>0.97</td>
<td>1.11</td>
<td>0.75</td>
</tr>
<tr>
<td>AmpC</td>
<td>Strain 90</td>
<td>0.61</td>
<td>0.81</td>
<td>0.85</td>
<td>1.13</td>
</tr>
</tbody>
</table>

Keyword:

$V_{max} = \text{maximum rates of hydrolysis}$  
$IC_{50} = \text{50% inhibition of enzyme}$
3.7 Detection of antibiotic resistance genes in *Acinetobacter* spp. via PCR.

3.7.1 Amplification of *bla*\textsubscript{IMP} genes.

PCR amplification of *bla*\textsubscript{IMP} gene among the 39 *Acinetobacter* spp. isolates using previously published primers by Yum *et al.* (2002) were positive in only two strains, S26 and S90. This PCR amplification gave an amplicon size of 448 bp as shown in Figure 3.19. However, these primers cannot amplify the entire coding region of *bla*\textsubscript{IMP} as the primers were only specific to *bla*\textsubscript{IMP-1} gene. Further PCR amplification was carried out using another set of primers which were designed to amplify the entire coding region of *bla*\textsubscript{IMP} gene. This PCR amplification gave an amplicon size of 795 bp as shown in Figure 3.20.

To complete the sequencing of the entire coding region of *bla*\textsubscript{IMP} gene, PCR products of S26 and S90 were sequenced with an internal reverse primer that we designed towards the upstream of the *bla*\textsubscript{IMP} region. The sequencing data showed that this gene has 99% amino acid sequence homology to IMP-4 gene of *A. calcoaceticus* (Genbank accession number AY795963). The detection of this gene only in two *A. calcoaceticus* strains, S26 and S90 showed that the IMP-type metallo-β-lactamases does not play a significant role in carbapenem resistance but suggests possibility of other resistance mechanisms.
Figure 3.20: Detection of \( \text{bla}_{\text{IMP}} \)-1 gene.

- **LANE 1:** 100 bp marker
- **LANE 2:** Negative control
- **LANE 3:** Strain 26
- **LANE 4:** Strain 90
- **LANE 5:** \( \text{bla}_{\text{IMP}} \) positive control strain (\textit{Acinetobacter} spp.), 448 bp

Figure 3.21: Detection of \( \text{bla}_{\text{IMP}} \) entire coding region.

- **Lane 1:** Negative control,
- **Lane 2:** Strain 26,
- **Lane 3:** 100 bp marker,
- **Lane 4:** Strain 90,
- **Lane 5:** \( \text{bla}_{\text{IMP}} \) positive control strain (\textit{Acinetobacter} spp.), 795 bp
3.7.2 Amplification of *bla*<sub>VIM</sub> genes.

As seen in section 3.6.1, only two among the 39 carbapenem resistant strains, both *A. calcoaceticus* harboured the IMP-4 gene. Therefore, further investigation was carried out to see if presence of other metallo-β-lactamases is involved in development of carbapenem resistance. In order to do this, PCR was carried out to detect the VIM-type metallo-β-lactamases using a set of VIM primers which were published previously by Poirel *et al.* (2000). Of the 39 carbapenem resistant strains that were screened, none harbored the *bla*<sub>VIM-2</sub> gene except for the positive control strain as shown in Figure 3.21. This suggests that VIM-type metallo-β-lactamases does not contribute to carbapenem resistance in these strains. However, positive PCR amplification of the control strain gave an amplicon size of 801 bp which matched to *bla*<sub>VIM-2</sub> gene of *A. baumannii* (Genebank accession number AF291420) with 99% amino acid sequence homology.

**Figure 3.22: Detection of *bla*<sub>VIM-2</sub> gene.**

![Image of gel electrophoresis](image_url)
3.7.3 Amplification of \textit{bla}_{OXA-23} gene.

Besides the detection of metallo-β-lactamases in 39 carbapenem resistant strains of 
\textit{A. baumannii} and \textit{A. calcoaceticus}, presence of another type of carbapenem-hydrolyzing 
\textit{bla}_{OXA-23} gene, was also detected in this study. I have only screened for the presence of 
\textit{OXA-23} as initially when I started my PhD in year 2004, \textit{OXA-23} was the predominant 
oxacillinases found in \textit{Acinetobacter} spp. PCR amplification to detect the presence of 
\textit{bla}_{OXA-23} gene using a set of \textit{OXA-23} primers which were published previously by Alfaz \textit{et al.} (2001) gave an amplicon size of 1058 bp as shown in Figure 3.22. Among the 39 
carbapenem resistance strain, 37 were detected positive for the presence of this gene. This 
PCR product of 1058 bp was sequenced and the data showed that this gene has 96% amino 
acid sequence homology to \textit{bla}_{OXA-23} (Genbank accession number AJ132105). Detection of 
\textit{OXA-23} gene in 37 carbapenem resistant strains indicates a higher prevalence of this gene 
among \textit{A. baumannii} strains of Malaysian isolates. This also may suggest that this gene 
plays a more significant role than metallo-β-lactamases towards the development of 
carbapenem resistance in this organism.

\textbf{Figure 3.23: Detection of \textit{bla}_{OXA-23} gene.}
3.7.4 Amplification of $bla_{AmpC}$ genes.

Detection of $bla_{AmpC}$ gene among carbapenem resistant strains of *A. baumannii* and *A. calcoaceticus* was also included in this study to detect the presence of cephalosporinases. PCR amplification to detect the presence of $bla_{AmpC}$ gene using a set of AmpC primers which were published previously by Bou and Martinez (2000) gave an amplicon size of 663 bp as shown in Figure 3.23. Among the 39 carbapenem resistance strains, 38 were detected positive for this gene including a strain of metallo-β-lactamase producer, S90. The PCR product of 663 bp was sequenced and the data showed that this gene has 98% amino acid sequence homology to $bla_{AmpC}$ (Genbank accession number EF016355). Detection of this gene in 38 of 39 *A. baumannii/calcoaceticus* strains is an evident of a naturally occurring β-lactamase as has been previously described by Poirel and Nordmann, 2006. The role of this naturally occurring β-lactamase towards carbapenem resistance is associated with the presence of other carbapenemases.

**Figure 3.24: Detection of $bla_{AmpC}$ gene.**
Table 3.7 shows the summarized distribution of β-lactamases in all the 49 strains which include Group A, B, C, and D (refer to Table 3.7). As a summary, studies on enzymatic mechanisms in *A. baumannii* and *A. calcoaceticus* strains showed that the involvement of multiple antibiotic resistance genes in these organisms contributed to carbapenem resistance and these genes were detected by PCR amplifications. Only two strains, S26 and S90 from Group D, were detected for the presence of IMP gene. However, none of the 49 strains were detected for the presence of VIM gene. All the 4 strains from Group A were found to carry OXA-23 and AmpC genes. Whereas only 3 strains from Group B and C, were carrying AmpC genes. Besides that, in Group D, all the 37 non-metallo-β-lactamase producing strains were found to carry AmpC and OXA-23 genes except one of the metallo-β-lactamase producer, S90 was found to carry AmpC gene. In this study, OXA-23 genes were present only if the strains were resistant to carbapenems. This suggests that besides IMP genes, the OXA-23 genes are also associated with carbapenem resistance in *A. baumannii* and *A. calcoaceticus*.

Table 3.7: Summary of number of isolates from each group that carries various types of resistance genes in *Acinetobacter* spp.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of isolates (n=49)</th>
<th>bla\text{IMP-4} (+ve)</th>
<th>bla\text{VIM-2} (+ve)</th>
<th>bla\text{AmpC} (+ve)</th>
<th>bla\text{OXA-23} (+ve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>35</td>
<td>2</td>
<td>0</td>
<td>34</td>
<td>33</td>
</tr>
</tbody>
</table>

Legend:

- **Group A**: IMP resistant; CAZ sensitive (n=4)
- **Group B**: IMP sensitive; CAZ resistant (n=4)
- **Group C**: IMP sensitive; CAZ sensitive (n=6)
- **Group D**: IMP resistant; CAZ resistant (n=35)
3.8 Location of genes encoding metallo-β-lactamase gene.

3.8.1 Characterization of plasmids.

Plasmids were extracted from both *A. calcoaceticus*, S26 and S90 (metallo-β-lactamase producers) by alkaline lysis method (Sambrook *et al.*, 1989) to study its profile. It was suggested previously by Livermore (1996) that β-lactamases may be plasmid- or chromosomally-mediated. Therefore, this study was carried out to determine whether the $bla_{IMP}$ gene was carried in the plasmids. The molecular weights for each plasmid were determined from a standard linear graph of $\log_{10}$MW of known plasmid versus their distance of migration (mm) from the wells of the gel. *E. coli* 39R861 containing plasmids of sizes 147 kb, 63 kb, 36 kb, and 7 kb was used to determine the molecular weights of plasmids. The plasmid profile of the 2 *A. calcoaceticus* isolates, S26 and S90, the metallo-β-lactamases producers showed that both of them had a similar pattern. They carried 3 large molecular weight plasmids of sizes 147 kb, 50 kb, 36 kb and with one small molecular weight plasmid at 7 kb (Figure 3.24).

In order to determine the location of the $bla_{IMP-4}$ genes in the 2 strains of *A. calcoaceticus*, S26 and S90 (metallo-β-lactamase producers), Southern blot hybridization was performed on these plasmids using a $bla_{IMP-4}$ PCR product as the probe. The plasmid that showed positive hybridization with the $bla_{IMP-4}$ gene was at about 50 kb. The Figure 3.25 shows the result of the plasmid DNA hybridization using $bla_{IMP-4}$ probe.
Figure 3.25: Plasmid profiles of $bla_{IMP-4}$ producing strains.

![Plasmid profiles image]

LANE 1: Lambda HindIII Marker
LANE 2: ATCC A. baumannii 15308
LANE 3: Strain 26
LANE 4: Strain 26
LANE 5: E. coli 39R861
LANE 6: Strain 90
LANE 7: Strain 90
LANE 8: Acinetobacter spp. $(bla_{IMP}$ positive control)

Figure 3.26: Plasmid DNA hybridization using $bla_{IMP-4}$ probe.

![Plasmid DNA hybridization image]

LANE 1: Lambda Hind III Marker
LANE 2: ATCC A. baumannii 15308
LANE 3: Strain 26
LANE 4: Strain 26
LANE 5: E. coli 39R861
LANE 6: Strain 90
LANE 7: Strain 90
LANE 8: Acinetobacter spp. $(bla_{IMP}$ positive control)
3.8.2 Detection and characterization of Class 1 integrons.

Apart from plasmids, there were also studies showed that IMP and VIM type of metallo-β-lactamases were found to be located in the integrons (D’Agata, 2004; Nordmann and Poirel, 2002). Therefore, in this study, integrons were also characterized in order to determine whether the \textit{bla}_{IMP} gene was carried in the integrons. PCR amplification using a set of primers which were published previously by Honang et al. (2003) gave an amplicon size of 569 bp as shown in Figure 3.26. A total of 31 strains out of 39 carbapenem-resistant strains were detected to carry the Class 1 integrase genes. Since, these primers were only specific to IntI gene which encodes the Class I integrase gene, in addition, another PCR amplification using the 5’ and 3’ conserved segment primers was carried out. These sets of PCR primers were used to detect the entire coding region of integron. PCR amplification of entire coding region of integron resulted in two bands of 2.5 kb and 3 kb in 31 out of the 39 carbapenem-resistant strains of \textit{Acinetobacter baumannii/calcoaceticus} (Figure 3.27). Restriction enzyme analysis with \textit{AluI} revealed 3 fingerprint patterns which designated profile 1, 2, and 3 (Figure3.28). Profile 1 and 2 had similar fingerprint patterns with 5 bands with the exception of an additional band of 200bp in profile 2 (Figure 3.28). Subsequent sequencing of the entire gene cassette representatives of each profile revealed that strains with profile 1 (2.5kb) contained the \textit{aacC1} gene, whereas the larger (3kb) integron cassette (profile 2) was identical to that of profile 1 with the exception of an additional copy of the \textit{orfX} gene. Both the \textit{bla}_{IMP-4} producing \textit{A. calcoaceticus}, S26 and S90, had a unique profile, 3. Nucleotide sequencing of the inserted gene cassettes revealed identical genes: \textit{bla}_{IMP-4}, \textit{qacG}, \textit{aacA4}, and \textit{catB3}. In addition, Southern blot hybridization of entire coding region of integron was carried out using \textit{bla}_{IMP-4} probe and as a result,
positive hybridization was observed (Figure 3.29). Thus, indicating that the $bla_{IMP-4}$ gene was carried on Class 1 integron.

**Figure 3.27: Detection of Intl gene.**

![Image showing gel electrophoresis with lanes labeled as follows: LANE 1: Negative control, LANE 2: Strain 6, LANE 3: Strain 12, LANE 4: Strain 13, LANE 5: Strain 14, LANE 6: Strain 15, LANE 7: 100 bp marker, LANE 8: Strain 22, LANE 9: Strain 30, LANE 10: Strain 26, LANE 11: Strain 29, LANE 12: *Acinetobacter* spp. ($bla_{IMP}$ positive control)]

**Figure 3.28: Detection of the entire coding region of the integron.**

![Image showing gel electrophoresis with lanes labeled as follows: LANE 1: Strain 26 (Duplicates), LANE 2: Strain 26, LANE 3: 1 kb marker, LANE 4: Strain 90, LANE 5: Strain 90, LANE 6: *Acinetobacter* spp. ($bla_{IMP}$ positive control)]
Figure 3.29: Restriction enzyme profiles of Class 1 integrons digested with *AluI* from clinical isolates of *Acinetobacter* spp.

Lane 1: Strain 6 (undigested PCR product) Lane 9: 100 bp;  
Lane 2: 1 kb; Lane 10: Strain 26;  
Lane 3: Strain 6; Lane 11: Strain 90;  
Lane 4: Strain 12; Lane 12: Strain 29;  
Lane 5: Strain 13; Lane 13: Strain 30;  
Lane 6: Strain 14; Lane 14: Strain 33;  
Lane 7: Strain 15; Lane 15: Strain 35;  
Lane 8: Strain 22; Lane 16: Strain 45.
Figure 3.30: Hybridization of the entire coding region of the integron with $bla_{IMP}$ probe.

1 2 3 4 5 6

LANE 1: Strain 26  Duplicates
LANE 2: Strain 26
LANE 3: 1 kb marker
LANE 4: Strain 90
LANE 5: Strain 90
LANE 6: Acinetobacter spp. $bla_{IMP}$ (+ve control)
3.8.3 Correlation of integron profiles with genotyping.

Data obtained from integron analysis above was subsequently analyzed to correlate the observed minor variations in integron profiles within each genotype with the macro-restriction profiles generated by PFGE. Analysis showed that 9 of the 10 isolates with profile 1 (2.5kb) had identical PFGE patterns, designated genotype B, whereas 11 from 19 isolates with profile 2 (3kb) had a PFGE pattern of genotype C (Table 3.8). The remaining isolates, one with integron profile 1 and eight with integron profile 2, had unique PFGE profiles and were classified as sporadic isolates. However, both the \( \text{bla}_{\text{IMP-4}} \) producing \( A. \) \( \text{calcoaceticus} \) strains, S26 and S90 with integron profile 3 (3kb) gave distinct PFGE profiles which differed from each other by more than seven bands.

Table 3.8: Correlation between Class 1 integron structure and its genotype.

<table>
<thead>
<tr>
<th>Integron</th>
<th>5’- and 3’-CS amplicon size (kb)</th>
<th>Inserted resistance gene cassettes within 5’- and 3’-CS</th>
<th>Genotype (No. of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.5</td>
<td>aacC1, adaAla, orf</td>
<td>B(9), K(1)</td>
</tr>
<tr>
<td>2</td>
<td>3.0</td>
<td>aacC1, adaAla, orf’, orf’</td>
<td>C(11), D(2), E(1), F(1), G(1), I(1), J(1), M(1)</td>
</tr>
<tr>
<td>3</td>
<td>3.0</td>
<td>( \text{bla}_{\text{IMP-4}}, \text{qacG}, \text{aacA4}, \text{catB3} )</td>
<td>A(1), L(1)</td>
</tr>
</tbody>
</table>
3.9 Determination of non-enzymatic mechanisms.

3.9.1 Detection of efflux pumps.

So far, findings from this study has shown that the presence of \textit{bla}_{IMP-4}, \textit{bla}_{OXA-23}, and Class 1 integron are three important factors leading to carbapenem resistance in \textit{A. baumannii} and \textit{A. calcoaceticus}, However, these are the enzymatic mechanisms found in these organisms. In order to study the non-enzymatic mechanisms, the involvement of efflux pumps in these organisms was analyzed. PCR amplifications were carried out using primers listed in Chapter 2 (Table 2.10) to detect presence of \textit{adeA}, \textit{adeB}, \textit{adeC}, \textit{adeR}, and \textit{adeS} genes which have been shown to play a role in antibiotic resistance particularly to aminoglycosides (Marchand et al., 2004). Among the 39 carbapenem resistant strains, 36 were positive for the presence of \textit{adeA}, \textit{adeB}, \textit{adeR}, and \textit{adeS} genes. However, for the \textit{adeC} gene, only 34 strains harbored this gene (Figure 3.30). In addition to this study, 3 representative carbapenem resistant strains; strain 1, strain 15, and strain 63 were chosen based on the gene association with resistance in these strains. Strain 15 and strain 63 harbored all the 5 \textit{ade} genes whereas strain 1 lack of \textit{adeC} gene. A representative gel image of strain 15 which harbor all the 5 \textit{ade} genes shown in Figure 3.30.

The disruption of each \textit{ade} gene by insertion-inactivation method was successful and was analyzed by PCR sequence determination. The resulting mutants were determined for antimicrobial susceptibility testing using various antibiotics. Comparing the MICs values of the wild-type strains of strain 1, strain 15, and strain 63, the derivative mutants showed lower MICs values than its parent strains (Table 3.9). The MICs values for all the mutants were 1 to 6 fold lower for ceftazidime, cefotaxime, gentamicin, amikacin, ciprofloxacin, amoxicillin and meropenem as shown in Table 3.9. The gene knockout studies show that MIC\textsubscript{AK}, MIC\textsubscript{CN}, MIC\textsubscript{CIP}, and MIC\textsubscript{CTX} were 4 to 6 fold lower in the mutant lacking \textit{adeB} gene as compared to \textit{adeA} gene, which only showed a 1 fold decrease.
in MICs when compared to the wild strain. Similar results are observed in gene knockouts of adeR gene and adeS gene, suggesting adeB gene as an efflux pump gene to be involved in antibiotic resistance with adeR gene and adeS gene as the two-component regulatory systems. The MIC\textsubscript{MEM} in each mutant lacking adeB gene, adeR gene, and adeS gene respectively showed a 2 fold decrease in MICs and only 1 fold decrease in MIC\textsubscript{CAZ}, and MIC\textsubscript{AMX}. However, all the mutants showed no differences in MICs values for imipenem as compared to wild-type strains.

**Figure 3.31:** Representative gel image of strain 15 which harbor adeA, adeB, adeC, adeR, and adeS genes.
Table 3.9: MICs of β-lactams for 3 mutants derived from the wild-type strains.

<table>
<thead>
<tr>
<th>Strain*</th>
<th>IMP (µg/ml)</th>
<th>MEM (µg/ml)</th>
<th>CAZ (µg/ml)</th>
<th>CTX (µg/ml)</th>
<th>CN (µg/ml)</th>
<th>AK (µg/ml)</th>
<th>CIP (µg/ml)</th>
<th>AMX (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (wild type)</td>
<td>32</td>
<td>64</td>
<td>8</td>
<td>64</td>
<td>16</td>
<td>4</td>
<td>8</td>
<td>&gt;512</td>
</tr>
<tr>
<td>1adeA'</td>
<td>32</td>
<td>32</td>
<td>4</td>
<td>32</td>
<td>8</td>
<td>2</td>
<td>4</td>
<td>512</td>
</tr>
<tr>
<td>1adeB'</td>
<td>32</td>
<td>16</td>
<td>4</td>
<td>2</td>
<td>0.125</td>
<td>0.25</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>1adeR'</td>
<td>32</td>
<td>16</td>
<td>4</td>
<td>2</td>
<td>0.25</td>
<td>0.125</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>1adeS'</td>
<td>32</td>
<td>16</td>
<td>4</td>
<td>1</td>
<td>0.25</td>
<td>0.125</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>15 (wild type)</td>
<td>32</td>
<td>64</td>
<td>&gt;512</td>
<td>32</td>
<td>128</td>
<td>8</td>
<td>64</td>
<td>&gt;512</td>
</tr>
<tr>
<td>15adeA'</td>
<td>32</td>
<td>32</td>
<td>256</td>
<td>16</td>
<td>64</td>
<td>4</td>
<td>32</td>
<td>256</td>
</tr>
<tr>
<td>15adeB'</td>
<td>32</td>
<td>16</td>
<td>256</td>
<td>2</td>
<td>16</td>
<td>0.5</td>
<td>4</td>
<td>256</td>
</tr>
<tr>
<td>15adeC'</td>
<td>32</td>
<td>64</td>
<td>256</td>
<td>64</td>
<td>&gt;512</td>
<td>8</td>
<td>64</td>
<td>256</td>
</tr>
<tr>
<td>15adeR'</td>
<td>32</td>
<td>16</td>
<td>256</td>
<td>2</td>
<td>32</td>
<td>1</td>
<td>2</td>
<td>256</td>
</tr>
<tr>
<td>15adeS'</td>
<td>32</td>
<td>16</td>
<td>256</td>
<td>2</td>
<td>32</td>
<td>1</td>
<td>2</td>
<td>256</td>
</tr>
<tr>
<td>63 (wild type)</td>
<td>16</td>
<td>32</td>
<td>&gt;512</td>
<td>64</td>
<td>128</td>
<td>4</td>
<td>32</td>
<td>&gt;512</td>
</tr>
<tr>
<td>63adeA'</td>
<td>16</td>
<td>16</td>
<td>256</td>
<td>32</td>
<td>64</td>
<td>2</td>
<td>16</td>
<td>256</td>
</tr>
<tr>
<td>63adeB'</td>
<td>16</td>
<td>8</td>
<td>256</td>
<td>2</td>
<td>8</td>
<td>0.25</td>
<td>2</td>
<td>256</td>
</tr>
<tr>
<td>63adeC'</td>
<td>16</td>
<td>32</td>
<td>256</td>
<td>64</td>
<td>512</td>
<td>8</td>
<td>64</td>
<td>256</td>
</tr>
<tr>
<td>63adeR'</td>
<td>16</td>
<td>8</td>
<td>256</td>
<td>2</td>
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<td>63adeS'</td>
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* The minus (-) sign shows created mutants lacking the respective genes

CLSI breakpoints: IMP, MEM and CN (S ≤ 4; I = 8; R ≥ 16); CAZ, and AMX (S ≤ 8; I = 16; R ≥ 32);
CTX (S ≤ 8; I = 16-32; R ≥ 64); AK (S ≤ 16; I = 32; R ≥ 64); CIP (S ≤ 1; I = 2; R ≥ 4)
Legend: IMP = imipenem; MEM = meropenem; CAZ = ceftazidime; CTX = cefotaxime;
CN = gentamicin; AK = amikacin; CIP = ciprofloxacin; AMX = amoxicillin.
3.9.2 Outer membrane permeability (OMP) analysis via SDS-PAGE.

3.9.2.1 Loss of 29-kDa outer membrane protein.

The outer membrane proteins (OMPs) have also been shown to play a role in development of antibiotic resistance including carbapenem resistance (Limansky et al., 2002; Bou et al., 2000; Clark, 1996). In this study, the OMP profile was determined by SDS-PAGE. As shown in Figure 3.31 which is a representative gel of selected strain that the carbapenem resistant *A. baumannii/calcoaceticus* had a missing OMP band at 29-kDa (red arrow). This probably suggests that the loss of a 29-kDa OMP would be one of the factors leading to carbapenem resistance among these *Acinetobacter* spp. isolates. The 29-kDa OMP band was only observed in ATCC strain *A. baumannii* 15308, *A. baumannii* and *A. calcoaceticus* carbapenem sensitive strains from this study.

**Figure 3.32 :** Representative gel of outer membrane protein profile via SDS-PAGE of selected strains.

<table>
<thead>
<tr>
<th>LANE</th>
<th>Description</th>
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<tr>
<td>1</td>
<td>Protein Marker</td>
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<tr>
<td>2</td>
<td>LANE 2 : ATCC <em>A. baumannii</em> (15308)</td>
</tr>
<tr>
<td>3</td>
<td>LANE 3 : Strain 30</td>
</tr>
<tr>
<td>4</td>
<td>LANE 4 : Strain 26</td>
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<td>5</td>
<td>LANE 5 : Strain 90</td>
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<td>6</td>
<td>LANE 6 : Strain 1</td>
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<td>LANE 7 : Strain 9</td>
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<td>8</td>
<td>LANE 8 : Strain 6</td>
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<td>9</td>
<td>LANE 9 : Strain 80</td>
</tr>
<tr>
<td>10</td>
<td>LANE 10 : Strain 55</td>
</tr>
</tbody>
</table>

Legend :  = 29-kDa outer membrane protein

Lane 1-6 and 8-10 = carbapenem resistant strains

Lane 7 = carbapenem sensitive strain
3.9.2.2 Potential target for antimicrobial therapy.

An additional study was carried out by raising polyclonal antibodies against specific outer membrane protein called Iron-regulated Outer Membrane Proteins (IROMPs). As mentioned before in Chapter 2, the rational of doing this experiment was to develop a potential target of antimicrobial therapy by raising polyclonal antibodies against IROMP. It was reported previously by Goel and Kapil (2001) that monoclonal antibodies produced against IROMPs were shown to have bactericidal effect. However, in this study, \textit{A.calcoaceticus} strain, S26 as representative of carbapenem resistant strain and with the loss of 29-kDa outer membrane protein was further investigated to study its survival when grown in iron deficient medium. Since the main focus of the study was carbapenem resistance, strain S26 was chosen as representative strain which had unique resistance mechanism, that is, the presence of IMP-4 metallo-\(\beta\)-lactamase. In this experiment, IROMPs with molecular weight of 45-50-kDa were observed when S26 was grown in iron deficient medium as shown in Figure 3.32.

Iron-regulated outer membrane proteins (IROMPs) bands were then excised from the SDS-PAGE gels, pooled and rabbit immunized to raise polyclonal antibodies. As reported by Goel and Kapil (2001), polyclonal antibodies produced by the rabbits were tested against \textit{Acinetobacter} spp. strains to determine whether these antibodies have bactericidal effect on these organisms. It was found that these polyclonal antibodies specifically killed or inhibited the bacteria grown in iron deficient medium as shown in Figure 3.33. The effect of these antibodies decreased with increasing dilution of the antibodies (5 fold versus 10 fold), shown in Figure 3.33. However, this difference was only seen after 3 hours post incubation, suggesting that the antibodies raised against iron-regulated outer membrane proteins (IROMPs) are bactericidal. In the presence of iron,
however, the polyclonal antibodies did have the bactericidal effect on *Acinetobacter calcoaceticus* growth but for a short period of 1.5 to 2 hours post incubation as shown in Figure 3.34. After 2 hours, however, the effect appeared to be nullified and the *Acinetobacter* spp. appeared to have recovered from this temporary bactericidal effect. This would be due to the presence of iron as nutrient in the medium which enabled the bacteria to acquire this essential nutrient for survival.

**Figure 3.33:** Iron-regulated outer membrane proteins (IROMPs) produced by strain S26 when this particular strain was grown in iron deficit medium.

Legend: Fe⁺ = strain grown in the presence of iron medium
Fe⁻ = strain grown in the absence of iron medium

↑ = 29-kDa outer membrane protein
↓ = Iron-regulated outer membrane proteins (IROMPs)
Figure 3.34: Effect of polyclonal antibodies raised against iron-regulated outer membrane proteins (IROMPs) with strain S26 grown in iron deficient medium.

Figure 3.35: Effect of polyclonal antibodies raised against iron-regulated outer membrane proteins (IROMPs) with strain S26 grown in iron medium.

Legend:

Antisera (1:5) = 5-fold dilution

Antisera (1:10) = 10-fold dilution
Chapter 4

Discussion and Conclusion
The emergence and spread of antibiotic-resistant bacteria causing infection is of great concern to clinicians. Since the seminal description of a penicillin-inactivating enzyme in *Escherichia coli*, the fierce 75-year struggle against these bacteria has been referred to as an “unwinnable war” (Abraham and Chain, 1940). Recent clinical attention has focused on the increasing frequency of non–lactose-fermenting gram-negative pathogens responsible for hospital-acquired infections (Gaynes and Edwards, 2005). In this group, *Acinetobacter* species and *Pseudomonas aeruginosa* are emerging as pathogens that frequently cause infections in patients in intensive care units (Wisplinghoff *et al*., 2004). In both genera of bacteria, resistance to multiple classes of antibiotics seriously compromises the ability to treat patients who are infected with these pathogens. In many instances, there are very few antibiotic choices against the infections. Hence, for the immunocompromised host, current effective therapy is a matter of survival. In this study, the molecular basis for antibiotic resistance in *Acinetobacter* spp. was highlighted specifically to carbapenem resistance mechanisms involved in this organism.

The incidence of carbapenem resistance is becoming a serious problem in *Acinetobacter baumannii* worldwide. There are several reports of carbapenem resistance *Acinetobacter baumannii* which have been associated with factors such as the presence of metallo-β-lactamases, oxacillinases, mobile genetic elements, and reduced expression of outer membrane proteins (Fernandez *et al*., 2003; Limansky *et al*., 2002; Riccio *et al*., 2000; Takashi *et al*., 2000; Poirel *et al*., 2000; Bou *et al*., 2000; Costa *et al*., 2000; Clark, 1996).

In this study, 49 strains of *Acinetobacter* spp. were isolated between August 2003 and March 2004 from University Malaya Medical Center and confirmed biochemically to belong to the *Acinetobacter baumannii/calcoaceticus* complex. Out of which, 39 of them
were carbapenem resistant strains. Of the 49 strains, 30.6% were isolated from the ICU, 12.2% isolated from geriatric units, 12.2% from patients associated with bone marrow transplant (BMT), 10.2% from the paediatric units and 34.7% from the surgical wards. This suggests that these strains may be a pathogen among immunocompromised patients and may be circulating in the hospital setting. These strains are also suggested to be colonizers rather than direct pathogens as it was mostly non-invasive isolates (89.8%).

There is no single biochemical test that enables ready differentiation of this genus between similar bacteria such as *A. baumannii* and *A. calcoaceticus*. However, the wide biochemical activities of the members of the genus makes them readily distinguishable from other bacteria at the genus level by the combination of nutritional tests applied to these organisms using commercially available diagnostic devices and systems such as API 20NE and an automated Biolog system. However, these systems are laborious and time-consuming. Therefore, an early detection of these microorganisms in clinical samples and blood cultures can result in more definitive antimicrobial therapy. One of the methods of achieving this is by Fluorescent *in-situ* hybridization (FISH).

FISH is a suitable method for the rapid and specific detection of pathogenic bacteria in clinical samples without time-consuming cultivation. The entire assay of FISH took less than 2 hours as compared to the conventional laboratory methods which require 1 to 3 days resulting in a time gain of almost 70 hours. Besides that, this technique delivers additional information concerning cell count and cell morphology and is an *in-situ* means of differentiation of mixed infections. This could allow for an early detection of these microorganisms and thus more definitive antimicrobial treatment of the infected patients could be adjusted 1 or 2 days earlier. This in turn could reduce the overall mortality among patients with Gram-negative bacteremia as has been documented in previous studies (Leone *et al.*, 2003). FISH has proven to be a powerful molecular method for
identification, visualization, and quantification of organisms of interest in microbial communities (Amann et al., 1995). Several reports show that FISH has already been successfully applied for the detection of *E. coli*, *H. pylori*, *Staphlococcus aureus*, and *Brucella* spp. (Welinghausen et al., 2006; Samarbaf et al., 2006; Hartmann et al., 2005; Regnault et al., 2000). However, this is the first report on detection of *Acinetobacter* spp. and *Pseudomonas aeruginosa* using FISH in Malaysia (Wong et al., 2007).

Several studies have documented the value of molecular techniques, including PCR for amplification and detection of microbial DNA or RNA in order to identify bacteria in clinical specimens (Sabet et al., 2007; Yoshimasa, 2002). Although PCR is a highly sensitive technique that can be used in direct identification of bacteria in blood (Sabet et al., 2007; Yoshimasa, 2002), it may not be appropriate for daily routine work as it is expensive and requires costly reagents and instruments, such as the LightCycler. In contrast, FISH may be a more suitable technique as it is inexpensive and has little demands concerning technical skills. The low cost also makes FISH affordable in small laboratories, even in less industrialized countries. Furthermore, the added advantage of FISH over PCR is that extraction of DNA from bacteria is omitted in the former. However, in some cases as described by Poppert et al. (2005), FISH might not be recommended for the diagnosis of samples with very low bacterial load due to its low sensitivity. In such cases, real-time PCR has an advantage over FISH as it is highly sensitive and specific thus suggesting that FISH and PCR are equally important and powerful tools for the bacterial identification.

In general, the genus *Acinetobacter* comprises at least 19 genospecies (Towner, 1997; Ehrenstein et al., 1996). Seven of the 19 genospecies have been given formal species names while the others are designated by numbers (Towner, 1997; Ehrenstein et al., 1996). Six of the genospecies (*A. calcoaceticus*, *A. baumannii*, genospecies 3, ‘between 1 and 3’, 13, and ‘close to 13’) are very similar phenotypically and are known as the *Acinetobacter*
calcoaceticus – baumannii complex (Acb complex) eventhough they are genetically distinct (Vaneechoutte et al., 1995). Identification of Acinetobacter spp. to the genospecies level using phenotypic techniques is difficult especially to identify strains belonging to the Acinetobacter calcoaceticus – baumannii complex (Acb complex). Restriction analysis of bacterial 16S rRNA (16S rDNA) genes has proved useful in taxonomic studies to distinguish between species belonging to the same genus (Jayarao et al., 1992) and this technique is called as amplified ribosomal DNA restriction analysis (ARDRA) (Vaneechoutte et al., 1995). In this study, ARDRA was carried out to confirm the identification of all the 49 Acinetobacter spp. isolates to species level.

Beta-lactamases are the most common and most important mechanism of resistance to β-lactam antibiotics where they are capable of hydrolyzing the four members of β-lactam antibiotics including penicillins, cephalosporins, monobactam and carbapenems. These β-lactamases may be plasmid- or chromosomally-mediated (Livermore, 1996). Generally, bacteria hydrolyze β-lactam antibiotics by using β-lactamases in an acylation and deacylation based process. By inactivating peptidoglycan transpeptidases, members of the family of penicillin-binding proteins, β-lactam antibiotics have been successfully used for many years to inhibit the peptidoglycan synthesis responsible for the biosynthesis of the bacterial cell wall (Lee et al., 2001; Fre`re, 1995). β-lactamases can be divided into four classes (A, B, C, and D) according to their sequence similarities (Ambler, 1980). On the basis of their different catalytic mechanisms, two groups have been established whereby the Class B enzymes are metallo-β-lactamases that require zinc for their activity, and Class A, C, and D β-lactamases contain serine groups in their active site (Majiduddin et al., 2002). Oxacillinases are Ambler class D β-lactamases with hydrolytic
activity against penicillins, extended-spectrum cephalosporins, methicillin, and aztreonam (Ledent et al., 1993).

The enzymes characterized from Class A enzymes include NmcA, Sme1-3, IMI-1, KPC1-3, and GES-2. However, several researchers have showed that these genes were only found in other Gram-negative bacteria but not in Acinetobacter spp. (Poirel et al., 2001; Yigit et al., 2001; Queenan et al., 2000; Rasmussen et al., 1996; Nordmann et al., 1993; Yang et al., 1990). Therefore, in this study, the identification of Class A enzymes in Acinetobacter baumannii/calcoaceticus isolates obtained from University Malaya Medical Centre was not carried out. On the other hand, other β-lactamases from Class B, Class C, and Class D enzymes such as IMP and VIM, AmpC, and OXA-23 genes were identified, respectively.

Among the 39 carbapenem resistance strains of A. baumannii/calcoaceticus, 38 were detected positive for the presence of AmpC gene except one strain of A. calcoaceticus, S26 (metallo-β-lactamase-producer). The chromosomally encoded cephalosporinase, AmpC enzyme (Class C), is a naturally occurring β-lactamases and therefore it was found almost in all the strains studied. Regarding this, evidence has accumulated that these cephalosporinases are genetically related (Beceiro et al., 2004; Corvec et al., 2003; Mammeri et al., 2003; Bou and Martinez, 2000; Lopez et al., 2001; Perilli et al., 1996; Vila et al., 1993; Blechschmidt et al., 1992; Joly-Guillo et al., 1988). Furthermore, phylogenetic analysis suggests that this cephalosporinase should be placed in a unique subgroup among the Class C β-lactamases (Hujer et al., 2005). To date, there has been no evidence to indicate that the chromosomal cephalosporinase is inducible (Hujer et al., 2005). In addition to the Class C cephalosporinase of AmpC, other β-lactamases also have been reported in A. baumannii. These include the TEM-1 type (Bou et al., 2000; Vila
et al., 1993), SHV type (Huang et al., 2004; Bergogne-Bérézin and Towner, 1996),
CTX-M type (Nagano et al., 2004), PER-1 (Yong et al., 2003; Poirel et al., 1999;
Vahaboglu et al., 1997), and VEB-1 (Carbonne et al., 2005; Poirel et al., 2003)
β-lactamases. Although they are important, it is difficult to assess their impact on
resistance in the presence of the AmpC cephalosporinase. This is because most
Acinetobacters naturally carry two intrinsic types of β-lactamases which include AmpC
(Bou and Martinez-Beltran, 2000) and also OXA genes (Heritier et al., 2005). Bou and
Martinez-Beltran (2000) have reported that an AmpC-type cephalosporinase was expressed
at a basal level and showed no reduction in the efficacy of expanded-spectrum
cephalosporins. This is probably due to the presence of the insertion sequence ISAbal
upstream of the \( \text{bla}_{\text{AmpC}} \) gene which enhances β-lactamase expression by providing
promoter sequences and resulted resistance to cephalosporins but not to carbapenems
(Heritier et al., 2006).

The second intrinsic β-lactamase in \( A. \text{baumannii} \) is an oxacillinase which
represented by the OXA-51/69 variants (Heritier et al., 2005; Brown et al., 2005). These
genesis are chromosomally located in \( A. \text{baumannii} \) isolates and share very weak identities
with other known oxacillinases. To date, there are 11 variants of OXA-51 that have been
identified in isolates from diverse geographical origins (Brown and Amyes, 2006; Brown
and Amyes, 2005). Among these variants, the OXA-51 and OXA-69 β-lactamases have
been studied in detail to elucidate their carbapenemase activities (Heritier et al., 2005;
Brown et al., 2005). However, it seems that the level of expression of the corresponding
genesis is quite low in most cases and, even after it has been cloned into high copy number
plasmids in \( \text{Escherichia coli} \) and \( A. \text{baumannii} \), OXA-69 has only a marginal impact on
susceptibility to all β-lactams, including carbapenems (Hérityer et al., 2005).
Together with these naturally occurring β-lactamases, several acquired β-lactamases have been identified as a source of carbapenem resistance in A. baumannii. These enzymes belong either to the Class B metallo-β-lactamases defined by Ambler et al. (1999) or to the Class D oxacillinases (Naas and Nordmann, 1999). To date, metallo-β-lactamases which include IMP-like, VIM-like, SIM-1, SPM-1 and GIM-1 have been reported, but only the first three of these groups have been identified in A. baumannii strains (Poirel and Nordmann, 2006). Besides that, oxacillinases such as OXA-23 to OXA-27, OXA-40, and OXA-58 have been also reported to be associated with carbapenem resistance in Acinetobacter baumannii (Perez et al., 2007).

The main objective of my study was to characterize carbapenem-hydrolyzing enzymes among the A. baumannii/calcoaceticus strains and to investigate its mechanisms of resistance involved. Among the 39 carbapenem resistant strains, all were detected positive for the presence of blaOXA-23 gene except the two A. calcoaceticus strains, S26 and S90 (metallo-β-lactamase producers). This gene was not detected in these two strains probably due to the presence of another type of carbapenem-hydrolyzing enzymes, blaIMP-4 (metallo-β-lactamase) as dominant mechanism of carbapenem resistance in these organisms. Besides that, in this study, OXA-23 gene was present only if the strain was resistant to carbapenems. This suggests that besides IMP-4 genes, the OXA-23 genes are also associated with carbapenem resistance in A. baumannii/calcoaceticus. The first description of such OXA-23 carbapenemase was obtained from a clinical isolate of A. baumannii found in Scotland in 1985 before the introduction of carbapenems (Brown and Amyes, 2006). Since then, this plasmid-encoded enzyme, initially named ARI-1 (acinetobacter resistant to imipenem) has been discovered in England, Brazil, Polynesia, Singapore, Korea, and China (Brown and Amyes, 2006; Jeon et al., 2005). Other types of
Oxacillinases with carbapenemase activity have been also identified in different parts of the world which include the OXA-24 to -27, OXA-40, and OXA-58 gens (Poirel et al., 2005; Naas et al., 2005; Brown et al., 2005; Girlich et al., 2004; Bou et al., 2000; Afzal-Shah and Livermore, 1998). In contrast to the naturally occurring oxacillinases such as OXA-51/-69 variants, most of the new OXA-type carbapenemases lack hydrolytic activity against oxacillin, cloxacillin, and methicillin but they display resistance to carbapenems (Afzal-Shah et al., 2001; Bou et al., 2000). Based on their amino acid sequence, these OXA-type carbapenemases can be grouped into eight different clusters (Walther-Rasmussen and Høiby, 2006). Moreover, these enzymes have been seen to be widely dispersed in some clinically relevant species, such as Acinetobacter baumannii in which four groups have been identified: OXA-23, OXA-24, OXA-51, OXA-58, and their variants (Walther-Rasmussen and Høiby, 2006).

The other enzyme which encodes the carbapenemases besides oxacillinases is the metallo-β-lactamases from Class B. Two major metallo-β-lactamases have been reported in A. baumannii which include the IMP- and VIM-types. The IMP-type metallo-β-lactamases were first described in a strain of P. aeruginosa found in Japan in 1988 (Watanabe et al., 1991) and now had spread around the world in different genera. In A. baumannii, IMP-type metallo-β-lactamases are usually detected as part of a Class 1 integron, as first discovered in the Far East. Currently, the IMP-type metallo-β-lactamases consists of 19 variants that clustered in seven phylogroups (Walsh et al., 2005). Six IMP variants which include IMP-1, -2, -4, -5, -6, -11 belonging to three different phylogroups have been identified in A. baumannii (Walsh et al., 2005; Gales et al., 2003; Da Silva et al., 2002; Chu et al., 2001; Takashi et al., 2000; Cornaglio et al., 1999). In addition, recently, IMP-4 has been identified in an Acinetobacter junii clinical isolate from Australia
(Peleg et al., 2006). VIM-type metallo-β-lactamases (Verona integron-encoded MBL), VIM-1 was first identified in Italy in 1997 in a *P. aeruginosa* isolate (Lauretti et al., 1999). However, VIM-type metallo-β-lactamases are rarely found in *A. baumannii* being represented only by VIM-2 reported in South Korea (Yum et al., 2002). This *bla*VIM-2 gene was found to be located on 2 newly described integrons (Class I integrons *In105* and *In106*) (Yum et al., 2002). Besides that, analysis of the genetic surroundings of the metallo-β-lactamases encoding genes identified in *A. baumannii* has revealed very similar structures since the *bla*IMP, and *bla*VIM genes are embedded in Class 1 integrons (Houang et al., 2003; Yum et al., 2002). The metallo-β-lactamase genes form part of the gene cassettes that are inserted between the 5’-conserved segment (5’-CS) and the 3’-conserved segment (3’-CS), together with other antibiotic resistance gene cassettes, mostly encoding aminoglycosidemodifying enzymes. In addition, the plasmid location of the metallo-β-lactamase genes explains their spread among *A. baumannii* and *P. aeruginosa* strains in specific region such as Italy and Korea (Poirel and Nordmann, 2006).

In a retrospective studies on resistant isolates collected as early as 1994 in Hong Kong and 1995 in Canada, it was determined that the carbapenem resistance was due to IMP-7 (*P. aeruginosa*) in Canada (Gibb et al., 2002) and IMP-4 (*A. baumannii*) in Hong Kong (Chu et al., 2001). The MBL gene *bla*IMP-4 was detected in 66 % of imipenem-resistant strains collected between 1994 and 1998 at the Prince of Wales hospital in Hong Kong (Chu et al., 2001). IMP-4 had 10 amino acids different from IMP-1 and 37 amino acids different from IMP-2. The *bla*IMP-4 was harboured on a plasmid and integron encoded along with three other resistance genes (*qacG2*, *aacA4*, and *catB3*) (Houang et al., 2003). *Acinetobacter* strains harboring IMP-4 were found in 1997 and 1998 at a prevalence of ~14% of all *Acinetobacter* strains and disappeared in 1999, but the reasons for this is not
clear (Houang et al., 2003). The blaIMP-4 was subsequently found in Citrobacter youngae and P. aeruginosa isolates from Guangzhou in mainland China. Guangzhou is close to Hong Kong, which suggests local dissemination of this IMP allele (Hawkey et al., 2001). IMP-4 has now been found in Australia in Escherichia coli, Klebsiella pneumoniae, and P. aeruginosa, possibly “imported” from Southeast Asia (Peleg et al., 2004; Poirel et al., 2004).

Concurrent with these findings, this study showed that out of these 39 carbapenem-resistant isolates of Acinetobacter baumannii/calcoaceticus, two strains were found to harbour a blaIMP-4 metallo-β-lactamases which were inhibited by EDTA and the remaining 37 were positive for blaOXA-23 gene. However, none of the isolates harboured blaVIM gene. The susceptibility testing using the agar dilution method showed that the metallo-β-lactamase producing strains, S26 and S90 generally had higher MICs to all the antibiotics tested compared to the non-metallo-β-lactamases, with higher MICs to meropenem than imipenem. Further analysis of the IMP-4 producing MBL strains showed that both enzymes had similar kinetic properties and isoelectric points with the exception of an additional band in strain S90 as shown in Chapter 3 (Figure 3.12). From the kinetics data, the IMP-4 enzyme from S26 appeared to be slightly more active compared to the enzyme from S90. Both enzymes had higher hydrolyzing activity towards meropenem than imipenem, which correlated with the MICs obtained. However these findings are in contrast with a previous study by Chu et al. (2001), whereby the IMP-type metallo-β-lactamases were shown to hydrolyze imipenem more rapidly than meropenem. In addition, in this study, the V_max for both these enzymes was higher for oxacillin than any of the other antibiotics tested which is in accordance with the findings of Afzal-Shah et al. (2001).
With regard to β-lactamases, integrons are mobile DNA elements with the ability to capture genes, particularly those encoding antibiotic resistance, by site-specific recombination (D’Agata, 2004; Nordmann and Poirel, 2002; Navia et al., 2002; Poirel et al., 2002; Poirel et al., 2001; Vila et al., 1997). Integrons have an integrase gene (int), a nearby recombination site (attI), and a promoter (Hall and Collis, 1998). There are at least three classes of integrons based upon the type of integrase gene they possess. Class 1 integrons have been examined the most extensively. They consist of a variable region bordered by 5'- and 3'- conserved regions. The 5'- region is made up of the int gene, attI, and the promoter which drives transcription of genes within the variable region. The 3'- region consists of an ethidium bromide resistance locus (qacED1), a sulfonamide resistance gene (sulI), and an open reading frame containing a gene of unknown function. The integrase of Class 2 integrons is located within the 3’ conserved region. Class 3 integrons have yet to be thoroughly studied. Integrons are widely distributed among clinical isolates of Acinetobacters. There have been several studies that have shown the association of antibiotic resistance, particularly aminoglycoside resistance with integrons (Seward and Towner, 1999; Gonzalez et al., 1998). In this study, among the 39 carbapenem resistant strains of A. baumannii and A. calcoaceticus, 31 strains including the two A. calcoaceticus, S26 and S90 (metallo-β-lactamase producers) were detected positive for the presence of Class 1 integron. PCR amplification and subsequent nucleotide sequence analysis revealed the presence of aacC1 and aadA1a resistance genes in integron profiles 1 and 2 (genotypes B-J, L) which confer resistance to aminoglycosides as shown in Chapter 3 (Table 3.8). The integrons in both S26 and S90 strains however, contained a blaIMP-4 allele along with the aacA4 and catB3 alleles, both of which encode for resistance to aminoglycosides. In this study, the blaIMP-4 metallo-β-lactamase gene was identified
within an integron that was located on a 50 kb plasmid in a clinical isolate of 
*A. calcoaceticus*. This is the second report of this kind, the first being in 2006 by Liu *et al.*, who reported a *bla*<sup>IMP-1</sup> in a plasmid-borne Class 1 integron in a clinical isolate of 
*A. baumannii*. Although identical cassette arrays were found in integrons of the IMP-4 
producing strains, PFGE analyses showed that S26 and S90 had different genotypes as 
shown in Chapter 3 (Table 3.8, Figure 3.11). These results suggest that carbapenem 
resistance in these strains of *A. calcoaceticus* could have been acquired via horizontal gene 
transfer which is concurrent with the findings of Gombac *et al.* (2002).

Generally, in Gram-negative bacteria, the efflux pump especially the resistance-
nodulation-cell division (RND) type has been demonstrated to be responsible for a wide 
range of antibiotic resistance (Marchand *et al.*, 2004; Magnet *et al.*, 2001; Poole *et al.*, 
2000; Li *et al.*, 1994). The efflux system as a mechanism of drug resistance that was first 
described in 1980 for tetracycline (McMurry *et al.*, 1980) and at that time was considered a 
curiosity. Today, efflux pumps are believed to contribute significantly to both intrinsic and 
acquired bacterial resistance because of the wide range of substrates they recognize. In 
addition, efflux pumps commonly are expressed in important pathogens, have synergistic 
effect with other resistance mechanisms, and play a role in the development of resistance. 
Magnet *et al.* (2001) had shown the AdeABC efflux pump which consist of *adeA, adeB,* 
*adeC* with two-component regulatory genes (*adeR* and *adeS*) to contribute to multidrug 
resistance in *Acinetobacter baumannii* including fluoroquinolones, tetracyclines, 
chloramphenicol, erythromycin, trimethoprim, and ethidium bromide. However, there is still 
a lack of information on efflux-mediated carbapenem resistance in *Acinetobacters*. Bou *et al.* (2000) has reported no differences in MICs in the carbapenem resistant *A. baumannii* 
isolates from Spain, suggesting no efflux mechanisms were involved. This finding was 
supported by Quale *et al.* (2003) which reported an efflux pump mediated resistance in
A. baumannii strains that did not appear to contribute to carbapenem resistance. Lately, Sinha et al. (2007), has reported that in their study, only two isolates obtained from one patient revealed efflux pump as a possible cause for carbapenem resistance. However, the author has only screened 9 isolates in total and demonstrated comparison of MICs assays using reserpine as efflux pump mechanism. The author also suggested screening of more isolates was essential in order to conclude that the efflux pump has a role in carbapenem resistance. To date, this is the only most recent publication that has discussed the role of efflux pumps in the development of carbapenem resistance in Acinetobacters. In the present study, an experiment was conducted to study the contribution of AdeABC efflux pump to carbapenem resistance in Acinetobacter spp. Screening of the adeA, adeB, adeC, adeR, and adeS genes in a total of 39 carbapenem resistance strains showed that 36 were positive for the presence of adeA, adeB, adeR, and adeS gene. However, for the adeC gene, only 34 strains harboured this gene. Disruption of ade gene individually resulted in 1 to 6 fold lower susceptibility for all the antibiotics including ceftazidime, cefotaxime, gentamicin, amikacin, ciprofloxacin, amoxicillin and also meropenem as shown in Chapter 3 (Table 3.9). However, no differences were observed in the MICs values of imipenem for all the transformants. This shows that resistance to meropenem has an effect on efflux pumps as compared to imipenem. This is probably due to meropenem utilizing a different mechanism and probably depends on upregulation of efflux pump as suggested by Livermore, (2001). Among the adeA, adeB, adeC, adeR, and adeS genes, only mutants derived from adeC insertion-inactivation resulted in no differences in antimicrobial susceptibility. This indicates that the adeC gene was not responsible for antimicrobial resistance to the above mentioned antibiotics in these organisms. Therefore, this finding suggests that the AdeAB pump probably may utilize other proteins such as outer membrane protein as suggested by Marchand et al. (2004).
Besides the involvement of antibiotic resistance in efflux pumps, several studies have reported the modification of outer membrane permeability (OMP) as another mechanism of resistance in *A. baumannii* (Limansky *et al.*, 2002; Bou *et al.*, 2000; Clark, 1996). Understanding the contribution of porins or outer membrane proteins (OMPs) to antibiotic resistance in *A. baumannii* has been a particular challenge. Unfortunately, it is difficult to accurately compare the loss of OMPs as there is variability in the number of observed OMPs (Fernandez-Cuenca *et al.*, 2003). In Japan, in the year 1991, Sato and Nakae have reported decreased band intensity of two outer membrane proteins with 44.5- and 46.5-kDa in *Acinetobacter calcoaceticus* isolates. Then, after 5 years, in 1996, in United States, Clark has reported reduced expression of a 33- to 36-kDa outer membrane protein in imipenem resistant *Acinetobacter baumannii*. This similar finding but with the loss of 31- to 36-kDa outer membrane protein was also reported in Brazil by Costa *et al.* (2000). Later, in the year 2002, in Argentina, Adriana *et al.*, has reported loss of a 29-kDa outer membrane protein in *Acinetobacter baumannii*. Most of these previous studies have only evaluated the role and function of outer membrane protein in carbapenem resistance *Acinetobacters* as one mechanism. There were another two more similar findings but with multiple mechanisms reported by Fernandez-Cuenca *et al.* (2003) from Spain and Siroy *et al.* (2005) from France. Fernandez-Cuenca *et al.* (2003) has shown the relationship between outer membrane protein and other carbapenem resistance mechanisms which include presence of metallo-β-lactamases, oxacillinases, and also increased expression of Class C cephalosporinases in *Acinetobacter baumannii*. Siroy *et al.* (2005) have described the channel formation of CarO, a 29-kDa OMP which confers resistance to both imipenem and meropenem in *A. baumannii*. In the study carried out by Bou *et al.* (2000) reported a loss of 22- and 33-kDa OMPs with the production of OXA-24 which resulted in resistance
to carbapenems was reported. Recently, a 43-kDa protein in *A. baumannii* was identified as a homologue of OprD; a well-studied porin which frequently associated with imipenem resistance in *P. aeruginosa* (Dupont *et al.*, 2005). In this study, the loss of 29-kDa OMP was found in all the 39 carbapenem resistance strains studied as shown also by Siroy *et al.* (2005). In addition to loss of 29-kDa OMP, all of these strains also had multiple mechanisms which include the presence of IMP-4, OXA-23, AmpC, and also AdeAB efflux pumps. Reports on loss of OMPs together with the production of beta-lactamases, have illustrated the interplay of several different mechanisms of resistance against one class of antibiotics particularly carbapenems. Therefore, establishing the relative contribution of the action of β-lactamases, β-lactam penetration through OMPs, interaction with other mechanisms of resistance, and the control of their expression presents formidable challenges. Regarding to these aspects, an additional experiment was carried out to investigate potential targets for antimicrobial therapy. This was done by raising polyclonal antibodies against specific outer membrane protein called Iron-regulated Outer Membrane Proteins (IROMPs) which also had a loss of 29-kDa OMP. In this experiment, *A. calcoaceticus* strain, S26 overexpressed IROMPs with molecular weight of 45- to 50-kDa when the strain was grown in iron deficit medium. Polyclonal antibodies raised against these IROMPs specifically killed or inhibited the bacteria grown in iron deficit medium, suggesting that these antibodies have bactericidal effect. In the presence of iron, however, the polyclonal antibodies did have the bactericidal effect on *Acinetobacter calcoaceticus* growth, but after 2 hours, they appeared to have recovered from this temporary effect probably due to essential nutrient obtained in the medium by the bacteria for its survival. Probably, as described by Goel and Kapil (2001), blocking the iron uptake pathways of the bacteria might reduce the uptake of iron, thus inhibiting the growth of the
bacterium. Therefore, this could be a good target for antimicrobial therapy as antibodies produced against IROMPs may have a role as protective antibodies in the course of infection in human host.

In conclusion, the increasing trend of carbapenem resistance in *Acinetobacters* especially *Acinetobacter baumannii* worldwide is a concern since it limits drastically the range of therapeutic alternatives. Polymixins which include colistin and polymyxin B could be used to treat *Acinetobacter* infections. However, the choice of polymixin therapy is further complicated by the toxicity of colistin. Metallo-β-lactamases, namely IMP and VIM have been reported worldwide, especially in Asia and western Europe, and confer resistance to all β-lactams. In this study, among the 39 strains, only two strains of *A. calcoaceticus*, S26 and S90 harbored the *bla*<sub>IMP-4</sub> gene, whereas the remaining 37 carbapenem resistance strains of which 1 *A. calcoaceticus*, and 36 *A. baumannii* strains harbored the *bla*<sub>OXA-23</sub> gene. This suggests that the most widespread β-lactamases with carbapenemase activity found in *A. baumannii* are carbapenem-hydrolysing Class D β-lactamases that are mostly specific for this species. These enzymes generally belong to three unrelated groups of clavulanic acid-resistant β-lactamases, represented by OXA-23, OXA-24 and OXA-58, that can be either plasmid- or chromosomally-encoded (Poirel *et al.*, 2005). Although Class I integrons are widely distributed among clinical isolates of *A. baumannii/calcoaceticus*, they do not seem to play a major role in the dissemination of carbapenem resistance as integron associated imipenem resistance was only detected in 2 of *A. calcoaceticus* strains, S26 and S90 out of the 39 clinical strains. However the location of the integron-borne *bla*<sub>IMP-4</sub> gene on a plasmid is cause for concern as previous studies have demonstrated the ease of transmission of carbapenem resistance through plasmid conjugation (Hawkey *et al.*, 2001). Thus, this study indicates that precautionary monitoring of integron-associated *bla*<sub>IMP-4</sub> in clinical strains of *Acinetobacter calcoaceticus* should be
carried out. In addition to the presence of β-lactamases and Class I integrons, carbapenem resistance in *A. baumannii/calcoaceticus* may also result from porin or penicillin-binding protein modifications. Several porins, including the 29-kDa CarO protein, that constitute a pore channel for influx of carbapenems, might be involved in carbapenem resistance (Siroy *et al.*, 2005). However in this study, all the 39 carbapenem resistant strains of *A. baumannii/calcoaceticus* showed loss of 29-kDa outer membrane protein. In addition, polyclonal antibodies raised against IROMPs showed that these antibodies have bactericidal effect on this organism, suggesting this could be a potential target for antimicrobial therapy. Besides the loss of 29-kDa outer membrane protein, reports on efflux pump, especially gene knockout of *adeB* gene in carbapenem resistance strains had led to decreased MICs level to meropenem, suggesting the contribution of this gene in carbapenem resistance in *Acinetobacter baumannii/calcoaceticus*. However, it will be an important approach in the near future if one attempts developing efflux inhibitors as possible development of new agents to control antimicrobial resistance in nosocomial pathogens such as *Acinetobacters*. Hence, an understanding of carbapenem resistance mechanisms might be crucial for the development of novel therapeutic strategies as it is likely that multiple mechanisms involved for carbapenem resistance in *Acinetobacter baumannii/calcoaceticus*. 
References


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Appendix

Gram staining procedure

1) **Crystal violet**
   Solution 1: Crystal violet 2 g
   Ethanol 95 % 20 ml
   Solution 2: Ammonium oxalate 0.8 g
   Distilled water 80 ml
   - Mix solution 1 and 2, and allow to stand for at least 24 hours, and filter before use.

2) **Lugol’s Iodine**
   Potassium iodide 2 g
   Iodine 1 g
   Distilled water 300 ml

3) **Diluted carbol fuschin**
   Stock solution: Safranine O 2.5 g
   Ethanol 95 % 100 ml
   
   Working solution: Stock solution 10 ml
   Distilled water 90 ml

Standard biochemical reagents

**Catalase reagent:**
Hydrogen peroxide 3 %
- Store in refrigerator at 4°C.

**Mc Farland 0.5 Turbidity Standard:**
0.048 M BaCl₂ 0.5 ml
0.35 M NH₂SO₄ 99.5 ml

**Blood Agar**
Tryptone / peptic digest of animal tissue 10 g
Brain heart infusion 500 g
Sodium chloride 5 g
Agar 15 g
Distilled water 1000 ml
- Sterilize by autoclaving.
- Cool media to 45-50°C, then add preheated (45°C) sterile blood.
Mueller Hinton Agar:
Beef infusion 300 g
Casein 17.5 g
Starch 1.5 g
Agar 17 g
Distilled water 1000 ml
- Sterilize by autoclaving.
- Blood may be added as described for blood agar.

Brain Heart Infusion (BHI) broth:
Calf Brain infusion 12.5 g
Beef heart infusion 5 g
Proteose peptone 10 g
Glucose 2 g
Sodium chloride 5 g
Disodium phosphate 2.5 g
Distilled water 1000 ml
- Sterilize by autoclaving.

Luria broth:
Tryptone 10 g
NaCl 10 g
Yeast Extract 5 g
- Sterilize by autoclaving.
* For making solid LB agar for plating, 15 g/L of agar is added to the above mixture.

Saline
Sodium chloride 0.9 g
Distilled water 100 ml
- Sterilize by autoclaving.

Tris-acetate EDTA (TAE) buffer (50X):
Tris base 242 g
Glacial acetic acid 57.1 ml
EDTA 18.61 g
Distilled water 1000 ml
- Sterilize by autoclaving.

Tris-borate EDTA (TBE) buffer (10X):
Tris base 121.2 g
Boric acid 61.8 g
EDTA 0.745 g
Distilled water 1000 ml
- Sterilize by autoclaving.
**Tris-EDTA (TE) buffer:**

1 M Tris-HCl, pH8.0  
1ml (final concentration = 10mM)

0.5 M EDTA  
200 µl (final concentration = 1mM)

Distilled water  
100ml

- Sterilize by autoclaving.

**0.5 M EDTA, pH7.0, 7.5, or 8.0**

Disodium EDTA.2H₂O  
18.61 g

Distilled water  
60 ml

- The pH is adjusted as desired with 1 M NaOH
- Top up with distilled water to 100 ml
- Sterilize by autoclaving.

* EDTA will not dissolve completely until the pH is adjusted with NaOH

**pH7.0**

1 M NaOH

NaOH  
4 g

Distilled water  
100 ml

- Sterilize by autoclaving.

**10 mM Tris-HCL, pH 7.5**

Tris base  
0.12 g

Distilled water  
80 ml

- The pH is adjusted as desired with concentrated HCL
- Top up with distilled water to 100 ml
- Sterilize by autoclaving.

**Phenol-chloroform mixture**

1) *Phenol* :

- water saturated redistilled phenol  
100 ml
- 0.5 M Tris-HCL pH8.0 containing 1mM EDTA  
100 ml
- Mix well and allow the layers to separate
- Remove upper aqueous phase and repeat step 2 and 3 once more
- 10 mM Tris-HCL pH8.0 containing 1 mM EDTA  
100 ml
- Mix well and layers to separate
- Remove upper aqueous phase and repeat step 5 and 6 once more
- 10 mM Tris-HCL pH8.0  
100 ml
- Store at 4°C in the dark

2) 24 :1 (v/v) chloroform-isoamyl alcohol

- Chloroform  
96 ml
- Isoamyl alcohol  
4 ml
- Store in a capped bottle.
3) Phenol-chloroform mixture
   - Lower phase of the phenol solution 5 ml
   - 24 :1 (v/v) chloroform–isoamyl alcohol 5 ml
   - TE, pH8.0 10 ml
   - Store at 4°C in the dark. Use the lower organic layer.

Phosphte-buffered saline (PBS)
NaCl 10 g
KCL 0.25 g
Na₂HPO₄ 1.43 g
KH₂PO₄ 0.25 g
   - Adjust to pH7.3
   - Distilled water 1000 ml
   - Sterilize by autoclaving.

Sodium dodecyl sulphate (SDS)
Sodium dodecyl sulphate 10 g
Distilled water 100 ml
Reagents and buffers for outer membrane assay

10X Sodium phaospbate buffer
NaH$_2$PO$_4$  25.0 g
Na$_2$HPO$_4$  59.3 g

Laemmli buffer
10 % (v/v) glycerol
5 % (v/v) 2- mercaptoethanol
3 % (v/v) sodium dodecyl sulphate
0.0625 M Tris-HCL, pH6.8
- Dissolved in double deionised water and stored at 4°C.

Protein reagent
Coomassie Brilliant Blue G-250  1.0 g
Ethanol (95 %)  47 ml
Phosphoric acid (85 %)  85 ml
- Dissolved Coomassie Brilliant Blue G-250 in ethanol and phosphoric acid and
  top up to 1L with double deionised water.
- Kept in dark bottle and stored at 4°C.

2 % Triton-X-100-10 mM phosphate buffer, pH7.2
Triton-X-100  20 ml
10mM phosphate buffer  980 ml
- Mixed well and stored at room temperature.

Acrylamide / Bis monomer solution (30 %T, 2.67 % Bis)
Acrylamide  58.4 g
Bis acrylamide  1.6 g
- Dissolved in double deionised water and the volume was made up to 200 ml.
- Filter sterilized and stored at 4°C up to one month only.

1.5M Tris-HCl, pH8.8
Tris base  18.171 g
Double deionised water  70 ml
- Dissolved in double deionised water and adjusted the to pH8.8 with
  concentrated hydrochloric acid.
- Top up the solution to 100 ml with double deionised water and stored at 4°C.

0.5M Tris-HCl, pH6.8
Tris base  6.057 g
Double deionised water  70 ml
- Dissolved in double deionised water and adjusted the to pH8.8 with
  concentrated hydrochloric acid.
- Top up the solution to 100 ml with double deionised water and stored at 4°C.
Coomassie blue staining solution
Coomassie Brilliant Blue R-250  0.1 g
Methanol  40 ml
Acetic acid  10 ml
- Dissolved Coomassie Brilliant Blue R-250 in methanol and acetic acid
- Top up the solution to 100 ml with double deionised water and stored at room temperature in dark bottle.

Destaining solution
Methanol  40 ml
Acetic acid  10 ml
- Top up the solution to 100 ml with double deionised water and stored at room temperature.

4X Loading dye
1 M Tris pH 6.8  0.6 ml
10 % SDS  4.0 ml
100 mM EDTA pH 7.0  0.1 ml
DTT  0.4 g
100 % glycerol  2.4 ml
1 % Bromo Phenol Blue in saturated urea  1.0 ml

Electrophoresis buffer
Tris  18.0 g
Glycine  14.4 g
10 % SDS  60 ml
- Top up to 6L with double deionised water and stored at room temperature.
Reagents and buffer for plasmid DNA isolation

Solution I
50 mM Tris-HCl (pH 8.0)
10 mM EDTA (pH 8.0)
- Dissolved in distilled water and sterilized by autoclaving.

Solution II
0.2 M NaOH
1% SDS
- The solution should be freshly prepared.

Solution III
5M potassium acetate 60 ml
Glacial acetic acid 11.5 ml
Distilled water 28.5 ml

70 % Ethanol
Distilled water 30 ml
Absolute ethanol 70 ml
Reagents and buffer for Southern Blot Hybridization

Prehybridization solution
6X SSC
0.05X BLOTTO
50 % Formamide

*1X BLOTTO
5 % Non-fat dried milk + 0.02% Sodium Azide, and dissolve in water

Wash Buffer 1
2X SSC
0.1 % SDS

Wash Buffer 2
0.2X SSC
0.1 % SDS

Wash Buffer 3
0.16X SSC
0.1 % SDS

Buffer 1
0.1 M Tris-HCl (pH7.5)
0.15 M NaCl

Buffer 2
3 % (w/v) BSA in buffer 1
3 g BSA in buffer 1

Buffer 3
0.1 M Tris-HCl (pH9.5)
0.1 M NaCl
50 mM MgCl₂

Tris-EDTA
20 mM Tris-HCl (pH7.5)
0.5 mM Na₂EDTA

- Filter the solutions to reduce background, except the 5 % SDS.
Reagents and buffer for Fish in situ Hybridation (FISH)

Fixing buffer
4 % Formaldehyde
96 % Ethanol

Hybridization buffer
20 mM Tris-HCl (pH7.2)
0.9 M NaCl
0.1 % SDS

Washing buffer
20mM Tris-HCl (pH7.2)
0.9 M NaCl

Probe preparation
Probe stock = 1mg/ml
[Probe] used = 10ng/ml
Reagents and buffer for Pulsed-field gel electrophoresis (PFGE)

Sodium Buffer (SB)
10 mM Tris, pH7.5
1 M NaCl
- Dissolved in distilled water and sterilized by autoclaving.

Lysis solution
10 mM Tris, pH7.6
50 mM NaCl
100 mM EDTA, pH7.6
0.2 % sodium deoxycholate
0.5 % sarcosyl
0.5 % Brij-58
- Dissolve in distilled water and sterilized by autoclaving.
- Before use, 500 µg/ml lysozyme and 2 µg/ml RNase were added into the solution.

ESP
0.5 M EDTA, pH8.0
1 % sarcosyl
- Dissolved in distilled water and 1 mg/ml Proteinase K was added in when the solution was used.

Tris-EDTA
10 mM Tris
1 mM EDTA, pH8.0
- Dissolved in distilled water and sterilized by autoclaving.

Digestion buffer mixtures
10X RE buffer 8.0 µl
Bovine serum albumin (100 mg/ml) 0.8 µl
Sterile distilled water 71.2 µl

1X TBE buffer
Tris base 10.77 g
EDTA 0.93 g
Boric acid 5.52 g
- Dissolved in distilled water and sterilized by autoclaving.

10 µg/ml ethidium bromide
Ethidium bromide 1 mg
Distilled water 100 ml
- Kept in dark bottle.
SCIENTIFIC MEETING ABSTRACTS & PUBLICATIONS

Publications:

1. **Wong Eng Hwa**, Geetha Subramaniam, Parasakthi Navaratnam and Shamala Devi Sekaran. Fluorescent in-situ Hybridization Assay for the detection of Non-Enterobactericeae in blood culture samples *(Accepted for Publication – Indian Journal of Medical Microbiology)*

2. **Wong Eng Hwa**, Geetha Subramaniam, Parasakthi Navaratnam, and Shamala Devi Sekaran. Detection and characterization of plasmid mediated Class 1 Integron among IMP-4 producing and non-producing carbapenem resistant *Acinetobacter* spp. in University Malaya Medical Centre (UMMC), Malaysia. *(Accepted for Publication - Journal of Microbiology, Immunology and Infection, Taiwan)*


Posters presented:

1. **Wong Eng Hwa**, Geetha Subramaniam, Shamala Devi Sekaran and Parasakthi Navaratnam. “Detection of IMP Metallo-β-lactamases in Multiresistant Acinetobacter spp. isolated in University Malaya Medical Centre” at the 5th Annual Scientific Meeting of the College of Pathologist, AMM July 2004, held at Damai Laut, Lumut, Perak.

2. **Wong Eng Hwa**, Geetha Subramaniam, Shamala Devi Sekaran and Parasakthi Navaratnam. “Molecular Epidemiology and Characterization of IMP-4 Metallo-Metallo-β-lactamases in Multiresistant Acinetobacter spp. isolated in University Malaya Medical Centre” Presented at the 9th Western Pacific Congress on Chemotherapy and Infectious Disease, at Bangkok, Thailand (1 – 5th December, 2004)


Oral presentation:

1. **Wong Eng Hwa**, Geetha Subramaniam, Parasakthi Navaratnam, Shamala Devi Sekaran. The role of AdeABC efflux pump in multidrug resistant *Acinetobacter* spp. isolated from University Malaya Medical Centre (UMMC), Malaysia. Biological Graduates Science Conference, 17-19th December, 2007, University of Malaya, Malaysia.
DETECTION OF IMP METALLO-β-LACTAMASES IN MULTIRESISTANT ACINETOBACTER SP. ISOLATED IN UNIVERSITY MALAYA MEDICAL CENTER.

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Introduction
Carbapenem-resistant Acinetobacter sp. have gained increasing significance as opportunistic pathogens in hospitalized patients. Reports of multidrug resistant isolates have increased considerably during the last decade and may partly be due to the extensive use of broad-spectrum antibiotics (Amyes et al., 1996). Carbapenem resistance is often associated with the loss and/or decrease in outer membrane protein and overexpression of multidrug efflux systems. However, carbapenem-hydrolyzing β-lactamases of Ambler Class B (metallo-enzymes) and Ambler Class D (oxacillinases) have also been detected in Acinetobacter sp. In this study, we describe the screening of carbapenem-resistant Acinetobacter sp. for the presence of carbapenemases.

Methodology
A total of 40 carbapenem-resistant clinical isolates of Acinetobacter sp. were obtained from inpatients of University Malaya Medical Center (UMMC) from August 2003 until March 2004. The identity of the isolates was confirmed using the API20NE system. Growth at 44°C was performed to differentiate between A. baumannii and A. calcoaceticus. The antibiotic susceptibility profiles to β-lactams were determined by the minimum inhibitory concentration (MIC) using the agar dilution method as described by the NCCLS. ATCC strains of Escherichia coli 25922 and Pseudomonas aeruginosa 27853 were used as controls. The antimicrobials used included imipenem, meropenem, ceftazidime, cefotaxime, and aztreonam. Preliminary screening for carbapenemase production was carried out using the Modified Hodge, EDTA and 2MPA double disc synergy tests (Lee et al., 2001; Arakawa et al., 2000). The isolates were then analysed for the presence of the blaIMP gene using PCR (Yum et al., 2002). The 448bp PCR products from blaIMP-positive isolates were sequenced to confirm the identity of the amplified products.

Results and Discussion
Among the 40 imipenem resistant strains, 36 were identified as A. baumannii whereas only 4 were identified as A. calcoaceticus. Only 3 strains out of the 40 strains were positive for metallo-beta-lactamase production using the double disc synergy and Modified Hodge tests. All 3 strains were A. calcoaceticus. However, 6 other isolates were characterized as equivocal by the Modified Hodge test although they were negative for metallo-beta-lactamase production using the double disc synergy test.
PCR amplification of the $bla_{IMP}$ gene was positive in the 3 $A.\ calcoaceticus$ isolates that were positive by both the Modified Hodge and double disc synergy tests. Preliminary sequencing data obtained from the 3 PCR products showed a 100% homology with $bla_{IMP}$-4. Amplification and sequencing of the entire $bla_{IMP}$ gene is being carried out to confirm the finding.

**Conclusion**

Although carbapenem resistance in *Acinetobacter* sp. in UMMC is a increasing problem, our findings indicate that only 7.5% of the isolates harboured the $bla_{IMP}$ gene. Screening for the presence of $bla_{VIM}$ has to be carried out to detect other carbapenemases. The findings suggest that the resistance to extended spectrum β-lactams in *Acinetobacter* sp. is predominantly due to non-enzymatic mechanisms such as altered permeability and efflux systems. Further studies have to be carried out in order to confirm this hypothesis. Strains classified as equivocal by Modified Hodge test but negative by double disc synergy test, may be low level producers of metallo-beta-lactamases. This is concurrent with the findings of Lee *et al.* (2001) that showed that the EDTA-disc synergy test is more specific for the detection of metallo-enzymes.
Presented at the 9th Western Pacific Congress on Chemotherapy and Infectious Disease, at Bangkok, Thailand (1 – 5th December, 2004).

Molecular Epidemiology and Characterization of IMP-4 Metallo-Beta-Lactamases in Multiresistant Acinetobacter spp. Isolated In University Malaya Medical Centre

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Background: Carbapenem-resistant Acinetobacter spp. have emerged as significant opportunistic pathogens in hospitalized patients. Such resistance is often associated with the loss and / or decrease in outer membrane protein and overexpression of multidrug efflux systems or production of beta-lactamases.

Objectives: In this study, we describe the molecular epidemiology and characterization of metallo-beta-lactamases in carbapenem-resistant strains of Acinetobacter spp.

Methods: A total of 40 carbapenem-resistant clinical isolates of Acinetobacter spp. were obtained from inpatients of University Malaya Medical Centre (UMMC) from August 2003 until March 2004. The identity of the isolates was confirmed using the API20NE system. Growth at 44°C was performed to differentiate between A. baumannii and A. calcoaceticus. The antibiotic susceptibility profiles to imipenem, meropenem, ceftazidime, cefotaxime, and aztreonam were evaluated by MIC determination using the agar dilution method (NCCLS). Preliminary screening for carbapenemase production was carried out using the Modified Hodge Test (Lee et al., 2001) and double disc synergy test (Arakawa et al., 2000). The isolates were then analysed for the presence of the blaIMP gene using PCR. Primers were designed flanking the entire coding region of blaIMP gene. The 795bp PCR product from blaIMP positive isolates were sequenced. Molecular epidemiology using pulsed-field gel electrophoresis was carried out to analyze the genomic profiles. The restriction enzyme use was ApaI.

Results: 36 isolates were A. baumannii and only 4 were A. calcoaceticus. By the metallo-enzyme screening test, only 3 of the strains, all A. calcoaceticus, were positive for metallo-beta-lactamase production. PCR amplification of the blaIMP gene was positive in these 3 strains. Preliminary sequencing data obtained from the PCR products showed a 100 % homology with blaIMP-4. The PFGE profiling showed that 2 of the blaIMP positive strains had an identical pattern and were clonally related.

Conclusions: Carbapenem resistance in Acinetobacter spp. in UMMC is an increasing problem, and our findings indicate that only 7.5 % of the isolates harboured the blaIMP gene. These findings suggest that the resistance to extended spectrum beta-lactams in Acinetobacter spp. isolated from UMMC is predominantly due to non-enzymatic mechanisms such as altered permeability and efflux systems.
Detection and characterization of plasmid mediated Class 1 Integron among IMP-4 producing and non-producing carbapenem resistance Acinetobacter spp. in University Malaya Medical Centre (UMMC), Malaysia.

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Background: The understanding of microbial resistance to the β-lactam class of antibiotics in the form of β-lactamases has come a long way since the early discoveries of narrow-spectrum penicillinases. Integron-borne β-lactamases co-occurring with a wide array of β-lactam resistance genes, particularly pose an increasing threat to the nosocomial environment, giving rise to multi-drug resistant microbes with complex resistance patterns. In our study, we describe our findings from screening 39 carbapenem resistant Acinetobacter spp. to determine the prevalence of metallo-β-lactamases.

Methods: 39 carbapenem-resistant clinical isolates of Acinetobacter spp. were obtained from University Malaya Medical Center (UMMC) from August 2003 until March 2004. MIC was evaluated using agar dilution method (CLSI). Preliminary screening for carbapenemases production was carried out and followed by PCR using blaIMP primers. Presence of integrons was determined by PCR amplification and initial comparison of the inserted cassettes in class I integron structures was determined by restriction enzyme analysis using AluI. Southern blot hybridization was performed to investigate the presence of IMP-4 producing metallo-β-lactamase gene on Class 1 integron using a digoxigenin-labeled blaIMP-4 probe. Molecular epidemiology using pulsed-field gel electrophoresis was carried out to analyze the genomic profiles.

Results: 31 strains were detected for the presence of Class 1 integron and only 2 strains of Acinetobacter calcoaceticus were detected for the presence of blaIMP-4 metallo-β-lactamases which was found located in the large plasmid of about 36 kb. Two different integron structures were found in that 31 isolates with 3 restriction patterns. Correlation was observed between carriage of Class 1 integron and epidemiological studies of these isolates. However, the 2 blaIMP-4 producing Acinetobacter calcoaceticus strains with similar integron structures (3kb) and identical restriction patterns, had different PFGE profile. This suggests that horizontal transfer of the entire integron structure could have taken place on different occasions and that, once acquired, the integrons are remarkably stable, and thus, strain differentiation may also have occurred after integron acquisition.

Conclusion: In conclusion, this study suggests that integron typing could be best used for characterizing isolates based on antibiotic resistance gene profile rather than for rapidly distinguishing between clones. Since the association of the blaIMP-4 gene in the integron was plasmid mediated, therefore this indicates that it has a high potential to spread.
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The role of AdeABC efflux pump in multidrug resistant Acinetobacter spp. isolated from University Malaya Medical Centre (UMMC), Malaysia.

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The AdeABC pump of Acinetobacter spp. confers resistance to various antibiotic classes. This pump is composed of the AdeA, AdeB, and AdeC proteins where AdeB is a member of the RND efflux pump superfamily. The adeA, adeB, and adeC genes are contiguous and adjacent to adeS and adeR, which are transcribed in the opposite direction and which specify proteins homologous to sensors and regulators of two-component systems, respectively. In this study an attempt is made to elucidate the role of AdeABC efflux pump in antibiotic resistance in Acinetobacter spp.

39 multidrug-resistant clinical isolates of Acinetobacter spp. were used. MIC was evaluated using agar dilution method (CLSI). Presence of AdeABC efflux pump genes were determined by PCR amplification. Subsequently, each gene was inactivated by plasmid insertion to study the contribution of these genes in developing antibiotic resistance and the resulting mutants were tested for their antimicrobial susceptibilities.

Among the multidrug-resistant strains, 36 strains had all the 3 (A,B,C) genes detected, while the remainder 3 strains had one or two of the genes detected. Inactivation of these individual genes showed decreased antimicrobial susceptibility indicating its contribution towards the development of antimicrobial resistance.

The presence of AdeABC multidrug efflux pump plays a major role in the development of antimicrobial resistance in Acinetobacter spp. The presence of either one or an interplay between these genes may have an effect on antimicrobial resistance in Acinetobacter spp.
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Carbapenem resistance mechanisms in *Acinetobacter* spp. isolated from University of Malaya Medical Centre (UMMC).

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**Background:** Carbapenem-resistant *Acinetobacter* spp. have gained increasing significance as opportunistic pathogens in hospitalized patients. Carbapenem resistance is often associated with the loss and / or decrease in outer membrane protein and overexpression of multidrug efflux systems. In this study, we describe a study on carbapenem resistance mechanisms involved in *Acinetobacter* spp. isolated from UMMC.

**Methods:** 39 carbapenem-resistant clinical isolates of *Acinetobacter* spp. obtained from inpatients at the University Malaya Medical Centre were used in this study. Preliminary screening for carbapenemase production was carried out and IEF was determined in the strains. The isolates were analyzed for the presence of the *bla*<sub>IMP</sub> gene using PCR and confirmed by Southern hybridization to obtain the location of this gene. Other resistance mechanisms such the presence of AdeABC efflux pump genes were also determined by PCR followed by inactivation by plasmid insertion and the resultant mutants were tested for their antimicrobial susceptibilities. Presence of outer membrane proteins were determined by SDS-PAGE. Iron-regulated outer membrane proteins (IROMPs) were expressed under iron deficit conditions as these are possible targets of antimicrobial therapy. Thus, antibodies against these IROMPs were raised and bactericidal activity of the strains was determined.

**Results:** Out of the 39 strains only two strains, S26 and S90, both *A. calcoaceticus* were positive for the presence of metallo-β-lactamases. Both these strains had similar MIC values for imipenem, cefotaxime, and aztreonam at 32, 512, and 64 μg/ml respectively. IEF analysis showed that both strains had a band of pl 8.0 which corresponded to that of *bla*<sub>IMP-4</sub>, while an additional band of pl 7.0 was present in strain S90. Strains, S26 and S90, were PCR positive for *bla*<sub>IMP</sub>, while the remaining 37 harbored *bla*<sub>OXA-23</sub>. Amplification and subsequent nucleotide sequencing of the entire coding region of *bla*<sub>IMP</sub> confirmed the identity of the *bla*<sub>IMP</sub> amplicon to be *bla*<sub>IMP-4</sub>. Plasmid analysis revealed that only the two strains, S26 and S90, carried plasmids: 147, 63, 36 in both strains with an additional 7kb plasmid in S26. Southern blot hybridization showed that the *bla*<sub>IMP-4</sub> gene was located on the 36 kb plasmid in strain S26 and was confirmed to be located on Class 1 integron. Screening and nucleotide sequencing of the Class 1 integron revealed identical genes: *bla*<sub>IMP-4</sub>, *qacG*, *aacA4*, and *catB3* in the 2 strains. However, PFGE analyses showed that S26 and S90 had different genotypes. Screening of efflux pump genes showed that 36 strains harboured all the 3 genes (*adeA*, *adeB*, and *adeC*). Inactivation of these individual genes showed decreased antimicrobial susceptibility indicating its contribution towards the
development of antimicrobial resistance. Besides that, all the strains showed loss of a 27 kDa OMP. The monoclonal antibodies produced showed bactericidal effect against the organism tested and it specifically killed the bacteria grown in iron deficit medium. This suggests that the outer membrane protein also plays an important role in carbapenem resistance in *Acinetobacter* spp.

**Conclusion:** Multiple mechanisms involved for carbapenem resistance in *Acinetobacter* spp. and therefore, understanding carbapenem resistance mechanisms might be crucial for the development of novel therapeutic strategies. However, it will be an important approach in the near future if one attempt to develop possible targets of new agents to control antimicrobial resistance in nosocomial pathogens such as *Acinetobacter* spp.
Patents:

1. Detection of IMP metallo-β-lactamases in multiresistant *Acinetobacter* spp.

2. Probes for rapid identification of *Acinetobacter* spp. and *Pseudomonas* spp.