Molecular epidemiological studies on sporadic and epidemic isolates of *Acinetobacter baumannii*

PhD thesis

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SUMMARY

Molecular typing of 110 *Acinetobacter baumannii* strains isolated in Abu Dhabi hospitals between March and November, 2008 revealed that 80 of them actually represent 11 clonal clusters within 3 lineages. As these strains apparently do have the capacity to stably maintain in hospital environments they were labeled as "epidemic" isolates. On the other hand, the 30 sporadic isolates represented fingerprints encountered only once during the study. Some of the epidemic clones were simultaneously present in hospitals and there were several clones encountered in more than one hospital. Sporadic isolates contained less than half as many resistance-related genes as the epidemic clones and were very heterogeneous. Several of these genes were significantly more common in the epidemic group (i.e. \( \text{bla}_{OXA23}, \text{bla}_{PER}, \text{armA} \) etc.). \( IS_Ab1 \)-linked- \( \text{bla}_{OXA23} \), unlike in strains isolated from other countries in the region, dominated local epidemic isolates, as being part of the core genotypes of all local clones.

Almost 100% of the epidemic strain was multi-drug resistant and non-susceptible to any of the first line drugs (ceftazidime, carbapenems, ciprofloxacin, gentamicin, trimethoprim-sulfamethoxazole) while remaining susceptible to colistin and tigecyclin. Sporadic isolates, although statistically more sensitive, also reached an alarming level of non-susceptibility.

It was shown that \( \text{bla}_{PER-7} \), a recently described allele of the PER β-lactamases and present in several of the local clones, can map, unlike previously described, also on a plasmid suggesting a possible path for its introduction into the species.

We have shown that the fast emerging \( \text{armA} \)-coded methylase is broadly present among the local isolates, and, at least in some cases, it is plasmid coded among them, too. The paradoxical amikacin susceptibility pattern, earlier associated with false reporting, was shown not to be related to a concentration-dependent expression of the methylase gene or to that of the \( \text{ade} \) or \( \text{abe} \) efflux pump genes.

We identified, cloned and sequenced the gene of a \( \text{bla}_{NDM-2} \) type MBL enzyme isolated from a patient earlier having received therapy in Egypt. The fingerprints and the similarities of the sequences of the regions flanking the \( \text{bla}_{NDM-2} \) gene to those of two other isolates from the region suggest that this clone is likely to have emerged in the Middle East and has spread subsequently to Europe.

Taken together our data show that epidemic strains are more resistant than their sporadic counterparts, particularly as far as broad-spectrum, first-line drugs are concerned. On the other hand, the type and susceptibility patterns of epidemic isolates may considerably differ at various regions emphasizing the effect of local factors (e.g. preferred drugs, antibiotic control etc). Apparently, Abu Dhabi hospitals have been penetrated by strains carrying emerging resistance genes (e.g. \( IS_Ab1\)-\( \text{bla}_{OXA23}, \text{bla}_{PER-7}, \text{armA} \)) but the region is also likely to serve as a reservoir for multi-drug resistant pathogens.
1. INTRODUCTION

Currently, one of the most, if not the most serious problem modern medicine is facing is the continuous rise of bacteria that are resistant to different antimicrobials. After having introduced the first antimicrobial agents into daily medical practice, for a short while, the discovery of the new compounds allowed us to successfully compete with this trend (Hawkey, 2008a). However, the genes responsible for the antibiotic resistance were already present, albeit at very low levels, before the introduction of antibiotics. The selective pressure created by the often imprudent use of these "magic bullets", not only in humans but also in companion and food animals and in the environment, have been responsible to provide an edge for the resistant cells and clones over susceptible ones (Hawkey, 2008a). The increasing mobility of people and goods, food and animals across the globe is another factor significantly contributing to the spread of resistant strains. Modern, scientific antibacterial therapy has started only over the last 60-70 years and by now become one of the utmost essentials of modern medicine. However, as stated by Hawkey: "As bacteria are thought to have evolved 3500 million years ago, 60 years is but ‘a second in a day’ in evolutionary time" (Hawkey, 2008b).

One of the most important hospital pathogen which has been added recently to the Infectious Diseases Society of America's dangerous pathogens hit-list is *A. baumannii* (Dijkshoorn et al, 2007). The emergence of multi drug resistance (MDR) among *A. baumannii* strains has been described worldwide. This prompted several microbiological and epidemiological studies leading to the formulation of interventions and infection control measures (Richet et al, 2001) to prevent the impact of this organism on health care.

This thesis, based on published and unpublished results, describes our findings on the resistance of *A. baumannii*, particularly against β-lactam and aminoglycoside drugs, among strains isolated in Abu Dhabi Emirate.
1.1. The genus *Acinetobacter* and *Acinetobacter baumannii*

During the past two decades, the *Acinetobacter* genus, and especially the species *A. baumannii* have gained enormous attention as one of the most important emerging bacterial pathogens in our times (Joly-Guillou, 2005; Murray and Hospenthal, 2005). Its emergence is a major concern because of its enormous capacity to adapt to a wide range of antibiotics, rapid transformation, and ability to persist in the environment for a very long time (Doughari et al, 2011).

The discovery of the genus *Acinetobacter* started in 1911 when Beijernick described a soil organism and named it as *Micrococcus calcoaceticus* (Henriksen, 1973). Within the next four decades, a number of bacterial species were discovered by different scientists and given different names to the same organism (Henriksen, 1973). In 1957, the genus *Acinetobacter* was proposed to include all the non-motile species belonging to *Achromobacter*. With the extensive comparative biochemical studies, finally the genus *Acinetobacter* was known and the species *Acinetobacter calcoaceticus* was suggested (Henriksen, 1973; Baumann et al, 1968).

*Acinetobacter* spp. are non-fermenting Gram-negative coccobacilli, non-motile, strict aerobic, oxidase negative and catalase positive. Its cells are often found in diploid formation or in clusters of variable length (Giamarellou et al, 2008). Its colonies are pale yellow to grayish white, like coliforms, on solid media and are sometimes strongly mucoid.

The first strain of *Acinetobacter* spp was isolated from soil in 1911 and referred to as *Micrococcus calcoaceticus* by Beijerinck (Henriksen, 1973). The *Acinetobacter* group was inappropriately defined for a long time and reclassified to different families and genera (Gordon and Wareham 2010). *Acinetobacter*, as a separate genus, was proposed in 1954, but since then it has gone through several comprehensive revisions. For a long time, the genus belonged to the family Neisseriaceae, but currently it is designated, together with *Moraxella* and *Psychrobacter*, to the family Moraxellaceae (Gordon and Wareham 2010) (TABLE 1.1).
TABLE 1.1. Taxonomy of *A. baumannii*

<table>
<thead>
<tr>
<th>UNIT</th>
<th>NAME</th>
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<tbody>
<tr>
<td>Kingdom</td>
<td>Bacteria</td>
</tr>
<tr>
<td>Phylum</td>
<td>Proteobacteria</td>
</tr>
<tr>
<td>Class</td>
<td>Gamma Proteobacteria</td>
</tr>
<tr>
<td>Order</td>
<td>Pseudomonadales</td>
</tr>
<tr>
<td>Family</td>
<td>Moraxellaceae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Acinetobacter</em></td>
</tr>
<tr>
<td>Species</td>
<td><em>A. baumannii</em></td>
</tr>
</tbody>
</table>

Currently over 30 genospecies are distinguished within the genus, i.e., groups with over 70% DNA-DNA hybridization rates and with $\Delta Tm$ (melting temperature) of $\leq 5^\circ C$ (*Bergogne-Berezin and Towner 1996*). Of these genospecies, only less than 20 were given a *bona fide* species name. The glucose-oxidizing, non-hemolytic complex made of closely related genospecies is often referred to as the *A. calcoaceticus-baumannii* complex. The *A. calcoaceticus-baumannii* complex can be identified and classified using molecular tools such as sequencing of the 16S rDNA, *gyrB* and *rpoB* genes as well as a variety of other methods (*Ehrenstein et al, 1996; Ecker, et al, 2006*). The most important genospecies of the genus is shown in TABLE 1.2 according to Towner (*Towner 2006; Peleg et al. 2008*).

The genus is made of ubiquitous organisms. After an enrichment culture, various acinetobacters could be retrieved from all samples collected from soil or water (*Seifert and Dijkshoorn, 2008*) as most representatives of the genus are thought to be environmental microorganisms (*Peleg at al. 2008*). In a study carried out in USA, they found that more than 95% of the samples collected from both soil and water were positive for some members of *Acinetobacter* genus (*Baumann, 1968*). Even from as extreme environment as the Andean lakes *Acinetobacter spp.* was isolated (*Ordonez et al, 2009*). In Kuwait, an *A. baumannii* strain has been thought to play a role in crude oil degradation since it was isolated from contaminated desert soil (*Obuekwe et al, 2009*).
Acinetobacters are also found as part of the human skin flora. Up to 43% of the individuals in the community carry them on their skin and mucous membrane. In Germany, in a study on the skin carriage rate of *Acinetobacter spp.* among healthy people *A. lwoffii* was recovered most frequently representing 58% of the isolates (Seifert et al, 1997). Similar findings were reported from the UK. In a study of 192

<table>
<thead>
<tr>
<th>GENOMIC SPECIES NUMBER</th>
<th>GENOMIC SPECIES NAME</th>
</tr>
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<tbody>
<tr>
<td>1*</td>
<td><em>Acinetobacter calcoaceticus</em></td>
</tr>
<tr>
<td>2</td>
<td><em>Acinetobacter baumannii</em></td>
</tr>
<tr>
<td>3</td>
<td><em>Acinetobacter pittii</em></td>
</tr>
<tr>
<td>13TU</td>
<td><em>Acinetobacter nosocomialis</em></td>
</tr>
<tr>
<td>4</td>
<td><em>Acinetobacter haemolyticus</em></td>
</tr>
<tr>
<td>5</td>
<td><em>Acinetobacter junii</em></td>
</tr>
<tr>
<td>6</td>
<td>Not named</td>
</tr>
<tr>
<td>7</td>
<td><em>Acinetobacter johnsonii</em></td>
</tr>
<tr>
<td>8</td>
<td><em>Acinetobacter lwoffii</em></td>
</tr>
<tr>
<td>9</td>
<td>Not named</td>
</tr>
<tr>
<td>10</td>
<td>Not named</td>
</tr>
<tr>
<td>11</td>
<td>Not named</td>
</tr>
<tr>
<td>12</td>
<td><em>Acinetobacter radioresistens</em></td>
</tr>
<tr>
<td>13BJ</td>
<td>Not named</td>
</tr>
<tr>
<td>14</td>
<td>Not named</td>
</tr>
<tr>
<td>15BJ</td>
<td>Not named</td>
</tr>
<tr>
<td>15TU</td>
<td>Not named</td>
</tr>
<tr>
<td>16</td>
<td>Not named</td>
</tr>
<tr>
<td>17</td>
<td>Not named</td>
</tr>
</tbody>
</table>

*Members of the *A. baumannii-calcoaceticus* complex are in bold and boxed by a double line*
healthy people they found that the *Acinetobacter* carriage rate was almost 44% (Berlau et al., 1999).

In contrast, in hospitalized patients, the rate of carriage of representatives of the genus can reach 75% (Seifert and Dijkshoorn, 2008). *A. baumannii* is considered the most important nosocomial species among the genus. In hospitals *A. baumannii* colonizes human skin, mucosal membranes as well as medical equipments such as tap water, sink traps, lotion dispensers, respiratory equipment, mattresses, bedside charts, pillows, bed curtains, blankets, door handles, telephones, steel trolleys, rubbish bins, and computers (Seifert and Dijkshoorn, 2008). The main factors thought to play a role in the persistence of *A. baumannii* in the hospitals are their resistance to antibiotics and disinfectants and the capacity to survive desiccation (Seifert and Dijkshoorn, 2008).

**1.2. Infections caused by *Acinetobacter baumannii***

Overall, members of the *Acinetobacter* genus are believed to have limited pathogenic potential to otherwise healthy people. On the other hand, in immunocompromised patients several species, in particular *A. baumannii* can cause severe, life threatening infections. Therefore it is considered as a typical opportunistic pathogen.

The various forms of infections by *A. baumannii* include bacteremia, urinary tract infection (UTI), meningitis, wound and burn infections, and most importantly nosocomial pneumonia, particularly in ventilated patients (Bergogne-Berezin and Towner 1996; Tomaras, et al. 2003; Lee, et al. 2006; Cevahir, et al. 2008; Gaddy, et al. 2009a; King, et al. 2009). These days, *A. baumannii*, combined with *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, represent the most important Gram-negative nosocomial pathogens (Landman, et al. 2007).

**1.2.1. Nosocomial bacteremia**

Bacteremia caused by *Acinetobacter* is associated with high mortality. The most common sources of bacteremia are infected intravascular and respiratory catheters, canules and tubes. To a lower extent surgical wounds, burns and infections of the
urinary tract are sources of bacteremia, while in 21–70% of the cases the origin remains unknown (Cisneros and Rodriguez-Bano 2002). Interestingly, compared to other pathogens, *A. baumannii* is more commonly found in mixed blood infections (Bergogne-Berezin and Towner 1996) and may be present in 10-15 % of all such mixed infections (Joly-Guillou, 2008).

1.2.2. Nosocomial pneumonia

Within the period from 1976 till 1990, the relative frequency of nosocomial pneumonia caused by *Acinetobacter* increased from less than 1% to 6% (McDonald et al. 1999). Currently, these bacteria represent one of the most important agents causing ventilator-associated pneumonia (VAP) together with *S. aureus*, *P. aeruginosa* and Enterobacteriaceae (Costa, et al. 2001; Luna and Aruj 2007; Joly-Guillou, 2008). There are several factors which contribute to acinetobacter-associated nosocomial pneumonia, such as head injury, neurosurgery, acute respiratory distress syndrome, aspiration, previous antibiotic therapy, deficiencies in the effectuation of infection control rules, and prolonged hospital stay (Luna and Aruj 2007).

1.2.3. Wound and burn infections

*A. baummanii* is also a common cause of hospital infection in burn patients (Joly-Guillou, 2008), but the infection caused is often less severe when compared to other organisms. Epidemic of this organism in burn units have often been linked to multidrug resistant strains and lasting epidemic is usually connected to the contamination of the hospital environment (Joly-Guillou, 2008). This bacterium gained a bad reputation among soldiers injured during the Iraqi war and it was named “Iraqibacter” due to clones spreading from Iraq to American military hospitals in Germany and US (Petersen, et al. 2007; Peleg, et al. 2008).

1.2.4. Meningitis

Meningitis is uncommonly caused by acinetobacter in the community. Few cases have been described after neurosurgical operations. The main cause of these cases is
thought to be a prolonged connection between the brain ventricles and the external environment, a ventriculostomy, or a cerebrospinal fluid fistula (Joly-Guillou, 2008).

1.3 Virulence factors of *Acinetobacter baumannii*

Until a few years back, *A. baumannii* had been considered as a harmless organisms with very little medical relevance, if any. Although by now the significance of this organism as an opportunistic pathogen is well appreciated, our knowledge about the mechanisms of the pathological processes it induces is still rudimentary (Braun, 2008). It possesses several factors and features related to its fitness, survival and disease-causing potential, such as toxins, enzymes, adhesins, surface hydrophobicity, porins, and quorum sensing capability that can secures its effective survival in often hostile environments (Braun, 2008).

For a bacterium to cause infections, the first step is to enter and colonize the host. Adhesion to surfaces is the capacity that secures the anchoring of the cell to mucosal surfaces and to cells of the host. However, the ability of *A. baumannii* to adhere to or even invade cells was generally found to be lower compared to other microorganisms such as *Campylobacter upsaliensis*, *Helicobacter pylori*, *Neisseria meningitidis*, *P. aeruginosa* or *Yersinia enterocolitica* (Mooney, et al. 2003; Capecchi, et al. 2005; Choi, et al. 2008). The low level of adhesion and invasion of *A. baumannii* is considered to correlate with reduced pathogenicity of this opportunistic pathogen (Dijkshoorn, et al. 2007; Peleg, et al. 2008).

Nevertheless, strains do possess adhesive capacity. Hydrophobicity can facilitate adhesion to plastic equipment such as catheters (Braun, 2008). In a study by Boujaafar el al. (1990), it was demonstrated that *A. baumannii* strains isolated from catheters and tracheal devices expressed higher surface hydrophobicity than strains obtained from normal skin (Braun, 2008).

As for many other microorganisms, pili have been identified as the main mediators of adhesion, but specific adhesins in *A. baumannii* are still to be described in details. In some cases, a strong link was found between antibiotic resistance, particularly between the presence of the *bla*<sub>PER-1</sub> beta-lactamase gene and the ability to adhere to
target cells (Lee, et al. 2008; Gaddy and Actis 2009). Others observed no difference between epidemic and non-epidemic strains in terms of adherence to epithelial cells (Lee, et al. 2006). In a study by Lee et al (2006), the presence of two different types of adherence in A. baumannii to human bronchial epithelial cells was observed: a dispersed adherence to the surface of the host cell, and a clustered type with bacteria forming microcolonies on the cell surface.

There is still a controversy on the relationship between epithelial adherence and the capacity to form biofilms. Some authors have found a clear connection between the two phenotypes (Lee, et al. 2008), while others reported the opposite (Sechi, et al. 2004).

Outer membrane proteins have also been shown to contribute to the virulence of A. baumannii. OmpA was implicated to facilitate adhesion (Choi, et al. 2008a), particularly to epithelial cells from the airways (Choi, et al. 2008b). Furthermore OmpA has the ability to localize in the mitochondria and in the nuclei after binding to the surface of the host cells and later induce nucleus cytotoxicity and death (Choi, et al. 2005; Choi, et al. 2008a; Choi, et al. 2008b). Another outer membrane protein, AbOmpA, induces maturation and activation of dendritic cells (DCs) and directs differentiation of the CD4+ cells toward Th1 polarity (Lee, et al. 2007).

The LPS of A. baumannii has been known to be a potent polyclonal activator (Symons, et al. 1982) and recently it was also described that heat-killed cells of the pathogen predominantly stimulates CD4+ T lymphocytes exhibiting a superantigen-like activity, while similar activity was not observed with E. coli cells (al-Ramadi, 2000).

Quorum sensing, i.e. the capacity of bacteria to communicate with each other and respond collectively to the changing environment (QS) (Whitehead, et al. 2001) has been described for acinetobacters, as well. More than half of the representative of the genus have been shown to produce some homoserine-lactone molecules, i.e. the crucial type of mediators of cell-to-cell communication: four in A. calcoaceticus and six varieties have been identified in A. baumannii (Bhargava, et al. 2010),
1.4. Epidemic potential and clonality of acinetobacter

*Acinetobacter baumannii* can easily be transmitted from patient-to-patient. In fact, there is a definite risk for a new patient acquiring the bacterium once admitted to the same hospital unit or ward occupied by patients colonized by acinetobacter (*Cisneros and Rodríguez-Baño, 2002*).

In the hospital setting, *A. baumannii* infections are present in two forms: sporadic infections and epidemics, members of the later group often exhibiting extensive resistance (*Dijkshoorn, et al., 2007; Gootz and Marra, 2008; Jain and Danziger, 2004; Giamarellou et al., 2008*). Epidemic strains of *A. baumannii* are highly clonal. The MDR strains causing major epidemics in hospitals all over the world represent a relatively few “successful”, “particularly fit to the task” clones, i.e. genetically related strains. The best-studied ones are called (pan-) European clones I-II and III (*Dijkshoorn et al., 1996; van Dessel et al., 2004*). Recently five more lineages have been identified and named as worldwide lineages (WW1 to WW8). The first three represent the European clonal lineages (*Higgins et al, 2010*).

1.5. Antibiotic resistance of *Acinetobacter baumannii*

One of the most interesting features of *A. baumannii* is the ease by which it can acquire resistance to various antibiotics. Mutations, new gene acquisitions, up-regulating expression of existing genes (should those be genes of drug-destroying enzymes or efflux pumps), or losing them, like in the case of porins, all may contribute to the broad spectrum resistance of these strains. Resistance of this organism can affect practically any drugs used in clinical practice. As a result of the rapid acquisition of resistance genes to different and multiple classes of antibiotics, several drugs have already been eliminated from treatment options for *A. baumannii* infections such as penicillins, cephalosporins, aminoglycosides, quinolones and tetracyclines (*Valencia et al, 2009*). Below is a brief review on two groups of antibiotics and the mechanism of bacterial resistance, i.e. β-lactams and aminoglycosides. We restrict our discussion to these two groups, as resistance to
them represents the most pressing problems and as we have been focusing on these problems during our work.

1.5.1. β-lactams

β-lactam antibiotics are named after the β-lactam ring present in their chemical structure. The basic structure of β-lactams consists of a thiazolidine ring known as the β-lactam ring. This core structure is essential to their antibacterial activity (Holten and Onusko, 2000; Samaha-Kfoury and Araj, 2003). The structures and the main groups are shown on FIGURE 1.1.

Inhibition of the bacterial growth, and eventual cell death is due to the interference with cell wall, specifically with the peptidoglycan synthesis. This polymer is assembled in a chain of enzymatic activities which involves at least 30 different enzymes. (Chambers, H. 2010). The antibiotics act by interfering with enzymes responsible for the formation of a peptide bridge within these polymers. Since some of these enzymes are inhibited by the simplest β-lactam, i.e. penicillin, they are misleadingly called penicillin binding proteins or PBPs (Chambers, H. 2010).

If β-lactams bind to these proteins, they inhibit their function and the synthesis of peptidoglycan stops, leading to cell death. Their use is rather safe in man and free of side effects since human cells lack the structure of peptidoglycan which these antibiotic target and hence their function is mainly bactericidal.
There are four major groups of drugs that belong to β-lactam antibiotics (FIGURE 1.1). Penicillins normally have a limited role in the treatment of acinetobacter infections. Ticarcillin and piperacillin, the so-called "anti-pseudomonal drugs", especially in combination with β-lactamase inhibitors (ticarcillin/clavulanic acid and piperacillin/tazobactam) are active against certain strains.

The cephalosporins used today are all semisynthetic derivative of the cephalosporin C molecule produced by the Acremonium fungus (Sykes 2000). Their basic structure is a β-lactam ring fused to six-member sulfur-containing dihydrothiazine ring. Cephalosporins are divided into generations based on their antimicrobial properties, and to a degree, to the time of their discovery (El-Shaboury, et al. 2007). It has been known that from the first till the fourth generation, the spectrum of cephalosporins has drifted towards Gram-negative cells with somewhat decreasing activity against Gram-positive organisms. The recent, fifth generation drugs are active against MRSA due to their increased affinity to PBPs. In therapy of acinetobacter infections, mostly 3rd and 4th generation drugs are used (Andes 2005).
Carbapenems, although still contain the group-defining β-lactam ring and have a similar mode of action, they differ in their structure and side chains from the rest of the group (FIGURE 1.1.). They bind with a great affinity to most high molecular weight PBP of both Gram-positive as well as Gram-negative bacteria (Chambers, 2010). Carbapenems can pass through the bacterial outer membrane barrier through the OprD, instead of OmpC or OmpF used mostly by cephalosporins or penicillins (Chambers, 2010). These unique features account for the wide antibacterial range of carbapenems and the absence of cross-resistance with other members of the β-lactam group. These also make them the preferred drug for the treatment of serious, life threatening Gram-negative, among them acinetobacter infections. However, not all carbapenems are equally effective: while imipenem and meropenem (i.e. group 2 carbapenems) are active on susceptible isolates, the species, similar to most non-fermenters, has a natural resistance to ertapenem (i.e. a group 1 carbapenem).

Monobactams are effective against Gram-negative aerobic bacteria only and aztreonam is the only drug found in the market currently. It has limited role, if any, in treating acinetobacter infections.

1.5.1.1. β-lactam resistance

Theoretically, there are four major mechanisms which may lead to resistance to β-lactam antibiotics: Destruction of the antibiotic by β-lactamases, modification of the target molecule (i.e. PBP), reduction of antibiotic uptake due to loss of porins and increased efflux of the drug through the outer membrane, respectively (Andes 2005; Chambers 2005; Vila, et al. 2007; Vila and Martinez 2008; Vila and Pachon 2008) (FIGURE 1.2.):
**Figure 1.2. Primary mechanisms of β-lactam resistance**

![Diagram of β-lactam resistance mechanisms](image)

*Adapted from Nordmann et al. 2012*

**β-lactamases**

β-lactamases are enzymes, which inactivate the β-lactams by splitting the amide bond of the β-lactam ring. These enzymes evolved allowing organisms to resist β-lactam compounds produced by various microorganisms in their natural habitat (mostly soil) and encountered by other, competing members sharing the same niche (*Opal and Pop-Vicas, 2010*). Eventually, these genes found their ways to organisms colonizing or infecting animals and humans.

The β-lactamase genes (*bla*) are found either chromosomally or on mobile elements commonly located within integrons, i.e. genetic elements with an integrase gene (*int*) capable of acquiring and expressing multiple resistance genes, facilitating their spread (*Opal and Pop-Vicas, 2010*).

Ambler classified β-lactamases according to the structure of their amino acids into four different classes, A to D (*TABLE 1.3.*).
Table 1.3. Ambler Classification of β-lactamases

<table>
<thead>
<tr>
<th>CLASS</th>
<th>ACTIVE SITE</th>
<th>ENZYME TYPE</th>
<th>SUBSTRATES</th>
<th>EXAMPLES</th>
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<td>A</td>
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<td>Penicillinases</td>
<td>Aminopenicillins</td>
<td>TEM1, SHV1</td>
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<td></td>
<td></td>
<td>Broad Spectrum</td>
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<td>Extended Spectrum</td>
<td>Broad spectrum</td>
<td>TEM-derived, CTX- derived</td>
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<td></td>
<td>(ESBL)</td>
<td>and aztroneam</td>
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<td>Carbapenemases</td>
<td>Extended spectrum</td>
<td>KPC1, KPC2</td>
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<td>plus cephamycins</td>
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<td>B</td>
<td>Zn^{2+}</td>
<td>Metallo-β-lactamases</td>
<td>Extended spectrum</td>
<td>IMP, VIM, GI M</td>
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<td>C</td>
<td>Serine</td>
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<td>D</td>
<td>Serine</td>
<td>Oxacillinases</td>
<td>Cloxacillin,</td>
<td>Oxa-family in P. aeruginosa</td>
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<td>methicillin</td>
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<td>Broad spectrum</td>
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<td>Extended spectrum</td>
<td>Broad spectrum</td>
<td>Oxa-derived in P. aeruginosa</td>
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<td>and monobactams</td>
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<td>Carbapenemases</td>
<td>Extended spectrum</td>
<td>Oxa-derived in Acinetobacter</td>
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<td>plus cephamycins</td>
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**Class A β-lactamases** include most of the *extended-spectrum β-lactamases (ESBLs)* such as the TEM, SHV and CTX-M enzymes. They are sensitive to β-lactamase inhibitors. They confer resistance to a broad spectrum of cephalosporins including 3rd and 4th generation drugs. The TEM- and SHV- like enzymes are the most commonly observed enzymes among ESBLs. Originally these groups had no broad-spectrum activities, but it gradually developed due to point mutations affecting the active site of the enzymes. Since 1980 more than 340 variants of theses enzymes have been observed (*Poirel et al, 2012*). Even though the *blaSHV*-like genes are mainly found in Enterobacteriaceae, some reports show their presence in *A. baumannii* and *P. aeruginosa* isolates, as well (*Hujer et al, 2006; Naas et al, 2007; Poirel et al, 2012*).

During the last two decades CTX-M like cefotaximases have started to emerge and currently they are the most important ESBLs in enteric bacteria (*Poirel et al, 2012*). Whereas CTX-M enzymes have been rarely identified in non-fermenters, a few A.
*Acinetobacter baumannii* isolates producing variants of CTX-M have been identified in different parts of the world (*Nagano et al., 2004; Poirel et al., 2012*).

While in Enterobacteriaceae GES-, PER- and VEB-like enzymes are less frequently reported, they are more commonly encountered in non-fermenters. The VEB-1 enzyme was found in a large number of isolates from France and Belgium (*Naas, et al., 2006a; Naas et al., 2006b*). PER-1 was originally identified in *P. aeruginosa* and hence the name. Different variants of the PER have been identified and grouped. The first subgroup consists of point mutation-derivatives of the PER-1 (PER-3, -4, -5, and -7). The second one includes PER-2 and PER-6 with 22 amino acid differences and 85% amino acid similarity with the PER-1 like (*Poirel et al. 2012*). PER-1 was found in many isolates in Turkey, Korea and USA (*Hujer et al., 2006; Poirel et al., 2005*). Recently, the *bla*<sub>PER-7</sub> gene encoding PER-7 (with four amino acid changes as compared to PER-1) was identified in an *A. baumannii* clinical isolate from France (*Bonnin et al. 2011*). The gene was associated with the ISCR1 element found inside a *sul1*-type integron located in the chromosome. These results revealed a new way for the spread of the PER gene, giving rise to the importance and the ability of ISCR1 elements to spread antibiotic resistance genes (*Bonnin et al. 2011*).

The detection of ESBLs in *A. baumannii* is often hindered, perhaps even more than in Enterobacteriaceae, by the presence of other β-lactamases, such as AmpC (*see later*) (*Peleg, Seifert et al. 2008*).

Class A carbapenemases are usually not-carbapenem specific, and often hydrolyze most (not all) compounds of the “lower β-lactam classes”, e.g. 3<sup>rd</sup> generation cephalosporins, albeit often with lower, sometimes with substantially lower efficacy (*Nordmann and Poirel 2002; Queenan and Bush 2007*). However, several enzymes have strong carbapenemase activity (*Poirel and Nordmann 2006*). The most important carbapenemases of this group are the KPC type enzymes (for *K. pneumoniae* carbapenemase) mostly found in Enterobacteriaceae (*Nordmann et al., 2012*).

**Class B β-lactamases** (often referred to metallo-β-lactamases (MBLs) require zinc as a divalent cation for their activity. They are capable of hydrolyzing all the β-
lactam antibiotics except the aztreonam (Walsh et al, 2005) and can be inhibited by EDTA or by other chelators (Walsh 2005; Pfeifer et al. 2010). They are often coded on plasmids as part of integrons, as well as on the chromosomes (Queenan and Bush 2007; Nordmann and Poirel 2002; Walsh 2005).

Since the 1990s, there has been a dramatic increase in the incidence of transferable MBL genes in Pseudomonas spp., Enterobacteriaceae and recently in A. baumannii (Nordmann et al., 2012). The most common ones are IMP, VIM, SIM, SPM, and GIM enzymes (Poirel and Nordmann 2006; Yang et al. 2009), the first three being the most frequently encountered also in A. baumannii especially in the Far East, South America and Greece (Jeong et al, 2006; Fritsche et al, 2005).

The newest member of the MBL family is the NDM group of enzymes (i.e. New Delhi metallo β-lactamase). It was discovered in Sweden from an Indian patient transferred from a hospital in New Delhi in 2008. Since then, it is spreading worldwide except in Central and South America. It has been suggested that the Middle East may act as a secondary source for the spread of NDM-1 (Nordmann et al., 2011). Remarkably, most of the strains carrying the NDM-1 gene are also resistant to other drugs (e.g. to aminoglycosides and fluoroquinolones) and found in conjunction with other resistance mechanisms, giving rise to a very narrow spectrum of therapeutic options (Nordmann et al., 2011). While most of the positive cases of NDM-1 are related to Enterobacteriaceae, the presence of the blaNDM-1 gene in A. baumannii has been emerging at various parts of the world (Peleg et al. 2008). Most of these isolates are multidrug resistant and some of them co-expresses other carbapenemases (i.e. blaOXA-23, blaOXA-181 and blaIMP) (Karthikeyan et al. 2010, Chen et al 2011, Pfeifer Y et al. 2011). Recently, the NDM-2 variant (Pro to Ala substitution at position 28) was described. This allele was first found in a multidrug-resistant A. baumannii strain isolated from a German patient having previously been hospitalized in Egypt (Kaase et al. 2011), while a subsequent one was isolated in Israel (Espinal et al. 2011).

**Class C β-lactamases** are often referred to as AmpC-type enzymes. They were found in Gram-negative bacteria, conferring resistance to amino-pencillins, to first, second, and some representatives also to third generation cephalosporins (Opal and
*Acinetobacter baumannii* in Abu Dhabi

Acinetobacter* baumannii* in Abu Dhabi

Pop-Vicas, 2010). Among *Haemophilus* and in some members of the Enterobacteriaceae family, as well as in acinetobacter the AmpC enzymes are typically chromosomally encoded, often repressed, expressed only when induced or de-repressed by mutations. Lately several plasmid-coded members have been spreading in various genera (*Jacoby, 2009*).

Recently, in *Acinetobacter*, this group of enzymes was re-named as *Acinetobacter*-derived cephalosporinases (ADC) (*Hujer et al, 2005*). Upon expression, these enzymes hydrolyze penicillins and the narrow spectrum cephalosporins. With the overexpression of the gene, the enzyme has the ability to destroy the extended-spectrum cephalosporins with the exception of the fourth generation (*Hujer et al, 2005*). In *A. baumannii*, expression of the ADC gene requires a strong promoter provided by the insertion sequence (e.g. ISAba1) upstream of the gene (*Corvec et al, 2003*). Recently a new extended spectrum AmpC enzyme was identified in *A. baumannii* which able to hydrolyze ceftazidime, cefepime and aztreonam (*Rodriguez-Martinez et al, 2010*).

It should be noted that several ADCs, similarly to some ESBLs may also exhibit limited carbapenemase activity. This, coupled with other resistance mechanisms, as porin loss or efflux (see later) may mount to clinically significant resistance.

**Class D β-lactamases** are primarily oxacillinases hydrolyzing oxacillin in a more efficient manner than benzylpenicillin and having varying spectrum of activity beyond this group of drugs. They are the most wide-spread β-lactamases in *A. baumannii*. Only a few variants can hydrolyze the extended-spectrum cephalosporins and carbapenems, and even those that can do exhibit usually moderate activity against the these extended spectrum of drugs.

In *A. baumannii*, the OXA-type class D β-lactamases are arranged into five families: the OXA-23-like, OXA-40-like, OXA-51-like, OXA-58-like and OXA-143-like groups, respectively (**TABLE 1.4**). It is noteworthy that the genes encoding for the OXA-51 like enzymes are intrinsic to the species, while for other families the genes have been acquired from other sources (*Poirel et al, 2009*).
TABLE 1.4. OXA-type beta lactamases in Acinetobacter baumannii

<table>
<thead>
<tr>
<th>GROUP</th>
<th>GROUP MEMBERS</th>
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<tbody>
<tr>
<td>OXA-23-like</td>
<td>OXA-23, OXA-27, OXA-49, OXA-73, OXA-102, OXA-103, OXA-105, OXA-133, OXA-134, OXA-146, OXA-165 to OXA-171</td>
</tr>
<tr>
<td>OXA-40-like</td>
<td>OXA-40, OXA-25, OXA-26, OXA-72 , OXA-139, OXA-160</td>
</tr>
<tr>
<td>OXA-51-like</td>
<td>OXA-51, OXA-64 to OXA-71, OXA-75 to OXA-80, OXA-82 to OXA-84, OXA-86 to OXA-95, OXA-98 to OXA-100, OXA-104, OXA-106 to OXA-113, OXA-115 - OXA-117, OXA-120 to OXA-127</td>
</tr>
<tr>
<td>OXA-58-like</td>
<td>OXA-58, OXA-96, OXA-97, OXA-164</td>
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<tr>
<td>OXA-143</td>
<td>OXA-143, OXA-182</td>
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The first oxacillinase gene was identified in A. baumannii on a plasmid, named ARI-1 but after its detailed characterization it was renamed OXA-23 (Paton et al, 1993). Afterwards, two enzymes were described from strains in Singapore and China and named as OXA-27 and OXA-49, both of them have 99% amino acid similarity with OXA-23 (Afzal-Shah et al, 2001). Since then, several enzymes have been identified in different places and from different species. Up to now the OXA-23-like group consists of seventeen enzymes (TABLE 1.4.).

Few members of the OXA-23 family, such as OXA-23, OXA-27 and OXA-49 showed the ability to hydrolyze carbapenems and also exhibiting some activity against oxyminio-cephalosporins, aminopenicillins and oxacillin (Paton et al, 1993; Afzal-Shah et al, 2001). However, the isolates harboring the OXA-23 gene showed resistance to ticarcillin, amoxicillin, imipenem and meropenem. Attempts to transfer the $\text{bla}_{\text{OXA23}}$ into susceptible A. baumannii strain showed a moderate level of resistance to carbapenems even in strains expressing the RND-type AdeABC efflux pump. Only the transformation of the entire plasmid containing the $\text{bla}_{\text{OXA23}}$ achieved
high level of resistance demonstrating that besides the OXA-23 expression, there are other factors required to achieve the clinically significant level of carbapenem resistance (Heritier et al, 2005). Epidemics with A. baumannii strains harboring the $\text{bla}_{\text{OXA23}}$ have been reported worldwide (Coelho et al, 2006; Heritier et al, 2005; Villegas et al, 2007; Evans et al, 2011). In a recent survey, it was found that the isolates carrying the $\text{bla}_{\text{OXA23}}$ are found globally and consistent with the clonal spread (Higgins et al, 2010).

After the discovery of the OXA-23 OXA-24, later renamed OXA-40, was the beginning of the second group of OXA-type $\beta$-lactamases and it was described first in a Spanish hospital (Bou et al, 2000a). Afterwards, five other enzymes were identified having more than 99% amino-acid similarity to OXA-40. Few members of the OXA-40 family showed weak activity against carbapenems, cephalosporins as well as penicillins (Afzal-Shah et al, 2001, Bou et al, 2000a). Comparison of the G+C ratio of the OXA-40 family with A. baumannii genome suggested that they are not intrinsic to the species and their origin is still to be revealed (Bergogne-Berezin and Towner, 1996).

All A. baumannii isolates harbor the intrinsic enzyme $\text{bla}_{\text{OXA51}}$ that belongs to the OXA-51 like family (Merkier and Centron, 2006; Turton et al, 2006a). The first OXA-51 enzyme was identified in 1996 from Argentinean strains and up to date the OXA51 family consists of 72 members (TABLE 1.4.) (Brown et al, 2005). Comparison of the G+C content showed 38.4-39.9 %, i.e. a value similar to that for the A. baumannii genome suggesting that the $\text{bla}_{\text{OXA-51-like}}$ gene is truly intrinsic to this species. It has been used as a tool for the identification of A. baumannii by molecular methods (Bergogne-Berezin et al, 1996).

The role of the OXA-51 family towards antibiotic resistance is still not clear. Among the 73 variants, the only well-studied genes are those coding for OXA-51 and OXA-69 (Brown et al, 2005; Heritier et al, 2005). Several studies showed that the expression of the $\text{bla}_{\text{OXA-51-like}}$ genes is very poor and that they play a role in resistance only if their expression level increased by a surrogate promoter provided by an insertion sequence (ISAba1) upstream of the gene (Turton et al, 2006b). It has been shown that specific members of the $\text{bla}_{\text{OXA-51-like}}$ subgroup are linked with
specific clonal lineages. For example the $bla_{OXA-69}$ gene is associated with the European clone I, and all the strains belonging to the European clone II carry the $bla_{OXA-66}$ gene (Evans et al, 2008).

In 2003, the OXA-58 like family has been described and the OXA-58 was the first member found in an MDR $A. baumannii$ isolate from a French hospital (Poirel et al, 2005). Currently, the OXA-58 family consists of four different enzymes (TABLE 1.4). $bla_{OXA-58}$ showed a weak activity against the penicillins and carbapenems and to some extent a variable activity against cephalosporins with the ability to hydrolyze cefpirome but not ceftazidime or cefotaxime (Poirel et al, 2005). Similar to the OXA-23 and OXA-40 families it has been found, based on its G+C ratio, that the OXA-58 family are imported from another source and they are not intrinsic to the species (Bogaerts et al, 2006; Poirel et al, 2006).

The very recent group of class D OXA-type $\beta$-lactamases is the OXA-143 family. The OXA-143 enzyme was described from isolates in Brazil on a plasmid and the gene was not associated with any insertion sequences. The enzyme showed ability to hydrolyze penicillins, carbapenems at low level and had no effect on cephalosporins (Higgins et al, 2009). The second enzyme of this family has been identified in 12 isolates from Korea (Kim et al, 2010).

The role of insertion sequences in resistance

In 2001, a new insertion sequence (IS) named IS$Abal$ was identified in $A. baumannii$ and it was located upstream of the gene encoding the $bla_{ADC}$ enzymes (Corvec et al, 2003). Later this IS was also found upstream of the $bla_{OXA-23}$ gene. The IS element consists of two overlapping open reading frames (ORF), followed by a frame-shift during translation which finally form an effective and functional transposase, responsible for the transposition activity of the IS (Mugnier et al, 2009).

It has been demonstrated that the IS$Abal$ element plays a role as a promoter once located upstream of different genes. For example, IS$Abal$ is found 9 base-pairs upstream of the $bla_{ADC}$ genes serving as a strong promoter for the overexpression of these enzymes (Heritier et al, 2006). Similarly, the investigation on the acquisition of
$\text{bla}_{\text{OXA-23}}$ showed that it is part of two transposon structures, each formed of two copies of insertion sequences, ISAb1 or 4, which not only promotes the acquisition of the gene but also serve as promoters for its expression (Corvec et al, 2007). Actually, this is the case observed with all the $\beta$-lactamases which requires the presence of a surrogate promoter provided by the IS element (FIGURE 1.3).

**FIGURE 1.3** ISAb1 and its role in expression of $\beta$-lactamase genes

*A: The own promoter of the bla gene is not strong enough to initiate sufficient transcription*

*B: A strong surrogate promoter provided by ISAb1 upstream of the bla gene results in a clinically significant expression of the gene*

**Alteration in target affinity**

In several bacterial species the modification of PBPs reducing the affinity to $\beta$-lactams plays a significant role in antibiotic resistance (Zapun et al, 2008). However, in *A. baumannii*, like on most Gram-negative organisms, this mechanism plays a minor role in $\beta$-lactam resistance (Poirel and Nordmann 2006). The decreased sensitivity to carbapenems was found to be associated with a reduced expression of PBP2 (Bou, et al. 2000b; Giamarello, et al. 2008) and the effect of alteration of
PBPs was also mentioned by others (*Limansky, et al. 2002*) but the resistance conferred is unlikely to reach clinical significance.

**Reduced uptake**

Another resistance mechanism, which is found in many bacteria, is the loss of OMPs, i.e. channels allowing certain molecules, including some antibiotics, to cross the cell membrane (*Delcour, 2009*). In *A. baumannii*, the mutations which affect the CarO OMP (*Poirel and Nordmann 2006*) can play a role in the reduced uptake of the antibiotics resulting in significant resistance to imipenem, even in the absence of carbapenemases (*Limansky, et al. 2002*). Loss of other proteins have also been implicated in various levels of carbapenem resistance, sometimes even in strains not producing any carbapenemases (*Poirel and Nordmann 2006; Vila, et al. 2007; Peleg, et al. 2008*). Nevertheless, the loss of porins plays the most important role if complementing the presence of β-lactamases having a limited carbapenemase activity on their own, such certain ESBLs and ADCs.

**Overexpression of efflux mechanisms**

Efflux systems pump several compounds, which including antibiotics, out of the bacterial cell and can play a major role in bacterial resistance to different classes of antibiotics (*Van Bambeke et al, 2000*). Pumps have a relatively broad spectrum of compound-specificity hence they may lower the intracellular concentration of various classes of drugs. This may lead, in case of some drugs, to significant resistance on its own; while in case of others it may contribute to significant resistance if other mechanisms (e.g. β-lactamases) are already functional. The latter situation is commonly seen among carbapenem resistant non-fermenters. An up-regulated efflux is often combined with overproduction of enzymes (e.g. cephalosporinases, AmpC beta lactamases) which, in synergism with the increased efflux, add up to resistance (*Poirel and Nordmann 2006*).

The efflux pump systems are grouped into different families. The resistance-nodulation-division (RND) family is the best studied among *A. baumannii* especially
the AdeABC group (Nemec et al, 2007). This group has been found to pump a wide range of antibiotics out of the cells including the β-lactams. Another two groups in the RND family were described in A. baumannii named AdeIJK and AdeFGH groups. The first group has the ability to expell carbapenems, while the second group was found to play a role in chloramphenicol, clindamycin and fluoroquinolone resistance (Coyne et al, 2010a; Damier-Poille et al, 2008; Coyne et al, 2010b).

Taken together, although the role of limited uptake and increased efflux β-lactam resistance of A. baumannii is undeniable, the major modes by which this bacterium counteracts these drugs are the production of a broad array of enzymes, i.e., various β-lactamases (Poirel and Nordmann 2006).

1.5.2 Aminoglycosides

Aminoglycosides have been introduced as antibacterial drugs since 1940s. They confer concentration-dependent bactericidal effect against many organisms such as P. aeruginosa, mycobacterium, etc. Their antimicrobial activity can be additive or synergistic with other drugs, such as β-lactams (Gilbert, and Leggett, 2010).

Aminoglycosides consist of an essential six-membered ring with amino group substituents called aminocyclitol. The aminocyclitol ring is connected to other amino or non-amino sugars through glycosidic bonds (FIGURE 1.4.). Streptomycin differs from the rest of the aminoglycosides in that it has a streptidine rather than 2-deoxystreptamine as the central aminocyclitol (Gilbert and Leggett, 2010)

Aminoglycosides are protein synthesis inhibitors. They act by binding to the 16S rRNA (Jana and Deb 2006; Lee, et al. 2006). Unlike that of other protein synthesis inhibitors (e.g. macrolides, chloramphenicol, tetracylines etc), the effect of aminoglycosides is generally bactericidal. The binding to the ribosomes results in significant decrease in protein synthesis as a consequence of mis-translation of mRNAs (Gilbert and Leggett, 2010).
1.5.2.1. Resistance to aminoglycosides

Resistance to aminoglycosides can be due to reduced entry, efflux, enzymatic modification of the drug or changing the target, i.e. the ribosomal binding site.

The efflux and porin mutations have been described above and they affect aminoglycosides, as well. In *A. baumannii*, efflux pumps have been known to contribute to aminoglycoside resistance. Several different pumps can be involved in conferring decreased susceptibility to aminoglycosides. The most important ones are the members of the RND family (AdeABC) and MATE (multidrug and toxic compound extrusion) family (AbeM) (*Vila, et al. 2007; Vila and Martinez 2008*). Mutations in the energy-dependent trans-membrane transport system across the cell membrane also limit aminoglycoside transport. Nevertheless, the importance of these mechanisms is secondary to that of enzymatic modification and target alteration in reaching clinically significant mutations (*Vila and Martinez 2008*).

The production of enzymes modifying and/or inactivating the aminoglycosides has been known for a long time (*Jana and Deb 2006*). The amino group of the drugs can be modified by N-acetyltransferases (AAC). The hydroxyl group can be modified by...
O-nucleotidyl (adenyl) transferases (ANT or AAD) or by O-phosphotransferases (APH) (Seward, et al. 1998; Jain and Danziger 2004; Bonomo and Szabo 2006). The modified drug binds poorly to the ribosome and hence the mechanism confers a significant level of resistance. An important feature of these enzymes is their drug specificity, which can be narrow, but can also include several aminoglycoside drugs (Jain and Danziger 2004).

The broadest pattern of aminoglycoside resistance is conferred by modifying the ribosomal target. This can be achieved either by mutations, e.g. as seen in case of streptomycin resistance in mycobacteria (Gilbert and Leggett, 2010). However, an emerging mechanisms resulting in a broad (involving practically all clinically useful aminoglycosides) and high-level resistance is the methylation of the target. These methylases have been playing an increasing role in aminoglycosides resistance worldwide. Since 2003, the 16S rRNA methylases emerged as a new mechanism of resistance and currently they consist of seven types (rmtA, rmtB, rmtC, rmtD, rmtE, npm and armA) (Cho, et al. 2009). Their genes are plasmid-encoded and have spread world-wide in different bacterial species such Enterobacteriaceae, P. aeruginosa and Acinetobacter spp. Of these types, only armA was found in A. baumannii (Yu, et al. 2007).

1.6. Treatment of Acinetobacter baumannii infections

In the relatively rare, antibiotic susceptible cases treatment follows disease-specific guidelines established for Gram-negative, in particular non-fermenter infections. Piperacillin-tazobactam, ceftazidime, often combined with aminoglycosides, or if susceptibility pattern allows, with ciprofloxacin are the key drugs in medication. Should the above β-lactams fail carbapenems (imipenem or meropenem) are administered, often in combinations with aminoglycosides. It is usually the loss of the extended β-lactams that results in grave situations. Often the only drugs left are the bacteriostatic tigecycline or the toxic colistin. Against both drugs resistance has already been emerging. In some countries sulbactam, a β-lactamase inhibitor, is used as a β-lactam drug by itself, and it has shown activities against some MDR strains (Levin 2002; Giamarellou, et al. 2008).
1.7. *Acinetobacter baumannii* in the Gulf region

Countries of the Arabian Gulf represent a very unique situation in the epidemiology of MDR pathogens. Several of these countries have a huge expatriate population (upto 80% as in the United Arab Emirates (UAE)) mostly arriving from regions heavily burdened by drug resistance. The Gulf countries are increasingly important trade and tourist hubs, as well as sources and, at the same time, targets of medical tourism. All these facts promote the intensive exchange of not only people, but at the same time microorganisms, as well. While the curative health care is of very high standards and ready to face the challenges implied, it is often not matched with an equally developed microbial/antibiotic surveillance system.

Although specific data are sparse, they indicate that *A. baumannii* is an increasing problem in the region. It was the most common (40.9%) of nosocomial MDR isolates from ICUs in Riyadh Military Hospital, more common than *K. pneumonieae* (19.4%) and *P. aeruginosa* (16.3%) (Saeed, et al. 2010). It was commonly found among infected diabetics in Saudi Arabia with a new clone carrying a new OXA-51 variant (OXA-131) dominating (Alsultan, et al. 2009). An epidemic of *A. baumannii* in a trauma ICU in Qatar was described with isolates resistant to all antibiotics except amikacin (El Shafie, et al. 2004). More recently, among the causative agents of bacteremia in Hamad General Hospital, Qatar 50% of all Acinetobacter spp. were found to be MDR (Khan, et al. 2010). In Oman *A. baumannii* was the second most common isolate from objects in hospital environment (21.3%) subsequent to *P. aeruginosa* (23%) and preceding *S. aureus* (6.6%) (Nzeako, et al. 2006). In the Kingdom of Bahrain a high rate of carbapenem resistance was associated with an extreme variety of carbapenem hydrolyzing enzymes (Mugnier, et al. 2009). Similarly high rate of carbapenem resistance, but susceptibility to tigecycline was found in Kuwait (Jamal, et al. 2009).

In the UAE, in Tawam Hospital *Acinetobacter spp.* was found to be the fourth most common agents of nosocomial bloodstream infections (7.8%), more common than *E. coli* (7.1%), *S. aureus* (6.0%) or *P. aeruginosa* (5.3%) (Jumaa and Neringer 2006). In 2006, a limited scale study found blaOXA-23 carrying strains present in the country. (Mugnier, Poirel et al. 2008). These data, although indicating the presence of the
problem but being short in molecular details prompted us to initiate an investigation aiming to study the molecular epidemiology of *A. baumannii* in one of the Gulf countries, in the UAE.
2. AIMS AND OBJECTIVES

The general, long term aims of our studies were to provide the first detailed insight into the molecular epidemiology of *A. baumannii* in the Abu Dhabi Emirate, UAE. By this way we anticipated to generate data not only for local use (i.e. to aid infection control practices in local hospitals) but also to provide data for a better understanding of the regional and global dynamics of spread of MDR *A. baumannii*.

The specific objectives were

- to compile a representative group of clinical isolates of *A. baumannii*
- to establish their antibiotic resistance
- to study the molecular background of the resistance phenotype, particularly as far as resistance to β-lactams and aminoglycosides are concerned
- to reveal any existing clonality among the strains with establishing indicators of inter-hospital transfers
- to compare the resistance and genetic make-up of sporadic and epidemic isolates
- and finally to investigate the presence of any of the fast-spreading metallo-beta lactamases, ESBLs and ribosomal methylases among local isolates
3. MATERIALS AND METHODS

3.1 Strain collection

Between March and November 2008, clinically relevant, non-repeat isolates of *A. baumannii* strains had been collected from five different hospitals in Abu Dhabi Emirate: Tawam, Al Ain, Mafraq, Sheik Khalifa and Al Rahba Hospitals, respectively. Initially, the strains were speciated by the laboratories of the participating hospitals using standard microbiological procedures (e.g. API or VITEK 2 identification panels). Clinical relevance (i.e. whether the strain was actually responsible for the symptoms of the patients or was a colonizer, only) was determined by the clinical microbiologists and attending physicians of the respective hospitals based on clinical details known only to them.

In order to look for the presence of MBLs, from 2009 till 2010 a second collection of *A. baumannii* strains had been compiled from Tawam hospital. This group contained clinically relevant carbapenem resistant isolates, only.

Upon receiving the strains the identity of the isolates was confirmed by a species specific PCR targeting the *bla*OXA51* gene (Turton, Woodford et al. 2006). All the strains were freshly suspended in Tryptic Soya Broth (TSB) (Oxoid) containing 10% glycerol and stored at -80 °C freezer.

3.2 Antibiotic sensitivity testing

The antibiotic disc diffusion tests were performed by the Kirby-Bauer method following the CLSI standards with discs purchased from MAST (CLSI 2010). Quantitative assays for imipenem, meropenem, ceftazidime, amikacin, gentamicin, tobramycin, kanamycin, streptomycin, netilmicin, spectinomycin, ciprofloxacin, tigecycline and colistin were conducted by E-tests (Biomérieux) according to the manufacturers instructions. In certain cases minimal inhibitory concentrations (MIC) were determined by microdilution carried out according to CLSI standards (CLSI
2010). For all assays *Escherichia coli* ATCC 25922 was used as a pan-susceptible control.

In case of colistin, for which no CLSI breakpoints exist, the EUCAST breakpoints (S ≤ 2 mg/L, R > 2 mg/L) were used (*EUCAST 2012*). In case of tigecycline, as neither CLSI nor EUCAST provides breakpoints, no susceptibility categories were established.

Non-susceptibility was defined as the combination of resistance and intermediate resistance.

3.3. Polymerase Chain Reaction (PCR)

Four-five colonies grown overnight on TSA plates were suspended in 200 µl of PCR quality distilled water (Sigma), vortexed for 10 seconds and centrifuged for 2 minutes at 13,000 rpm. The supernatant was used as the sample in PCR reactions directly or after being stored at -30 °C. PCR reactions were performed for 30-40 cycles at various annealing temperatures depending upon the melting temperature of the primer set used. The reactions were performed using the Applied Biosystems 2700 thermocycler with MicroAmp tubes. Amplicons were analyzed on 1-2 % agarose gels in the presence of ethidium bromide. The gels were photographed and scanned using the Biometra gel documentation system (Biometra, Gottingen, Germany).

The primers used in this study are shown in TABLE 3.1.
**TABLE 3.1. Primers used in the study**

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENCES (5' TO 3')</th>
<th>GENE</th>
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<td>Group1ompAF 306</td>
<td>GATGGCGTAAATCGTGTA</td>
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<td>Turton et al., 2007</td>
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<td>blaPER</td>
<td>Libisch et al. 2008</td>
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Acinetobacter baumannii in Abu Dhabi

Acinetobacter baumannii

PER R  GCGTCCATCAGGCAACA
OXA-69 A  CTAATAATTTGATCTACTCAAG
OXA-69 B  CCAGTGGATGGATGGATGATTATC
blaIMP-F  CTACCGCAGCAGAGTCTTTTG
blaIMP-R  AACCAGTTTGTCTTACCAC
blaVIM-F  AGTGGTGAGTATCCGACAG
blaVIM-R  ATGAAAGTGCGTGAGAGAC
NDM1- Fo  TGGCCAGCGACTTGGCCTTG
NDM1-Re  ACCGATGACCAGGCGCCCA

TO DETECT GENES OF AMINOGLYCOSIDE MODIFYING ENZYMES

aadA-F  TGATTTGCTGGTTACGGTGAC
aadA-R  CGCTATGTTCTCTTGCTTTTG
ant(2')-Ia-F  ATCTGGCCGCTCTGGAT
ant(2')-Ia-R  CGAGCCTGTAGGACT
aph(3')-VI-F  CGGAAACAGCGGTTTTAGA
aph(3')-VI-R  TTCCTTTTGTCAGGTC
rrn-F  GAGGAAGTTGGGATGACGT
rrn-R  AGGCCCGGAAGCAGTATTCA
aac(3)-Ia-F  GACATAAGCCTGTTCGTT
aac(3)-Ia-R  CTCCGAACTCACGACGA
aac(3)-Iia-F  ATGCATACGCGGAAGGC
aac(3)-Iia-R  TGCTGGCACGATCGGAG
aac(6')-Ib-F  TATGAGTGGCTAAATCGAT
aac(6')-Ib-R  CCCCGCTTTCTGACCA

DETECT GENES OF 16S RIBOSOMAL SUBUNIT MODIFYING ENZYMES

armA-F  TATGGGGTCTTACTATTCGCTCT
armA-R  ATTCCTACTATTCGTCT

armA-F  TCTTCCATTTCTCTCCTTCTT
armA-R  TCTTCCATTTCTCTCCTTCT

rmrA-F  CTAGCGTCCATTTCTCT
rmrA-R  TTTGCTTCCATTTCTCT

rmrB-F  TCAACGATGCCTACCTCTCTC
rmrB-R  GCGGGCAAGGTAACCTCC

rmrC-F  GCCAAAGTACTCAAGGTG
rmrC-R  CTCAGATCTGACCAACAAG

rmrD-F  CTGTTGAGCCAGCGGAGAC
rmrD-R  GCGCTCCATCCATGCTGATAG

Heritier et al. 2005
Turton et al. 2004, Senda et al. 1996
Turton et al. 2004
Ghazawi et al. 2012
Clark, et al. 1999
Akers, et al. 2010
Turton et al 2004, Senda et al. 1996
Ghazawi et al, 2012
Fritsche, et al. 2008

- 33 -
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FOR MULTILOCUS SEQUENCE TYPING (MLST)

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PRIMERS USED FOR EFFLUX PUMPS.

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Srinivasan et al. 2009
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**MISCELLANEOUS PRIMERS**

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<td>Strain clonality</td>
<td>Duan et al, 2009</td>
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3.4. Plasmid profile analysis

For routine detection and isolation of large and small plasmids the method of *Kado and Liu 1981* was used with minor modifications. The strains were inoculated on a half TSA plates to obtain confluent growth and incubated overnight at 37°C. Next day cells were collected by toothpicks from an area of approximately 4 cm² and suspended gently into 250 µl of lysing solution (3% SDS, 50 mM Tris, pH 12.57), and mixed by gentle agitation until the suspension became homogenous and viscous. The lysed cells were incubated at 60°C in a thermoblock for 45 minutes with gentle mix every 15 minutes. After the incubation period one volume of 1:1 phenol-chloroform was added. The solution was emulsified by gentle shaking. The emulsion was broken by centrifugation at 13,000 rpm for 15 minutes. The top aqueous layer (approximately 60 µl) was transferred to clean tubes without disrupting the precipitate at the interface. Samples were subjected to electrophoresis for 3 hours at 120 V in 0.8% agarose gel. The gels were stained with ethidium bromide, destained and scanned using the Biometra gel documentation system.

3.5. Detection of megaplasmids by S1 nuclease digestion

For very large plasmids the methods described by *Basta et al. 2004* and *Barton et al. 1995* were followed with modification. In brief, the strains were grown overnight in 15 ml TSB at 37°C with shaking with or without antibiotics, as needed. Next day under photometrical control at 600 nm the density of the cultures was set and approximately 4 x10⁹ cells were collected by centrifugation. The cells were washed with washing buffer containing 1 M NaCl in 10 mM Tris-HCL and resuspended in 500 µl of EC buffer (1 M NaCl, 100 mM EDTA, 6 mM Tris-HCL, 0.5% N-lauroyl sarcosine, 0.2% deoxycholate). The cell suspensions were mixed with an equal volume of 1% PFGE sample preparation agarose (Sigma) containing 20 µg/ml RNase and 1 mg/ml lysozyme. Five hundred microliters of the mixture was immediately transferred to 1 ml syringes. After 10 min at 4°C, the solidified plugs were sliced by a razor blade into 1 mm thick slices. To lyse the cells the plugs were incubated in 1 ml of EC buffer (which, at this time contained 20 µg/ml RNase and 1 mg/ml lysozyme) at 37°C for 1 hour. Subsequently, the plugs were incubated at 50°C.
C overnight in ES buffer (1% N-lauroylsarcosine in 0.5 M EDTA) supplemented with 1mg/ml proteinase K). The ES buffer and the proteinase were inactivated by washing the plugs twice at 37° C for 45 min in 1ml of 1mM phenylmethylsulfonyl fluoride (PMSF, Sigma) prepared in TE buffer (10 mM Tris, 1 mM EDTA). To remove the traces of PMSF, the plugs were washed twice with TE buffer at 37° C for 45 minutes.

In order to linearize the circular megaplasmids, the incorporated DNA within the plugs was digested with 1 U of S1 nuclease (Sigma) in 200 µl of S1 buffer (50 mM NaCl, 30 mM sodium acetate [pH 4.5], 5 mM ZnSO4) for 10 min at 37° C subsequent to an initial incubation in 10 mM Tris for 15 minutes at room temperature (RT). The reaction was stopped by adding 100 µl of cold ES buffer to the plugs. After 15 minutes of incubation on ice, the plugs were loaded into wells of a 1% horizontal agarose gel (PFGE running agarose, Sigma). Lambda concatamer (New England Biolabs) was used as size marker loaded into the two lateral wells of each gel. The gel was placed in 0.5X TBE at 14°C in a contour-clamped homogeneous electric field of a CHEF DNA Mapper apparatus (BioRad) and electrophoresed for 18 hrs on 6 V/cm constant voltages with a pulse time increasing from 5 sec to 25 sec. After electrophoresis, gels were stained with ethidium bromide for 20 min, destained in 500 ml of sterile Milli-Q water for 20 min and scanned under UV light.

3.6. Conjugation

For conjugation experiments *E. coli* RAZ (Na-azid resistant in-house derivative of the rifampin resistant J53) and an inhouse derivative of *A. baumannii* BM4547 rif (kindly provided by Dr. Patrice Nordmann, Paris) and also made resistant to Na-azid (BM4547 RAZ) were used as recipients. Conjugation was attempted as follows: 4 hour old fresh TSB cultures of the donor and recipient were combined in 1: 5 ratio in a 50 ml Falcon Tube (BD) in a 5 ml final volume. The mixture was incubated without shaking for another 4 hours, then it was centrifuged at 3500 rpm for 15 minutes. The pellet was resuspended in 200 µl TSB and added as a drop to the center of TSA plate without any antibiotics. The next day, the growth was harvested in 5 ml PBS, centrifuged and washed once in PBS. The pellet then was resuspended in 3 ml of PBS, serially diluted and plated onto plates containing 100 µg/ml Na-azide and the
appropriate antibiotics. Next day colonies were collected and subjected to repeated antibiotic susceptibility tests, plasmid electrophoresis, PCR and, if necessary, to ERIC and PFGE to confirm that indeed, transconjugants, instead of mutants of the donor or recipient were obtained. If needed, conjugation was repeated at various temperatures (30° and 37° C).

3.7. Molecular fingerprinting of the isolates

For the macrorestriction analysis of the strains by pulsed field gel electrophoresis (PFGE) the method of Seifert et al. was followed (Seifert, et al. 1994) with minor modifications. Bacteria cultured in 5 ml TSB overnight at 37 °C were harvested by centrifugation. The pellet was re-suspended and washed 3 times in 1 ml 50mM Na-EDTA. After the last wash the pellet was re-suspended in 1 ml EC buffer (10 mM TRIS-HCl, 1 M NaCl, 100mM Na-EDTA, pH 8.0.). Two-hundred µl of that cell suspension was mixed with 300 µl of melted 1% PFGE sample preparation agarose (SIGMA) and immediately filled into 1 ml syringes. After 10 min at 4°C, the solidified plugs were sliced by a razor blade into approx. 1 mm thick slices.

To lyse the cells and to liberate the un-fragmented chromosome plug slices were transferred one by one into Eppendorf tubes containing 475 µl of cell lysis buffer (50 mM Tris-HCl, 50 mM EDTA pH 8,0, 1% Sarcosyl) and 25 µl of proteinase K (GIBCO) and were incubated overnight at 55 ºC. After cell lysis, agarose plugs were transferred into 50 ml centrifuge tubes and washed twice at 50 oC with 20 ml of sterile, pre-heated Milli-Q water followed by four times washing with 10 ml of sterile pre-heated TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Each washing step lasted 15 minutes. The plugs were stored in 5 ml of TE buffer at 4°C.

Chromosomal DNA in the blocks was digested with 40 U ApaI restriction enzymes (New England Biolabs) overnight at 25°C in 100 µl of enzyme buffer provided with the enzyme. After digestion, the plugs were washed in 250 µl of 0.5X TBE for 5 min and were transferred into wells of a 1.4% horizontal agarose gel (PFGE running agarose, Sigma). Lambda concatamer (New England Biolabs) was used as size marker loaded into the two lateral wells of each gel. The gel was placed in 0.5X
TBE at 14°C in a contour-clamped homogeneous electric field of a CHEF DNA Mapper apparatus (BioRad) and electrophoresed for 22 hrs on 6 V/cm constant voltage with a pulse time increasing from 0.45 sec to 30 sec. After electrophoresis, the gel was stained with ethidium bromide for 20 min, destained in 500 ml of sterile Milli-Q water for 20 min and photographed under UV light. The genomic patterns were analyzed with the GelCompar II program (Applied Maths). The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree graphically showing the level of relatedness between the isolates was created based on the Dice similarity coefficient (SD) (Dice, with 1% position tolerance) (Tibayrenc and Ayala 2000). Strains showing patterns with SD >= 85% were arbitrarily considered to represent a pulsotype.

**Multilocus Sequence Typing (MLST)** of the isolates was carried out routinely by the method of Bartual et al. (2005). Genomic DNA was prepared by boiling 4-5 colonies in 200 µl of sterile water. PCRs for the seven housekeeping genes, *gltA, gyrB, gdhB, recA, cpn60, gpi*, and *rpoD*, were performed in 25-µl volumes containing 5 µl Q buffer, 2.5 µl 10x buffer supplemented with 1.5 mM MgCl2, 800 µM PCR nucleotide mix, and 1.25 U Taq DNA polymerase (Qiagen). The primers are listed in **TABLE 3.1.**

The products were purified using the Exo-SAP (Usb) and were sequenced in both directions using 3130X genetic analyzer (Applied Biosystems). The sequence results were analyzed using the online BLAST ([http://www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)). The isolates were then assigned to sequence types (STs) using the tools on the *A. baumannii* MLST webpage ([http://pubmlst.org/abaumannii/](http://pubmlst.org/abaumannii/)). It should be noted that in one case the MLST type of our NDM-2-expressing isolate was also determined by an alternative typing system (Diancourt et al, 2010) by our collaborators in Paris.

**The Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR)** approach was used to quickly verify differences between donor, recipient and transconjugants or transformants. It was carried out by using the ERIC2 primer (**TABLE 3.1**) with amplification parameters as described (Duan et al, 2009). Comparison between amplicon patterns was done by visual inspection.
3.8 Cloning of bla<sub>NDM</sub>

Genomic DNA was purified using the Qia Amp DNA mini kit (Qiagen), and partially digested with HindIII (New England Biolabs) for 30 seconds, 1, 2, 4, and 10 minutes. The resulting fragments were purified using the PCR purification kit (Qiagen) and eluted in 50 µl of elution buffer. The purified product was then ligated into pUC19 using the T4 DNA ligase (NEB) overnight at 16° C. Before ligation the vector had been linearized by HindIII and treated with calf intestinal alkaline phosphatase (NEB).

The ligation mixture was transformed into <i>E. coli</i> DH5α competent cells. In brief, the ligation mixture was added to the thawed competent cells. Incubated on ice for 20 minutes, the cells were heat shocked at 42 ° C for 2 minutes, followed by adding them to 1 ml of TSB and shaking at 37° C from 45 minutes to 1 hour. The cultures were plated onto plates containing 8 µg/L ceftazidime. Next day, the colonies were screened for bla<sub>NDM</sub> by PCR, and the positive clones were digested with HindIII to estimate the size of the insert.

Of a clone carrying the plasmid p132LigB with an insert of ca. 10 kb containing the bla<sub>NDM</sub> gene was identified. A 3675-bp long partial sequence of the insert covering the flanking regions of the bla<sub>NDM</sub> gene (GenBank accession number JN112341) was generated by primer walking and subsequently confirmed by direct, bidirectional sequencing with primers ASndm1 (5’- GTCGCAAAGCCCAGCTTCGCA-3’) and ASndm2 (5’- GCCTCGCATTTGCGGGGTTTTTA-3’).

3.9. Sequencing

PCR products were purified either by the EXO-SAP or PCR purification kit (Qiagen) prior to sequencing. Sequencing was done using the Big Dye Cycle Terminator V.3.1 (Applied Biosystems) according to the manufacturer's instructions. The samples were sequenced in both directions using the 3130X Genetic Analyzer (Applied Biosystems). Sequences were analysed by using the MEGA4
Acinetobacter baumannii in Abu Dhabi

(http://www.megasoftware.net/mega4/mega.html) and Clone Manager (Sci-Ed software) softwares.

3.10. Southern blot

Plasmid gels (0.8-1.2%), prepared either by the methods of Kado (Kado and Liu 1981) or by S1 digestion (Basta et al. 2004, Barton et al. 1995) were stained by ethidium bromide and photographed and scanned using the Biometra gel documentation system with rulers placed around the gel for subsequent identification of bands.

The gels were depurinated in 0.25 M HCl, followed by denaturation in 0.5 M NaOH, 1 M NaCl and finally neutralized in 1M Tris, 0.6 M NaCl. All incubations were carried out at room temperature, twice, 15 minutes each with gentle shaking. The gel was capillary-transferred to Hybond N+ membranes (Amersham) by soaking overnight in 20X SSC. The next day the membranes were UV cross-linked at 70,000 micro-joules and the hybridization was performed by using the DIG DNA labeling and detection kit (Roche).

Probes for hybridization were generated by PCR amplification and gel purification, followed by quantitation using the ND-1000 spectrophotometer (Nano Drop Technologies, USA). The DNA fragments were labeled using the DIG DNA labeling kit. In brief, 200 ng of purified fragment was boiled for 10 minutes in a boiling water bath and quickly chilled on ice. The denatured DNA was conjugated according to the manufacturer’s directions. The blot was pre-hybridized at certain hybridization temperature calculated depending on the probe size and GC ratio (following the company's instruction) in a pre-warmed hybridization buffer (5X SSC 1% blocking solution, 0.1 % N-lauryl sarcosine 0.02% SDS) for 30 minutes followed by the addition of the buffer containing the probe. The blot was hybridized overnight at 48 °C with gentle shaking.

The next day, blots were washed twice with 2XSSC / 0.1% SDS at room temperature for 5 minutes followed by two subsequent washing in 0.1X SSC / 0.1 % SDS at 68 °C for 15 minutes with constant agitation. The membrane was briefly rinsed in
washing buffer at room temperature, incubated in blocking solution for 30 minutes, and again incubated in antibody solution provided with the kit for 30 min. The blot was washed twice in washing buffer, equilibrated in detection buffer and finally incubated without shaking in the color substrate solution for various lengths of times in the dark till the desired spot or band intensity was visualized. The blot was digitized using the Biometra gel documentation system.

3.11. Gene expression assay

In all assays targeting 16S ribosomal RNA genes was used as a constitutively expressed control. Bacterial RNA was isolated from cultures inoculated in 2 ml TSB at 37°C overnight with or without antibiotics using the Trizol Max bacterial RNA Isolation kit (Invitrogen Life Technologies). Bacterial cells were spun down, resuspended in 200 µl of preheated enhancement reagent (provided with the kit), and incubated at 95°C for 4 minutes. The cells were then lysed in 1 ml of Trizol. Briefly, cell lysates in Trizol were incubated at room temperature for 5 minutes followed by the addition of chloroform, vigorous shaking for 15 seconds and incubation at room temperature for 2-3 minutes. Phases were separated by centrifugation at 12,000 g for 15 minutes, and 90% of the aqueous phase (clear in color) was transferred to fresh Eppendorf tubes containing 500 µl of isopropanol to precipitate RNA. RNA was precipitated by mixing and incubating the tubes at room temperature for 10 minutes followed by centrifugation at 12,000 X g for 10 minutes. The pellets were washed once with 70% ethanol and centrifuged at 7,500 g for 5 minutes. Pelleted RNA was re-suspended in nuclease free water, its concentration determined spectrophotometrically in ND-1000 (Nano Drop Technologies, USA).

2.5 µg of the RNA was DNase-treated using 2-3 units of amplification grade DNase I (Invitrogen Life Technologies) at 37° C for 91 minutes. The DNase was heat inactivated at 65° C for 10 minutes after the addition of EDTA to 2.5mM. One µl of the DNase-treated RNA samples were tested in PCR using 16S primers (TABLE 3.1.) to confirm the absence of DNA prior to cDNA synthesis.

This was followed by cDNA synthesis by first denaturing the RNA in the presence of dNTPs and a random hexamer primer for 5 minutes at 65° C, followed by quick
cooling on ice for 5 minutes. After spinning the components to the bottom of the tube, cDNAs were generated by a reverse transcription reactions with 100 U of Moloney Murine Leukemia virus (MMLV) reverse transcriptase (Invitrogen Life Technologies) in the presence of 40 U of RNasin RNase inhibitor (Promega) for one hour at 42° C. cDNAs were amplified using the Qiagen Taq polymerase kit (Qiagen). PCR reactions were performed for 20-30 cycles at various annealing temperatures depending on the melting temperature of the primer set used.

3.12. Statistical analysis

The assessment of the association of specific genes and features with sporadic and epidemic isolates was carried out by the likelihood score test. As members of the epidemic clones represented closely related isolates they were re-weighted by the weight equal to the inverse of the size of the particular clone. The calculations were done using the Logistic Regression Module of the SPSS 19.0 program (Hosmer and Lemeshow 2000) by Professor Nico Nagelkerke (FMHS, UAEU).

3.13. Arbitrary definitions used

Strains were considered “multidrug resistant” if they were non-susceptible to carbapenems and to at least two non-beta lactam classes. A “sensitive” isolate was susceptible to carbapenems and to at least 3 other classes, while a “resistant isolate”, i.e. strains in between, were carbapenem susceptible, but non-susceptible to 3 other classes. A strain was regarded to have and epidemic potential ("epidemic strain" for this study) if at least one other isolate exhibited the same PFGE profile (with ≥85% similarity in the banding pattern) and carried the same blaOXA51-like gene. Strains clustered by these features were regarded as clones, while all other isolates were considered sporadic.
4. RESULTS

4.1. Molecular epidemiology of A. baumannii in Abu Dhabi hospitals

4.1.1. Establishment of the strain collection

After eliminating repeated and clinically non relevant isolates, altogether 110 A. baumannii strains recovered from Abu Dhabi hospitals between March and November, 2008 were included in the study. The distributions of the strains according to hospitals and according to sample types are shown on TABLES 4.1. and 4.2., respectively.

TABLE 4.1. Hospitals providing the strains

<table>
<thead>
<tr>
<th>HOSPITALS</th>
<th>Tawam</th>
<th>SKMC</th>
<th>Mafraq</th>
<th>Al Ain</th>
<th>Rahba</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>40</td>
<td>26</td>
<td>26</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>%</td>
<td>36.4</td>
<td>23.6</td>
<td>23.6</td>
<td>13.6</td>
<td>2.7</td>
</tr>
</tbody>
</table>

TABLE 4.2. Distribution of clinical samples

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Respiratory</th>
<th>Wound</th>
<th>Circulation*</th>
<th>Urine</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>38</td>
<td>33</td>
<td>16</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>%</td>
<td>34.5</td>
<td>30.0</td>
<td>14.5</td>
<td>11.8</td>
<td>9.1</td>
</tr>
</tbody>
</table>

*Circulation – blood culture + iv. lines, catheters

4.1.2. Clustering of the isolates and identification of epidemic strains

The genomic DNA of the strains was subjected to digestion with ApaI followed by pulsed field gel electrophoresis. Based on the banding patterns obtained, and using
an arbitrarily chosen 85% similarity threshold, 14 clusters (A-N) containing minimum two isolates were identified (FIGURE 4.1.).

The clusters altogether contained 87 strains while 23 isolates formed singletons. One-one clusters contained 19, 12 and 10 isolates, and two had 11 strains each, respectively. Of the eight smaller clusters two-two contained four, and five clusters contained 2 isolates, each.

PFGE patterns are determined by the physical location of restriction sites on the genomic DNA, only. Therefore, in order to establish groups containing isolates likely to represent real clones, this typing method was complemented by revealing the allelic type of the $bla^{OXA51-like}$ enzymes indigenous to all A. baumannii isolates (Heritier et al, 2005). Only those strains were considered to form a clone, which, beyond belonging to the same macrorestriction group, also carried the same allele of the gene indicating that they are member of the same lineage (Evans et al, 2008).

Thirty-eight strains belonging to 5 clusters carried $bla^{OXA69}$, 14 isolates grouped into 2 clusters carried $bla^{OXA64}$ and 28 strains forming four clusters had $bla^{OXA66}$. It was noteworthy that clones carrying the same $bla^{OXA51-like}$ alleles were found next to each other on the PFGE similarity tree (FIGURE 4.2.). Seven isolates, originally recorded among cluster-forming strains, were, as not fulfilling the above criteria, re-assigned to the singletons' pool.

Based on these data we had 80 isolates forming 11 clones we considered to have epidemic potentials as their representative have been repeatedly isolated during the study period. There were single clones with 19, 12 and 11 members, and two-two clones with 10, 4, 3 and 2 strains, each, respectively. These isolates will be referred to as "epidemic" strains. On the contrary, the 30 singletons, as representing the only isolates recovered exhibiting their respective types, will be called "sporadic" isolates in this thesis (FIGURE 4.2.).
FIGURE 4.1. Macrorestriction clusters of the isolates
FIGURE 4.2. Distribution of epidemic clones and sporadic isolates


The bla_{OXA51} alleles are shown. Dotted line marks 85% similarity threshold.
4.1.3. Duration of clonal presence and indications of inter-hospital transfer

Representatives of the larger clones (i.e. clones D, F, H, J and N) have been present for several months in Abu Dhabi hospitals although some, e.g. J and F exhibited clear peaks (FIGURE 4.3.).

FIGURE 4.3. Distribution of epidemic clones over time
The distribution of epidemic and sporadic strains according to hospitals and clinical samples are seen on TABLES 4.3. and 4.4. The majority of strains from the three large tertiary care hospitals (Tawam, SKMC and Mafraq) qualified as epidemic isolates, while from Al Ain and Raqba hospitals mostly sporadic isolates were recovered.

TABLE 4.3. Distribution of epidemic and sporadic isolates in the hospitals

<table>
<thead>
<tr>
<th>HOSPITALS</th>
<th>Tawam</th>
<th>SKMC</th>
<th>Mafraq</th>
<th>Al Ain</th>
<th>Rahba</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>%*</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Epidemic</td>
<td>30</td>
<td>19</td>
<td>24</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>75.0</td>
<td>73.1</td>
<td>92.3</td>
<td>40.0</td>
<td>33.3</td>
<td></td>
</tr>
<tr>
<td>Sporadic</td>
<td>10</td>
<td>7</td>
<td>2</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>25.0</td>
<td>26.9</td>
<td>7.7</td>
<td>60.0</td>
<td>66.7</td>
<td></td>
</tr>
</tbody>
</table>

* % within the particular hospital, SKMC: Sheik Khalifa Medical City

With the exception of doublet clones (B and L), members of all other clones were recovered from multiple hospitals. Nevertheless, for each of the larger clones a "preferred-hospital" (i.e. where it was mostly isolated from) could clearly be identified. Members of clones D and H have mostly been recovered from Tawam Hospital, those of clone F came mostly from Mafraq Hospital, while clones J and N contained mostly Sheik Khalifa Hospital isolates. (FIGURE 4.2.).

Almost 40% (38.8%) of the epidemic strains derived from respiratory samples and 81.6% of all respiratory isolates represented types repeatedly isolated during the study period. On the other hand approximately the same percentage (36.7%) of the sporadic isolates came from wound samples, but still, the majority of the strains of this origin (22, 66.7% of this sample type) also represented epidemic isolates. The specimen numbers for other sample types were too low to recognize clear trends but even with the larger groups no statistically significant association was observed with the epidemic/sporadic nature of the isolates and the sample type (TABLE 4.4.).
4.1.4. Genotypes of the isolates

Based on 24 genes and gene-combinations (e.g. ISAb1-bla\textsubscript{OXA23}) related to resistance to β-lactam and aminoglycoside antibiotics the genotype of strains was established. The eleven epidemic clones represented 9 core genotypes, i.e. a set of genes present in >50% of the members of the cluster. Clones C and D, and E and F shared the same core genotypes (TABLE 4.5.). On the contrary, the 30 sporadic isolates exhibited a considerable genetic variability carrying 21 genotypes (TABLE 4.6.). Of the 24 genes and gene-associations tested, while epidemic clones carried, as an average 7.9 of them, the respective figure for the average genotype of the sporadic strains was 3.8.

The distribution of the various genes is shown in TABLES 4.7. and 4.8., respectively. No bla\textsubscript{OXA-24}, rmt and npm genes were detected and bla\textsubscript{OXA28} was found only in three sporadic isolates. Of the β-lactam resistance genes bla\textsubscript{OXA-23}, blaper, while of the aminoglycoside resistance genes aac(3)-Ia, aph(3')-VI, ant(2')-Ia, armA were significantly more characteristic to epidemic isolates. Similarly, int and ISAb1 were significantly more commonly found among epidemic strains than among their sporadic counterpart. However, if the presence of ISAb1 in a sporadic isolate was accompanied by the presence of bla\textsubscript{OXA-23} they were linked in all three isolates. While of the β-lactam resistance genes only the ISAb1-linked bla\textsubscript{AmpC} was found in approximately equal ratios in both groups, several of the aminoglycoside genes were
encountered with the same frequencies in the isolates irrespective of their epidemiological fitness.
**Table 4.5.** Genotypes of epidemic strains

<table>
<thead>
<tr>
<th>Clone</th>
<th>CORE GENOTYPE*</th>
<th>ADDITIONAL GENES **</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B</strong></td>
<td>OXA23 Int ISAb1 ISAb1-23 aadA aac(3)-Ia (aac(6')-Ih)</td>
<td>aph(3')-Ia aph(3')-VI ant(2')-Ia</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>OXA23 Int ISAb1 ISAb1-23 aadA aac(6')-Ih aph(3')-Ia aph(3')-VI</td>
<td></td>
</tr>
<tr>
<td><strong>D</strong></td>
<td>OXA23 Int ISAb1 ISAb1-23 aadA aac(6')-Ih aph(3')-Ia aph(3')-VI</td>
<td></td>
</tr>
<tr>
<td><strong>E</strong></td>
<td>OXA23 ISAb1 23-ISAb1-23 aph(3')-VI ant(2')-Ia</td>
<td></td>
</tr>
<tr>
<td><strong>F</strong></td>
<td>OXA23 ISAb1 23-ISAb1-23 aph(3')-VI ant(2')-Ia</td>
<td>Int aac(6')-Ih aph(3')-Ia</td>
</tr>
<tr>
<td><strong>H</strong></td>
<td>OXA23 Int ISAb1 ISAb1-23 Per aac(3)-Iia armA strAB</td>
<td></td>
</tr>
<tr>
<td><strong>I</strong></td>
<td>OXA23 Int ISAb1 ISAb1-23 Per armA strAB</td>
<td>aac(3)-Iia</td>
</tr>
<tr>
<td><strong>J</strong></td>
<td>OXA23 Int ISAb1 ISAb1-23 ISAb1-AmpC aadA aac(6')-Ib</td>
<td>aph(3')-Ia armA strAB ISAb1-51 aac(6')-Ih</td>
</tr>
<tr>
<td><strong>L</strong></td>
<td>OXA23 Int ISAb1 ISAb1-23 ISAb1-AmpC aadA aac(6')-Ib aac(6')-Ih</td>
<td>aph(3')-Ia armA strAB</td>
</tr>
<tr>
<td><strong>M</strong></td>
<td>OXA23 Int ISAb1 ISAb1-23 ISAb1-AmpC Per aac(3)-Ia</td>
<td>strAB aadA aac(6')-Ib aph(3')-Ia armA</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>OXA23 Int ISAb1 ISAb1-23 ISAb1-AmpC aac(3)-Ia</td>
<td>strAB Per aac(6')-Ib aph(3')-Ia armA</td>
</tr>
</tbody>
</table>

* Genes present in >50% of the clone members was considered as the core genotype of the clone

**Additional genes present in a few isolates of the clones, only
### TABLE 4.6. Genotypes of sporadic isolates

<table>
<thead>
<tr>
<th>TYPE</th>
<th>GENOTYPES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>aadA</td>
</tr>
<tr>
<td>3</td>
<td>OXA 58</td>
</tr>
<tr>
<td>4</td>
<td>aph(3')-VI</td>
</tr>
<tr>
<td>5</td>
<td>Int</td>
</tr>
<tr>
<td>6</td>
<td>ISAb1</td>
</tr>
<tr>
<td>7</td>
<td>aac(6')-Ih</td>
</tr>
<tr>
<td>8</td>
<td>ISAb1</td>
</tr>
<tr>
<td>9</td>
<td>aac(6')-Ib</td>
</tr>
<tr>
<td>10</td>
<td>OXA 23</td>
</tr>
<tr>
<td>11</td>
<td>ISAb1</td>
</tr>
<tr>
<td>12</td>
<td>OXA 23</td>
</tr>
<tr>
<td>13</td>
<td>ISAb1</td>
</tr>
<tr>
<td>14</td>
<td>OXA 23</td>
</tr>
<tr>
<td>15</td>
<td>ISAb1</td>
</tr>
<tr>
<td>16</td>
<td>Int</td>
</tr>
<tr>
<td>17</td>
<td>Int</td>
</tr>
<tr>
<td>18</td>
<td>Int</td>
</tr>
<tr>
<td>19</td>
<td>OXA23</td>
</tr>
<tr>
<td>20</td>
<td>Int</td>
</tr>
<tr>
<td>21</td>
<td>OXA23</td>
</tr>
</tbody>
</table>
TABLE 4.7. Distribution of $\beta$-lactam resistance genes, genes related to drug resistance

<table>
<thead>
<tr>
<th></th>
<th>blaOXA-23</th>
<th>Int</th>
<th>ISAb1</th>
<th>ISAb1-blaOXA-23</th>
<th>ISAb1-blaAmpC</th>
<th>blaPER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n %</td>
<td>n %</td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>ALL</td>
<td>84 76.4</td>
<td>69  62.7</td>
<td>93  84.5</td>
<td>81 98.8</td>
<td>34 31.8</td>
<td>16 14.5</td>
</tr>
<tr>
<td>SPORADIC</td>
<td>5.0 16.7</td>
<td>9   30.0</td>
<td>13  43.3</td>
<td>3 100</td>
<td>7  25.9</td>
<td>0  0.0</td>
</tr>
<tr>
<td>EPIDEMIC</td>
<td>79 98.8</td>
<td>60  75.0</td>
<td>80  100.0</td>
<td>78 98.7</td>
<td>27 33.8</td>
<td>16 20.0</td>
</tr>
<tr>
<td>$p*$</td>
<td>&lt;0.0001</td>
<td>0.007</td>
<td>0.003</td>
<td>1.000</td>
<td>0.571</td>
<td>0.008</td>
</tr>
</tbody>
</table>

* significance, ** of those carrying both genes

TABLE 4.8. Distribution of aminoglycoside resistance genes

<table>
<thead>
<tr>
<th></th>
<th>aadA</th>
<th>aac(3)-Ia</th>
<th>aac(3)-Ila</th>
<th>aac(6')-Ib</th>
<th>aac(6')-Ih</th>
<th>aph(3')-Ia</th>
<th>aph(3')-VI</th>
<th>ant(2')-Ia</th>
<th>armA</th>
<th>strAB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n %</td>
<td>n %</td>
<td>n %</td>
<td>n %</td>
<td>n %</td>
<td>n %</td>
<td>n %</td>
<td>n %</td>
<td>n %</td>
<td>n %</td>
</tr>
<tr>
<td>ALL</td>
<td>38  34.5</td>
<td>14  12.7</td>
<td>7  6.4</td>
<td>16  14.5</td>
<td>25  22.7</td>
<td>39  35.5</td>
<td>39  35.5</td>
<td>25  22.7</td>
<td>30  27.3</td>
<td>51  46.4</td>
</tr>
<tr>
<td>SPORADIC</td>
<td>7   23.3</td>
<td>0  0.0</td>
<td>0 0.0</td>
<td>2  6.7</td>
<td>6  20.0</td>
<td>7  23.3</td>
<td>3  10.0</td>
<td>1  3.3</td>
<td>2  6.7</td>
<td>9  30.0</td>
</tr>
<tr>
<td>EPIDEMIC</td>
<td>31  38.8</td>
<td>14  17.5</td>
<td>7  8.8</td>
<td>14  17.5</td>
<td>19  23.8</td>
<td>32  40.0</td>
<td>35  43.8</td>
<td>24  30.0</td>
<td>28  35.0</td>
<td>42  52.5</td>
</tr>
<tr>
<td>$p*$</td>
<td>0.207</td>
<td>0.015</td>
<td>0.143</td>
<td>0.262</td>
<td>0.507</td>
<td>0.236</td>
<td>0.036</td>
<td>0.044</td>
<td>0.020</td>
<td>0.234</td>
</tr>
</tbody>
</table>

* significance
4.1.5. Antibiotic resistance of the isolates

In general, the strains were highly resistant to antibiotics. With the exception of colistin, at least 50% of all the strains were non-susceptible to any antibiotics for which break-point values were available (i.e. this excludes tigecycline) (TABLE 4.9.). Regarding colistin, only one non-susceptible epidemic strain with a slightly elevated MIC (i.e. 3 mg/L) was encountered. While non-susceptibility was common among the strains, it was significantly more frequently seen among epidemic isolates for some drugs reaching (ciprofloxacin), for several others (ceftazidime, meropenem, imipenem, gentamicin, trimethoprim-sulfamethoxazole) approaching 100%. As a consequence, 97.5% of the epidemic strains were considered MDR compared to 20% of the sporadic isolates, i.e. a highly significant difference.

The comparison of the MIC distribution of the isolates confirmed these observations. While in case of most drugs the MIC90 figures barely discriminated between the sporadic and epidemic group, the MIC50, and in few cases (ceftazidime, meropenem, imipenem, ciprofloxacin, gentamicin, spectinomycin) even the MIC10 figures clearly exhibited higher figures in the latter group (TABLE 4.10.).

It was noteworthy that against colistin there was practically no resistance, even the MIC10 figures were the same in both groups. For tigecycline, for which we could not assess clinically relevant susceptibility due to the lack of break-point values a small but notable difference between epidemic and sporadic existed. The former group required 3 mg/L for inhibiting 50%, and 1 mg/L for inhibiting 10% of the isolates, respectively contrasting the respective figures (0.75 and 0.25 mg/L) of the sporadic strains.
TABLE 4.9. Rate of antibiotic non-susceptibility

<table>
<thead>
<tr>
<th>STRAINS</th>
<th>CAZ (n%)</th>
<th>MER (n%)</th>
<th>IMI (n%)</th>
<th>AKC (n%)</th>
<th>GMC (n%)</th>
<th>TOB (n%)</th>
<th>CIP (n%)</th>
<th>DXT (n%)</th>
<th>STX (n%)</th>
<th>COL (n%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL</td>
<td>86 (78.2)</td>
<td>84 (76.4)</td>
<td>84 (76.4)</td>
<td>70 (63.6)</td>
<td>86 (78.2)</td>
<td>54 (49.1)</td>
<td>90 (81.8)</td>
<td>68 (61.8)</td>
<td>90 (81.8)</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>SPORADIC</td>
<td>8 (26.7)</td>
<td>6 (20.0)</td>
<td>5 (20.0)</td>
<td>7 (23.3)</td>
<td>3 (10)</td>
<td>10 (33.3)</td>
<td>10 (33.3)</td>
<td>11 (36.7)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>EPIDEMIC</td>
<td>78 (97.5)</td>
<td>78 (97.5)</td>
<td>65 (98.75)</td>
<td>79 (63.75)</td>
<td>51 (100)</td>
<td>80 (72.5)</td>
<td>79 (98.75)</td>
<td>1 (1.25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>0.023</td>
<td>0.001</td>
<td>0.718</td>
<td></td>
</tr>
</tbody>
</table>

CAZ - ceftazidime, MER - meropenem, IMI - imipenem, AKC - amikacin, GMC - gentamicin, TOB - tobramycin, CIP - ciprofloxacin, DXT - doxycycline, STX - trimethoprim-sulphamethoxazole, COL - colistin
TABLE 4.10. Distribution of MIC values of sporadic and epidemic strains

<table>
<thead>
<tr>
<th></th>
<th>CAZ</th>
<th>MER</th>
<th>IMI</th>
<th>CIP</th>
<th>AKC</th>
<th>GMC</th>
<th>STR</th>
<th>KAN</th>
<th>NET</th>
<th>SPEC</th>
<th>TOB</th>
<th>COL</th>
<th>TGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>3</td>
<td>0.25</td>
<td>0.25</td>
<td>0.13</td>
<td>3</td>
<td>0.5</td>
<td>12</td>
<td>2</td>
<td>1</td>
<td>16</td>
<td>0.75</td>
<td>0.38</td>
<td>0.5</td>
</tr>
<tr>
<td>SP</td>
<td>1</td>
<td>0.19</td>
<td>0.19</td>
<td>0.13</td>
<td>1.5</td>
<td>0.25</td>
<td>8</td>
<td>1.5</td>
<td>0.75</td>
<td>16</td>
<td>0.5</td>
<td>0.38</td>
<td>0.25</td>
</tr>
<tr>
<td>EPI</td>
<td>32</td>
<td>12</td>
<td>16</td>
<td>&gt;32</td>
<td>6</td>
<td>24</td>
<td>64</td>
<td>3</td>
<td>6</td>
<td>96</td>
<td>1.5</td>
<td>0.38</td>
<td>1</td>
</tr>
<tr>
<td>MIC50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>256</td>
<td>128</td>
<td>&gt;1024</td>
<td>&gt;256</td>
<td>8</td>
<td>256</td>
<td>3</td>
<td>0.5</td>
<td>3</td>
</tr>
<tr>
<td>SP</td>
<td>4</td>
<td>0.38</td>
<td>0.25</td>
<td>0.19</td>
<td>3</td>
<td>0.75</td>
<td>16</td>
<td>3</td>
<td>1.5</td>
<td>24</td>
<td>1</td>
<td>0.5</td>
<td>0.75</td>
</tr>
<tr>
<td>EPI</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;256</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;256</td>
<td>12</td>
<td>&gt;1024</td>
<td>192</td>
<td>0.5</td>
<td>3</td>
</tr>
<tr>
<td>MIC90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;256</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;256</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>SP</td>
<td>256</td>
<td>&gt;32</td>
<td>12</td>
<td>&gt;32</td>
<td>48</td>
<td>48</td>
<td>&gt;1024</td>
<td>&gt;256</td>
<td>6</td>
<td>&gt;1024</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>EPI</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;256</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;256</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

CAZ- ceftazidime, MER- meropenem, IMI- imipenem, CIP- ciprofloxacin, AKC- amikacin, GMC- gentamicin, STR- streptomycin, KAN- kanamycin, NET- netilmicin, SPEC- spectinomycin, TOB- tobramycin, COL- colistin

SP - sporadic strains, EPI- epidemic isolates
4.2. Aminoglycoside resistance associated with \textit{armA} ribosomal methylase

The presence of the \textit{armA} gene in several (30) isolates (TABLE 4.8.) coding for a ribosomal methylase raised the possibility of broad-range aminoglycoside resistance among these strains. The gene was part of the core genotype in clones H, I, J and L, respectively and several members of clones M and N, as well as 2 sporadic isolates (NM 83 and NM 109) also carried it (TABLES 4.5. and 4.6.). Therefore, for these isolates, the quantitative susceptibility testing was extended to other drugs of this class (i.e. streptomycin, kanamycin, netilmicin, and spectinomycin) although, in case of \textit{A. baumannii}, currently no CLSI interpretation criteria exist for them. The distribution of the quantitative figures for these drugs is also included in TABLE 4.10.

Of the 30 isolates (2 sporadic and 28 epidemic strains, respectively) 27 expressed a uniform aminoglycoside susceptibility pattern, shown in TABLE 4.11.

**TABLE 4.11.** Quantitative susceptibility figure of 28 out of 30 \textit{armA} carrying isolates

<table>
<thead>
<tr>
<th>AKC</th>
<th>GMC</th>
<th>STR</th>
<th>KAN</th>
<th>NET</th>
<th>SPEC</th>
<th>TOB</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;256</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
</tr>
</tbody>
</table>


As the presence of the \textit{armA} gene was associated earlier with paradoxical amikacin susceptibility pattern in \textit{A. baumannii} (Krahe et al. 2010), we tested our isolates for this phenomenon. Indeed, all 27 strains carrying the \textit{armA} and expressing the susceptibility pattern shown in TABLE 4.11. exhibited the halo of susceptibility
around a zone of resistance implying as if the bacterium resisted to a higher concentration (close to the disc) while was susceptible to a lower concentration far from the disc (FIGUER 4.4).

**FIGURE 4.4. Paradoxical amikacin susceptibility**

![GM - gentamicin, AK - amikacin](image)

The phenomenon was present only with amikacin. No other genes, but armA (e.g. no aminoglycoside degrading enzymes) could be associated with its expression. The two isolates carrying the armA gene but not exhibiting the susceptibility pattern characteristic to the expression of ribosomal methylases (TABLE 4.11.) did not show the paradoxical zone formation. When retesting subcultures taken from various parts of the culture (i.e. far from the zone, close to the zone of inhibition, from colonies within the zone of inhibition and from near the disc) the phenomenon was equally present (data not shown).

Attempts were made to correlate the phenomenon also with the presence of efflux pumps. Using primers specific to a variety of pump genes commonly found in *A. baumannii* (TABLE 3.1.) components of the adeABC and ABeM pumps (Vila, et al. 2007; Vila and Martinez 2008) were found in all strains exhibiting this pattern of amikacin susceptibility. As the nature of the phenomenon suggested concentration dependent response it was investigated whether the expression of the genes of armA, or those of the efflux pumps were affected by the concentration of amikacin. When testing the cDNA of epidemic strain NM 99 by semi-quantitative RT-PCR no effect was found on the amount of specific mRNA produced in response to exposure to
various concentrations of amikacin, neither in case of armA (FIGURE 4.5.) or any of the efflux genes tested (data not shown).

FIGURE 4.5. Expression of armA in response to various amikacin concentrations

As the armA gene may locate on a plasmid in A. baumannii (Cho et al. 2009) attempts were made to conjugally transfer the gene either into E. coli or into A. baumannii. However, irrespective of the conjugation temperature used, no transconjugants were obtained. Nevertheless, we tried to detect any plasmids in the two sporadic armA positive strains, and in representatives of various armA carrying clones (FIGURE 4.6.).
FIGURE 4.6. Plasmid profile and Southern hybridization of armA positive strains

While the sporadic strains and members of clones J, L, M, N carried plasmids of varying sizes, none of these, neither the chromosome gave a signal with the probe. On the other hand members of clones H and I contained a plasmid of ca. 200 kb which clearly gave a strong signal indicating the location of armA.
4.3. Plasmid-localization of \( bla_{PER7} \)

One member of the collection, an epidemic strain from clone H (NM55) had a pair (NM 128) having been isolated from the same patient four months apart. As coming from the same patient and exhibiting the same PFGE patterns (FIGURE 4.7.) they were considered isogenic and NM 128 was excluded from the previously presented analysis. However, while NM55 expressed the same resistance pattern as the rest of its clone, NM128 was more susceptible. It was particularly noteworthy that although both strains were resistant to carbapenems, it was only NM 55, which also exhibited resistance to ceftazidime (TABLE 4.12.).

Comparing the genotypes of the two isolates revealed that NM 55, like the majority of the members of its clone, carried the \( bla_{PER} \) gene while NM128 did not. As this gene codes for an ESBL with considerable 3\(^{rd}\) generation cephalosporinase activity we assumed that its loss in NM128 might be responsible for its susceptibility to cephalosporins, while its carbapenem resistance was maintained by \( bla_{OXA-23} \) still carried by the strain (TABLE 4.13.).

FIGURE 4.7.

Macro-restriction patterns of NM 55 and NM 128

\( A \) and \( D \) - MW controls, 
\( B \) - NM55, \( C \) - NM128
### TABLE 4.12. Antibiotic susceptibility of NM55 and NM128.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>CAZ</th>
<th>MER</th>
<th>IMI</th>
<th>TGC</th>
<th>CIP</th>
<th>COL</th>
<th>AKC</th>
<th>GMC</th>
<th>STR</th>
<th>KAN</th>
<th>NET</th>
<th>SPEC</th>
<th>TOB</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM 55</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>1</td>
<td>&gt;32</td>
<td>0.5</td>
<td>&gt;256</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>96</td>
<td>&gt;1024</td>
</tr>
<tr>
<td>NM 128</td>
<td>2</td>
<td>24</td>
<td>32</td>
<td>0.5</td>
<td>&gt;32</td>
<td>0.38</td>
<td>32</td>
<td>512</td>
<td>&gt;1024</td>
<td>&gt;256</td>
<td>256</td>
<td>128</td>
<td>48</td>
</tr>
</tbody>
</table>


### TABLE 4.13. Genotype of NM55 and NM128

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>int</th>
<th>ISAba1-blaOXA-23</th>
<th>blaper</th>
<th>aac(3)-Iia</th>
<th>armA</th>
<th>strAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM 55</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NM 128</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Meanwhile, by sequencing the gene, our collaborators in Edinburgh established that its actual allelic type was $\text{bla}_{\text{PER-7}}$.

We attempted to localize the gene $\text{bla}_{\text{PER-7}}$. Experiments to prove the possible plasmid localization of the gene by transferring it into a suitable recipient repeatedly failed. However, by conventional gel electrophoresis plasmids of around 200 kb (slightly less in NM128) were visible. In NM55 this plasmid strongly hybridized with a $\text{bla}_{\text{PER}}$ oligo probe indicating the localization of the gene (FIGURE 4.8. - A and B.) As simple plasmid extraction gels are unsuitable to accurately size plasmids in this molecular mass range, we assessed the size of these plasmids by S1 nuclease digested preparations separated in pulsed electric field. The gels revealed that NM 55 indeed carry a plasmid of ca. 200 kb, while the one in NM 128 was approximately 20 kb less (FIGURE 4.8. - C.).

**FIGURE 4.8.** Localization of $\text{bla}_{\text{PER-7}}$ on a plasmid

Panel A - Plasmid extract (Kado and Liu 1984), Panel B - $\text{bla}_{\text{PER-7}}$ Southern hybridization, Panel C – S1 nuclease digestion
4.4. \textit{bla\textsubscript{NDM-2}} in \textit{Acinetobacter baumannii}

4.4.1. Identification of \textit{bla\textsubscript{NDM}} carrying \textit{Acinetobacter baumanii}

To reveal whether the \textit{bla\textsubscript{NDM}}, i.e. a rapidly emerging MBL among Enterobacteriaceae penetrated the population of \textit{A. baumannii} strains in Abu Dhabi we screened a 155 strong pool of carbepenem resistant isolates collected between 2008 and 2010 in Tawam hospital, Al Ain, UAE. Using an in-house primer pair (TABLE 3.1.) two \textit{bla\textsubscript{NDM}} positive strains were identified.

Both strains were recovered from a 55-year old Egyptian female with a congenital single kidney. She had been treated for a metastatic colon carcinoma in 2004 in Egypt but due to the recurrence of the disease in 2006 she had received subsequent treatments at multiple centres in Cairo (Egypt), Beirut (Lebanon), Dubai and Al Ain (UAE). In March 2008, due to a urinary obstruction from the pelvic spread of the malignancy, she underwent surgery in Egypt with insertion of a metallic ureteric stent. This was followed by recurrent urinary tract infections caused by an ESBL-producing \textit{E. coli} and a multidrug-resistant \textit{P. aeruginosa}. She received treatment with ceftriaxone and subsequently with meropenem. In April 2009, she was admitted to Tawam Hospital because of an accidental dislodgement of the left solitary kidney nephrostomy. The first urine culture yielded \textit{A. baumannii}. During the following months, the same strain was repeatedly isolated from urine samples, but no antibiotics were given because she remained afebrile and clinically stable. The two isolates studied were from May (AG132) and August 2009 (AG124).

The two strains revealed the same susceptibility profile (TABLE 4.14.). Both isolates harbour the naturally occurring \textit{bla\textsubscript{OXA-70}} gene and were positive for \textit{IS\textsubscript{Aba1}}, which was not, however, identified upstream of the \textit{bla\textsubscript{OXA-70}} nor of the \textit{bla\textsubscript{AmpC}} genes. Both isolates were negative for all other carbapenemase genes tested by PCR (i.e. \textit{bla\textsubscript{OXA-23}}, \textit{bla\textsubscript{OXA-24}}, \textit{bla\textsubscript{OXA-58-like}}, \textit{bla\textsubscript{OXA-48/-181}}, \textit{bla\textsubscript{VIM}} and \textit{bla\textsubscript{IMP}}). Attempts to detect any of the ribosomal methylases genes, not infrequently associated with NDM-producing organisms (Karthikeyan et al, 2010) also yielded negative results.
Table 4.14. Antibiotic susceptibility of the $bla_{NDM}$ positive isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>IMI (mg/L)</th>
<th>MER (mg/L)</th>
<th>CAZ (mg/L)</th>
<th>CIP (mg/L)</th>
<th>GM (mg/L)</th>
<th>AM (mg/L)</th>
<th>COL (mg/L)</th>
<th>TG (mg/L)</th>
<th>SAM (mg/L)</th>
<th>AZT (mg/L)</th>
<th>DOX (mg/L)</th>
<th>CHL (mg/L)</th>
<th>STX (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG132</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>0.125</td>
<td>32</td>
<td>0.125</td>
<td>0.19</td>
<td>18</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>AG124</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>0.125</td>
<td>48</td>
<td>0.094</td>
<td>0.094</td>
<td>23</td>
<td>9</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*IMI* - imipenem; *MER* - meropenem; *CAZ* - cefazidime; *CIP* - ciprofloxacin; *GM* - gentamicin; *AM* - amikacin; *COL* - colistin; *TG* - tigecycline; *SAM* - ampicillin/sulbactam; *AZT* - aztreonam; *DOX* - doxycycline; *CHL* - chloramphenicol; *STX* - trimethoprim-sulphamethoxasol.

The two isolates exhibited the same macro-restriction pattern (FIGURE 4.9.). They belonged to the same MLST types irrespective of the typing system used, i.e. ST253 (Bartuel et al, 2005) or ST103 (Diancourt et al, 2010). Both of the isolates contained multiple plasmid bands ranging from 7- to 140-kb.

FIGURE 4.9. Pulsed field gel electrophoresis of the $bla_{NDM}$ positive isolates
4.4.2. Transferring \( \text{bla}_{\text{NDM}} \) from \textit{Acinetobacter baumanii}

Attempts were made to conjugally transfer the \( \text{bla}_{\text{NDM}} \) gene. Repeated experiments carried out at different temperatures to mobilise the gene into an \textit{E. coli} recipient were not successful. However, when using an \textit{A. baumannii} recipient (BM4547, kindly provided by Prof. P. Nordmann, Paris) made Na-azid resistant (BM4547-RAZ) transfer of the \( \text{bla}_{\text{NDM}} \) gene with concomittant increase of the carbapenem MIC was achieved (TABLE 4.15.).

Table 4.15. Carbapenem susceptibilities of the donors, the recipient and a few derivatives

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>NOTE</th>
<th>IMIPENEM</th>
<th>MEROPENEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG124</td>
<td>Donor</td>
<td>32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>AG132</td>
<td>Donor</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>BM4547 RAZ</td>
<td>Recipient</td>
<td>0.125</td>
<td>1</td>
</tr>
<tr>
<td>BM4547RAZ-AG124/2</td>
<td>Derivative</td>
<td>8</td>
<td>&gt;32</td>
</tr>
<tr>
<td>BM4547RAZ-AG124/4</td>
<td>Derivative</td>
<td>16</td>
<td>&gt;32</td>
</tr>
<tr>
<td>BM4547RAZ-AG132/2</td>
<td>Derivative</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>BM4547RAZ-AG132/3</td>
<td>Derivative</td>
<td>8</td>
<td>32</td>
</tr>
</tbody>
</table>

However, while the derivatives were all positive by PCR targeting \( \text{bla}_{\text{NDM}} \) (FIGURE 4.10. - C) and exhibited the same PFGE pattern as the recipient (FIGURE 4.10. - B), this was not accompanied by a simultaneous transfer of any detectable plasmid (FIGURE 4.10. - A).

The S1 nuclease digestion method to reveal any plasmids in the derivative did not yield a positive result, either (FIGURE 4.11.).
FIGURE 4.10. Transfer of $\textit{bla}_{\text{NDM}}$

Panel A - Plasmid extract (Kado and Liu 1984), Panel B - $\textit{bla}_{\text{NDM}}$ PCR, Panel C - Pulse Field Gel Electrophoresis (PFGE). D - donor, R - recipient, D1 and D2 - derivatives

FIGURE 4.11. Plasmid detection in $\textit{bla}_{\text{NDM}}$ - carrying derivatives by the S1 nuclease method

Da and Db - donors, R - recipient, D1-3 - derivatives
In the absence of detectable plasmid transfer accompanying the uptake of the \( \textit{bla}_{\text{NDM}} \) gene naturally occurring transformation from DNA from the disintegration donor during co-incubation with the recipient was assumed. However, repeated attempts to model the transformation with DNA prepared by a variety of methods and using recipient made competent by different techniques, and even applying electroporation remained unsuccessful.

4.4.3. Cloning and characterization of the \( \textit{bla}_{\text{NDM}} \) gene from \textit{Acinetobacter baumannii}

In order to determine the genetic structure surrounding the \( \textit{bla}_{\text{NDM}} \) gene, DNA from strain AG132 was partially digested with HindIII (New England Biolabs NEB, USA) and ligated using T4 DNA ligase (NEB) into pUC19. The ligation mixture was transformed in \textit{Escherichia coli} DH5\( ^{\alpha} \) followed by plating onto plates containing 8 \( \mu \text{g/L} \) ceftazidime. A clone carrying the plasmid p132LigB with an insert of c. 10 kb containing the \( \textit{bla}_{\text{NDM}} \) gene was identified. \textbf{TABLE 4.16.} shows the antibiotic susceptibility of the clone in comparison with pUC19 and AG132.

\textbf{TABLE 4.16.} Antibiotic susceptibility of the transformant

<table>
<thead>
<tr>
<th>STRAINS</th>
<th>NOTE</th>
<th>MIC (mg/L)</th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IMI</td>
<td>MER</td>
<td>CAZ</td>
<td></td>
</tr>
<tr>
<td>AG132</td>
<td>Donor</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;256</td>
<td></td>
</tr>
<tr>
<td>pUC19</td>
<td>Recipient</td>
<td>( \leq )0.125</td>
<td>( \leq )0.125</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>p132LigB</td>
<td>Transformant</td>
<td>1</td>
<td>2</td>
<td>&gt;256</td>
<td></td>
</tr>
</tbody>
</table>

\textit{IMI} - imipenem, \textit{MER} - meropenem, \textit{CAZ} - ceftazidime
A 3675-bp long partial sequence of the insert covering the flanking regions of \textit{bla} \textsubscript{NDM} (GenBank accession number JN112341) was generated by primer walking and subsequently confirmed by direct, bidirectional sequencing.

Detailed sequence analysis identified that the strain carries the \textit{bla} \textsubscript{NDM-2} allele (Kaase \textit{et al} 2011) which, however, did not contain the silent A $\rightarrow$ G substitution at position 468 found in the Israeli isolate (Espinal \textit{et al}, 2011). Insertion sequence ISAba125 was found upstream of the NDM-2 gene while downstream a putative bleomycin resistance gene and a phosphoribosyl anthralinate isomerase gene were found (FIGURE 4.12.).

**FIGURE 4.12.** Genetic surrounding of the \textit{bla}NDM-2 gene in \textit{A. baumannii} AG132

\[
\begin{align*}
\text{P} & \quad \text{IS} \quad \text{ble} \quad \text{\Delta trpF} \\
\text{ISAba125} & \quad \text{bla} \text{NDM2} & \quad \text{ble} & \quad \text{\Delta trpF} \\
3675 \text{ bp} & \\
680 \text{ bp} & \\
\end{align*}
\]

\text{P} - promoter, IS – Insertion sequence, ble – Bleomycin, \Delta trpF - phosphoribosyl anthralinate isomerase

The sequence of the \textit{bla}NDM-2 gene, as well as that of its flanking regions was deposited to GenBank with accession No. JN112341
5. DISCUSSION

In 2000, the WHO issued a rather dramatic warning regarding the drugs, i.e. antibiotics, having been considered for long by many as the "magic bullets" ending all misery brought onto mankind by infections. It said: "Drug resistance threatens to reverse medical progress" (WHO 2000). It came just barely 50 years after the introduction of the first antibiotics into clinical practice. By now drug resistance is widespread, affecting every corner of the world and almost all medically important bacterial species sparing a few, only, if any.

Antibiotic resistance is present in the community among bacteria causing primarily community acquired infections. While this already represents a serious problem (e.g. penicillin and macrolid resistance in Streptococcus pneumoniae, ESBL producing urinary tract pathogens - just to name a few) the situation in our hospitals is really affecting and already limiting the way health care is (and can be) delivered.

A list of organisms have recently been identified by the Infectious Diseases Society of America as being responsible for the majority of these hospital acquired, drug resistant infections. These organisms are the members of the notorious "ESKAPE" list standing for Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter sp. (Rice 2008; Boucher, et al. 2009).

The situation is particularly serious regarding the Gram-negative members of this list. For the Gram-positive organisms there are some new therapeutic alternatives even if options having been considered "last reserves" for a long time (e.g. vancomycin) had been exhausted (Livermore 2003; Livermore 2004; Livermore 2009). For life-threatening Gram-negative infections traditionally 3rd generation cephalosporins and/or fluoroquinolones, often combined with aminoglycosides, had had represented the treatment of choice. The spread of ESBL producing bacteria, however, pushed us towards the extensive use of carbapenems. Before long this lead to the emergence and spread of carbapenem resistant clones both among Enterobacteriaceae and in non-fermenters, such as P. aeruginosa and A. baumannii.
(Livermore and Woodford 2006). Lately, with the frequent loss of even these drugs options remaining are rather limited. Colistin is a relatively toxic drug (Evans, et al. 1999), tigecycline is a bacteriostatic agent, i.e. a disadvantage when using in immunocompromised patients (Karageorgopoulos, et al. 2008) and sulbactam has a limited spectrum only; not to mention that resistance is already well-known against all of these agents, too (Pachon and Vila 2009; Michalopoulos and Falagas 2010).

As there are no promising new drugs in the pipeline to replace currently available ones for Gram-negative pathogens, and no broad-coverage vaccines are available against these infections either, the only way to mitigate their effect is to control their spread. This, however, is attainable, only if we have a clear understanding of the reasons, dynamics and complexity of the spread of these organisms. To contribute to this knowledge was the purpose we initiated our studies.

The target of our studies was A. baumannii. During the past 15 years this organism gained a tremendous attention arriving into the main stage of clinical microbiology from relative obscurity. This is well illustrated by the fact that up to the end of 1989 the search string "Acinetobacter baumannii" on PubMed resulted in 11 hits only. Although this is likely to be affected by the early confusion in nomenclature, the number of hits all during the 1990ies was still 343, only. However, this was to be followed by as many as 1635 hits for the next 10 years, i.e. almost 5-times increase. And during the past 28 months, actually from the 1st of January, 2010, till today (i.e. the 20th of May) more than one paper a day has been dedicated to this organism (the actual hit number for this short period was already 1080).

It is the remarkable capacity of this organism to survive in hospital environments with the consequent impact on morbidity and mortality, which has been attracting so much attention. It is particularly noteworthy, as this organism has relatively limited pathogenic potential on its own (Braun and Vidotto 2004). It is speculated that the success of this organism in hospitals is likely to relate to its considerable fitness (Gordon and Wareham 2010; Neonakis, et al. 2010).

Nosocomial infections may present in two forms: sporadic infections and epidemics. This is definitely true for A. baumannii (Dijkshoorn, et al. 2007). It is logical to
assume that strains causing sporadic cases should have the capacity to colonize and infect individual patients. But to establish themselves temporarily in a given environment extra features might be required (Weber, et al. 2010). However, no differences between sporadic and epidemic *A. baumannii* isolates have been found regarding their capacity to survive in dry environment, i.e. a logical characteristic one may consider to give an edge to persist in hospitals (Jawad, et al. 1998). Similarly, some found no difference in the resistance against commonly used disinfectants when comparing the two groups of isolates (Wisplinghoff, et al. 2007), although others described some association between resistance to disinfecting agents and that to antibiotics (Kawamura-Sato, et al. 2010). Antibiotic resistance, however, has been considered a tool of survival in an environment soaked as much in antibiotics, as hospitals are. Indeed, increased resistance to therapeutic drugs has been described among epidemic strains, although with considerable differences according to geographical locations (Dijkshoorn, et al. 1996; van Dessel, et al. 2004).

5.1. Comparison of sporadic and epidemic isolates


The 110 isolates collected may not represent all clinically relevant isolates during the study period as we had no control over the regularity hospital laboratories informed us about the isolation of a strain. Nevertheless, previous publications from Tawam Hospital indicate that this organism is responsible for 7.8% of nosocomial bloodstream infections (Jumaa and Neringer 2006) and similar data coming from other regional hospitals of similar sizes suggest that this pathogen is likely to be a serious health risk in our hospitals, as well (Alsultan, et al. 2009; Jamal, et al. 2009).
To group strains as sporadic and epidemic isolates we choose an arbitrary definition, i.e. clustering two or more isolates of the same type were considered strains with epidemic potentials as being present for an extended period of time. We had no access to clinical data allowing us to investigate the presence or absence of direct contacts or possible common sources shared by patients shedding the strains of the same type. Therefore our approach emphasized more the capacity of these isolates to repeatedly infect susceptible hosts without uncovering the actual routes of transmission. Nevertheless, as this assumes extra fitness compared to those strains causing single infections only, we believe that this does not influence the validity of our analysis.

One should keep in mind however, that this categorization is still subject to some limited bias. Strains recovered only once during the study may, in fact, represent isolates with real epidemic potential if the same type had been surfacing beyond, i.e. before or after the study period. As, however, this had an impact on the specificity of criteria for sporadic, rather than that on epidemic isolates (i.e. real epidemic isolates might have been grouped, wrongly with sporadic strains but not the opposite) we disregarded this bias.

We used two criteria for categorization. Determining the macro-restriction pattern is a broadly used fingerprinting method proven for several pathogens. Major alterations in this pattern are unlikely to take place by a single genetic event. However, as it is based on the mere physical presence or absence and location of specific restriction sites, independent isolates may express the same pattern. Therefore we decided to combine it with the determination of the \( \text{bla}_{\text{OXA-51-like}} \) allele type, i.e. the variety of the oxacillinase gene intrinsic to the species. This approach was thought to be appropriate as specific alleles of this gene are linked to clonal lineages of the organism (Evans et al, 2008) and in this way a marker of global epidemiology was combined with one more suited to trace local differences between strains (i.e. PFGE pattern). Using other genes to delineate epidemics was disregarded as some of the genes we tested our isolates for (TABLE 3.1.) may localize on mobile genetic elements in combinations. Hence, a single genetic event may result in considerable changes in the type.
By these criteria we identified 11 clones of varying sizes and containing 80 (72.7%) of the isolates. It was clear that the eleven clones clustered into 3 major lineages according to their \textit{bla}\textsubscript{OXA51-like} alleles: one with \textit{bla}\textsubscript{OXA69} (clones B, C, D, E and F), one with \textit{bla}\textsubscript{OXA64} (clones H and I) and one with \textit{bla}\textsubscript{OXA66} (clones J, L, M and N), respectively (\textbf{TABLE 4.2.}). Based on the data available it is not possible to decide whether the various PFGE types (representing the clones) were independently introduced or they are the results of local microevolution of the three lineages.

Revealing the type of enzymes sheds some light onto possible international epidemiological connections as some of these \textit{bla}\textsubscript{OXA51-like} alleles are associated with epidemic lineages (\textit{Evans, et al. 2008}). OXA-66 β-lactamase are often found in isolates belonging to an \textit{A. baumannii} lineage including the prevalent European clone 2, while those clustered around the OXA-69 enzyme are found in another lineage encompassing European clone 1. This latter lineage has been causing widespread infections lately in Kuwait (\textit{Al-Sweih, et al. 2012}). The most commonly identified enzymes are those of the OXA-66 cluster, which are particularly highly represented in South America and Asia (\textit{Merkier and Centron, 2006, Koh et al., 2007, Evans et al., 2008}). Enzymes of the OXA-69 cluster are also common, particularly in Eastern Europe (\textit{Evans et al., 2008}).

Establishing the \textit{bla}\textsubscript{OXA51-like} allele of the sporadic isolates was beyond the limits of this study and is currently in progress. Preliminary data show a considerably variety of alleles present \textit{(unpublished observation)}.

Irrespective of any possible sources, it was clear that representatives of the three lineages have been stably and simultaneously established in Abu Dhabi hospitals (\textbf{FIGURE 4.2.}). In case of the larger clonal groups (i.e. D, F, H, J, N) it was clear that each had a "favored" hospital, i.e. the majority of the members have been recovered from a specific health care setting. For instance clones D and H (a \textit{bla}\textsubscript{OXA69} and a \textit{bla}\textsubscript{OXA64} clone, respectively) were characteristic to Tawam Hospital, while M dominated Mafraq and J and N did so with Sheik Khalifa Hospital. Nevertheless, clear indications in all cases existed that representatives of the same clones were present in other hospitals, as well.
Although the number of strains analyzed were too small to establish a rule, but it was noticed that the strains isolated not from the "favored" hospital of the clone often mapped on, or towards the side on the PFGE similarity tree of the particular clone (FIGURE 4.2.) showing a relative distance from the rest of the group. This may indicate that members of a clone may encounter different selective pressures in different hospitals resulting in local derivatives of the main group. In the absence of detailed information regarding patients' history and movement, the fact of patient transfers, as possible explanation of exporting clones could not be established. Nevertheless, our data definitely highlights this possibility and should warn local authorities.

The fact that nearly three quarter of the strains represented isolates fit to be present permanently did not apply to all hospitals. Only in case of the three major, tertiary care hospitals showed this pattern. From Al Ain Hospital the majority of the isolates were sporadic ones. From Rahba, while the trend was the same, the number of strains was too low to support any conclusion (TABLE 4.3.). Nevertheless, and not surprisingly, it is clear, that hospitals with hemato-oncology and large intensive care facilities are more prone to experience nosocomial epidemics.

A considerably high percentage (81.6%) of respiratory and 66.7% of wound isolates were epidemic strains, representing 38.8 and 27.5% of these samples, much higher figures than for other sample types. We believe that this is not surprising as on one hand these are the sample types which spread infections most easily, and they are the commonest type of *A. baumannii* infections, as well (Zanetti, *et al.* 2007; Munoz-Price and Weinstein 2008; Murray and Hospenthal 2008).

Some of the larger clones (D, H and N) have been present for several months (FIGURE 4.3.) suggesting extended, instead of explosive epidemics, while for others (J and F) a clear peak was identifiable. Particularly the former pattern suggests the extended survival of the organism in the inanimate hospital environment (Morgan, *et al.* 2010; Thom, *et al.* 2011).

The independent nature of sporadic isolates was well reflected by the fact that 21 different genotypes (20 + one without any genes targeted) were distributed among
the 30 strains (TABLE 4.6.). On the other hand the 11 clones represented 9 core
genotypes (TABLE 4.5.), only. To make it sensible for clones with two strains, only,
we defined core genotype as consisting of genes present in at least half of the
members. However, it should be noted that all of the core genes in all the large
clones were missing only from a very few isolates, i.e. indeed that core genes were
characteristic of the clones (data not shown). In some clones certain genes appeared
with low frequencies, only i.e. being present in a minority among the clone-
members. The possibility that practically any of these can be plasmid-coded would
explain their low-frequency presence. Nevertheless, as this did not affect the
grouping of the strains, this option was not investigated further.

As the genes targeted during genotyping were all directly (resistance genes), or
indirectly (int, ISAb1) related to drug resistance it was not surprising that more than
twice as many of these genes were present in epidemic, than in sporadic isolates (7.9
vs. 3.8).

It was noteworthy that no blaOXA24 (lately re-named blaOXA46) and only a very few
sporadic isolates with blaOXAS8 were encountered among the local isolates. It is
interesting because not only that these enzymes, particularly the former one, has
been encountered world-wide (Castanheira, et al. 2008; Mendes, et al. 2009a;
Mendes, et al. 2009b) and are fast spreading, but they were also found as close to our
region, as Iran and India (Feizabadi, et al. 2008; Karunasagar, et al. 2011).

When comparing the distribution of genes between sporadic and epidemic strains,
four patterns emerged. Some of the β-lactam resistance and resistance-related genes
were not only significantly more frequent in the latter group but were present in all,
or in almost all epidemic isolates as if being essential to cause epidemics locally.
These were the blaOXA-23 gene and the ISAb1 sequence which, on the other hand,
were missing from the majority of the sporadic isolates (TABLE 4.7.). As the
expression of the blaOXA-23 gene depends on the presence of surrogate promoters
(Turton, et al. 2006) (Heritier, et al. 2006) it was not surprising that in all but one
strains, irrespective of being sporadic or epidemic the ISAb1 was located upstream of
the gene. This meant that all epidemic clones present in these Abu Dhabi hospitals
during the study period, irrespective of their $bla_{OXA51}$-like-based lineage, carried $bla_{OXA23}$. Epidemics with strains carrying this gene has been reported around the globe (Heritier, et al. 2005; Marque, et al. 2005; Coelho, et al. 2006; Villegas, et al. 2007; Zhou, et al. 2007; Evans, et al. 2011; Higgins, et al. 2009) and even this overpowering dominance of this enzyme type among local isolates have been reported (Villegas, et al. 2007; Liakopoulos, et al. 2012; Peymani, et al. 2012). Interestingly, in Kuwait (Al-Sweih, et al. 2012) no such dominance of the enzyme type was observed, while it was completely missing from Saudi isolates (Alsultan, et al. 2009). These observations suggest that while global trends can be observed, the actual incidences of strain types are likely to be driven by local factors.

Although often plasmid-coded, it is still not clear why and how the gene of this enzyme has become so widespread. Not only that it requires a surrogate promoter (Turton, et al. 2006; Heritier, et al. 2006) for any measurable expression, but it has a relatively weak carbapenemase activity (Afzal-Shah, et al. 2001), i.e. both factors would argue against its success. Further studies should reveal the unique advantages clones carrying the gene of this enzyme may carry to fully understand the reasons of its spread (Mugnier, et al. 2010).

The second type of pattern was shown by the $bla_{PER}$ and $aac(3)$-Ia genes. While they were completely absent from sporadic strains (and significantly more common in the epidemic group), they were restricted to certain clones, only. In case of $bla_{PER}$ it was present in clones (H, I and M, and in few isolates of N) (TABLES 4.6. - 4.7.). The class A PER (Pseudomonas Extended Resistance) type ESBLs, particularly PER-1, are widespread among non-fermenters and among some Enterobacteriaceae causing resistance to 3rd generation cephalosporins all over the world (Poirel, et al. 2012). Recently, a new type, PER-7 was described in A. baumannii (Bonnin, et al. 2011).

It was noteworthy that the clones carrying $bla_{PER}$ were distributed between the $bla_{OXA64}$ (H and I) and $bla_{OXA66}$ (M and N) lineages. On the other hand clone J, being within the same lineage group (i.e. $bla_{OXA66}$) as clones M and N, did not contain the gene. The genes of both PER-1 and PER-7 were initially described as chromosomally located (Bonnin, et al. 2011; Poirel, et al. 2012). Although not
impossible with such genetic make-up, but, at least retrospectively, this distribution of \textit{bla}_{\text{PER}} among local isolates, would have been looked more consistent with plasmid location - as it was subsequently demonstrated (see later).

The \textit{aac(3)-Ia} gene codes for a 3-N-aminoglycoside acetyltransferase acetylating the aminoglycoside molecule on the 3' aminogroup (Davies and Wright 1997). It has been shown to be one of the most common aminoglycoside-degrading enzymes with a narrow (gentamicin) specificity (Shaw, et al. 1993) and is plasmid-coded (Seward, et al. 1998). Its location on a mobile element was well-reflected by its distribution among our epidemic strains, as being present in clones B, M and N (TABLE 4.5.), i.e. clusters belonging to different lineages (FIGURE 4.2.) while absent from others.

The third pattern of difference between sporadic and epidemic strains was seen with the \textit{int, aph(3')-VI, ant(2')-Ia} and \textit{armA} genes, respectively. These genes, although found significantly more common in epidemic isolates, they were not exclusive for them and were seen with some regularity among sporadic isolates, as well (TABLES 4.6. - 4.8.).

Integrons, through their \textit{att} site and with the help of their integrase (coded by the \textit{int} gene) capture and express multiple resistance genes and hence play an important role in the development of resistance of acinetobacter (Partridge, et al. 2009), particularly among strains causing epidemics (Ploy, et al. 2000; Koeleman, et al. 2001; Gombac, et al. 2002; Fournier, et al. 2006; Kraniotaki, et al. 2006). Therefore it was not surprising that with the exception of two clones (E and F, TABLE 4.5.) the \textit{int} gene was part of the core genotype of epidemic isolates.

\textit{aph(3')-VI} is a phosphotransferase affecting primarily kanamycin and neomycin. It has commonly been found in \textit{Acinetobacter} earlier (Shaw, et al. 1993; Seward, et al. 1998), as well as more recently (Cho, et al. 2009). The \textit{ant(2')-Ia}-coded adenylating enzyme is widespread among all Gram negative bacteria, including \textit{Acinetobacter}, and mostly affects gentamicin, tobramycin and kanamycin (Shaw, et al. 1993). Both genes have been described to be transferable by conjugation (Seward, et al. 1998).
The presence of armA, an emerging ribosomal methylase within various genera of Gram-negative organism (Yu, et al. 2007; Zhou, et al. 2010) is an alarming phenomenon. Apparently it has penetrated at least two of the locally prevalent lineages represented by clones H, I and J, L, respectively becoming part of their core genotype, while being present in some members of clones M and N, as well. While the armA methylase was broadly represented, the rmt type enzymes were not encountered in our A. baumannii collection, similarly to the experience of other studies (Cho, et al. 2009). The implications of the presence of armA, as well as our further results regarding armA positive strains will be discussed later.

The genes present in both groups without any significant difference represented the forth pattern of distribution (TABLES 4.7. and 4.8.). Of this group two genes are of particular interest. The ISAb1-linked blaAmpC was present in approximately 30% of the strains in both groups. This is noteworthy as this enzyme is, or rather this group of enzymes is getting increasing attention lately (Jacoby 2009). Nevertheless, its presence was apparently not a "must" to confer the advantage needed to get permanently established in our hospitals. The same applies to the strAB gene, although the latter one was the most frequently encountered aminoglycoside resistance gene present in 46.4% of all, and 52.5% of epidemic isolates. While carried by several sporadic isolates, it was present in all blaOXA64 and blaOXA66 lineage clones but missing from those of the blaOXA69 lineage. As expected of this narrow spectrum enzyme (Shaw, et al. 1993), all strains expressing it exhibited very high resistance (MIC >1024 mg/L) against streptomycin.

Taken together it seems that while ISAb1-promoted blaOXA23 among local strains was near to clearly distinguish between the epidemic and sporadic nature of the strains, other genes, even if some were significantly more common in the former group, were not so characteristic. Either because they characterized a few clones only while missing from other epidemic clones (e.g. blaPER), or were more mixed between the two groups (e.g. aac(3)-Ia, aph(3')-VI, ant(2')-Ia and armA). In some cases the uniform distribution reached the point that the difference was not even significant between the two groups (e.g. blaAmpC). This results show that there were no uniformly present, stable link between blaOXA23 and any other genes studied.
As expected, the differences in genes were more or less reflected in the susceptibilities of the isolates, as well although often being modulated by overlapping resistance mechanisms. Note, that all during the analysis strains were characterized only as sensitive and non-susceptible combining resistant and intermediately resistant strains into the same category. We decided to do so since EUCAST does not use the intermediate category, at all (EUCAST 2012) and it is missing for some drugs (e.g. colistin, trimethoprim-sulfamethoxazole) in the CLSI system, as well (CLSI 2010).

At a first look the entire collection appeared extremely resistant, as more than 75% of all strains were non-susceptible to 3rd generation cephalosporins, carbapenems, gentamicin, ciprofloxacin and trimethoprim-sulfamethoxasole - practically the entire spectrum of first line drugs to treat life threatening Gram-negative infections (TABLE 4.9.). However, one should remember that these general figures were highly biased by the fact that epidemic isolates were more numerous than sporadic ones. Indeed, once analyzing the results comparatively with a statistical test accounting for the number and clonal nature of epidemic isolates a more detailed picture emerged. For all antibiotics tested, except colistin, epidemic strains were significantly more frequently non-susceptible than sporadic isolates reaching (ciprofloxacin) or approaching (ceftazidime, carbapenems, gentamicin, trimethoprim sulfamethoxasole) 100%. On the other hand, for sporadic strains non-susceptibility slightly exceeded 30% only in case of ciprofloxacin, doxycycline and trimethoprim sulfamethoxasole and barely, or did not exceed 25% for the most important agents as cephalosporins, carbapenems and aminoglycosides (TABLE 4.9.).

It was noteworthy that at the time of the strain collection only a single colistin non-susceptible isolate was encountered. Colistin is one of the key drugs recommended if first line drugs fail (Zavascki, et al. 2007). Regretfully, since the time of the study, colistin non-susceptible strains started appearing in Abu Dhabi, as well (personal communication from different hospitals, unpublished).

These inequalities between the two groups were well reflected by the quantitative data, as well (TABLE 4.10). The fact that several sporadic strains were also resistant was shown by the lack of discrimination of MIC90 in case of beta-lactams and
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ciprofloxacin. This fact should be interpreted as even in the case of sporadic isolates using these drugs without the confirmation of the actual susceptibility carries a considerable risk. To distinguish between the two groups values, as MIC50 or MIC10 were needed (even though, they carry little practical consequences in therapy and hence not calculated routinely).

Even though there are no published breakpoint values for tigecycline we included this drug in the quantitative analysis as one of the possible second-line alternatives (Gilad and Carmeli 2008; Giamarellou and Poulakou 2009). In this case also the MIC10 and MIC50 figures were higher for the epidemic isolates. Applying the values recently suggested, i.e. susceptible ≤2 mg/L, intermediate 4 mg/L and resistant ≥8 mg/L, (Liu, et al. 2010) the MIC90 for both groups reached the non-susceptible range.

Based on these figures, alarming by all international comparison (Saeed, et al. 2010, Alsultan, et al. 2009, El Shafie, et al. 2004, Khan, et al. 2010, Nzeako, et al. 2006, Mugnier, et al 2009, Jamal, et al. 2009), it was not surprising that almost all (97.5%) of the epidemic strains qualified as MDR compared to 20% in the sporadic group. Currently there is no universally accepted definition for MDR (Falagas, et al. 2006). Actually the definition used by us ("non-susceptibility to carbapenems and to at least two non-beta lactam classes") was very close to what was suggested just a few months ago by a panel of international experts, i.e. MDR: "non-susceptible to ≥1 agent in ≥3 antimicrobial categories" (Magiorakos, et al. 2012).

This part of the study allows us to entertain some speculations. Obviously, theoretically it is still a possibility that epidemic strains are more resistant because they are present and are exposed to antibiotics for longer period of time. However, to us the alternative explanation sounds more realistic, i.e. they can survive in this unique environment since they are resistant. What makes them able to become more resistant, whether they are more flexible, more receptive to foreign genes or having a higher mutation rate remains to be elucidated.

It is interesting to contemplate whether resistance to different antibiotics has equal power to secure a nosocomial epidemic potential? Based on our data, we surmise that, at least locally, resistance to carbapenem, ciprofloxacin and at least some of the
aminoglycosides appears to be a clear must to secure the fitness needed to survive in Abu Dhabi hospitals. As more and more local hospitals are introducing strict and well-controlled stewardship programs it will be interesting to see in the future what impact this will have on the susceptibility patterns of local epidemic isolates.

5.2. armA-mediated aminoglycoside resistance and its consequences


Similarly to several reports described the plasmid location of armA (Zhou, et al. 2010) we could also locate it on a plasmid in two of the positive clones (FIGURE 4.6.). Currently we do not have an explanation why we repeatedly failed to get a signal with the other strains and sporadic isolates while being positive by PCR. As no signal was received on the chromosome, one possible explanation is that in these strains the gene is located on a megaplasmid too large to be strong enough with the plasmid extraction and electrophoresis method used. It should be noted that some of these strains, when tested, did not hybridize when using the S1 nuclease method, either (data not shown). Alternatively we cannot exlude the possibility that in these strains an allele of the gene is present. While providing an amplicon with the primers used, the internal sequences could be sufficiently different to prevent hybridization. To prove this hypothesis experiments are currently in progress.

Beyond the therapeutic difficulties it causes, the presence of armA is also a diagnostic problem. In 2010 it was reported that the VITEK-2 automated system may give false readings regarding the amikacin susceptibility of Acinetobacter declaring several strains susceptible which, in fact were resistant (Akers, et al. 2010). The authors failed in their attempts to correlate the mechanism with a particular resistance gene present, i.e. with a particular resistance mechanism. It should be
noted, however, that \textit{armA} was not among the genes tested (\textit{Akers, et al. 2010}). At the same time, another study did correlate false testing results with the presence of the \textit{armA} gene (\textit{Jung, et al. 2010}). They also associated the phenomenon with a unique pattern of "double zone inhibition" in the disc diffusion test also observed and linked to \textit{armA} by others (\textit{Krahe et al. 2010}).

This paradoxical phenomenon has first been observed and named after the author "Eagle-phenomenon" (\textit{Eagle 1948}). Although its mechanism is still not understood it has been detected with several organisms and drugs (\textit{Goldstein and Rosdahl 1981; Kondo, et al. 2001}).

Our finding that the majority (27 out of 30) of the \textit{armA} positive isolates actually showed the phenomenon (\textbf{FIGURE 4.4.}) confirms these observations. As all this 27 isolates, unlike the three negative ones, exhibited the high level and broad-spectrum resistance to aminoglycosides characteristic to ArmA methylase (\textbf{TABLE 4.11}), we surmise that in the negative isolates the gene was not expressed.

However, as our results show the expression of \textit{armA} does not directly correlate with the Eagle phenomenon. Once exposing strains to various concentration of amikacin, as they supposedly are at various distances form the disc, no difference in the expression of the \textit{armA} gene (\textbf{FIGURE 4.5.}) or the \textit{ade} and \textit{abe} efflux genes (\textit{data not shown}) was observed. Our data still does not completely exclude the direct involvement of the ArmA methylase in the phenomenon. Its activity still can be modulated at the translational, or post-translational level - options beyond the scope of our study.

One other argument also favors an \textit{armA} independent explanation. While \textit{armA} - mediated resistance affects almost all aminoglycosides (\textit{Zhou, et al. 2010}), the phenomenon was observed with amikacin, only (\textbf{FIGURE 4.4.}).

On a completely speculative basis, based on the data currently available it is still possible that the Eagle phenomenon, and the consequent false readings in automated systems could be caused by a gene linked to, but different from \textit{armA}. Should this be the case this gene and the mechanism is still to be identified. However, whatever
the background and the actual mechanism is, our data clearly proved that the explanation considered the most plausible, i.e. concentration-dependent expression of the armA, is not the reason behind it.

5.3. Plasmid-localization of blaPER7

In A. baumannii, resistance to cephalosporins is mediated by the overexpression of a family of AmpC β-lactamases, lately called ADC (Acinetobacter-derived cephalosporinases) (Rodriguez-Martinez et al., 2010), or from acquisition of extended-spectrum β-lactamases (ESBLs) (Bonnin et al, 2011). The ESBL genes that have been identified in A. baumannii are blaPER-1, blaPER-2, blaGES-11, blaGES-14, blaVEB-1, blaVEB-1a, blaTEM-92 and blaCTX-M-2. (Bonnin et al, 2011; Poirel, et al. 2012). Recently, a new variant of the Ambler class A PER-type β-lactamases, PER-7, showing a broad activity against cephalosporins was identified in A. baumannii (Bonnin et al, 2011). It mapped on the chromosome and was associated with a mosaic class 1 integron structure, belonging to the ISCR1 family.

Using a general \( \text{bla}_{\text{PER}} \) primer we have identified several clones carrying the gene (see Discussion above). An unexpected event, i.e. coming across with an isolate from a patient from whom we already had one strain, guided us to look into the genetic background of this enzyme. While the first isolate which was part of the surveillance study (NM 55, clone H) was expressing the highly resistant profile characteristic to the clone, its isogenic counterpart, NM 128 was susceptible to ceftazidime but retaining resistance to carbapenems (TABLE 4.12.). The efficacy of the resistant mechanism lost (i.e. a ceftazidime MIC in NM 55 >256 mg/L) suggested that it is likely to be enzymatic. This hypothesis was confirmed by the genotyping of the isolates, i.e. NM 55 carrying, while NM 128 missing \( \text{bla}_{\text{PER}} \), while both contained the IS\text{Ab}1-linked \( \text{bla}_{\text{OXA-23}} \) (TABLE 4.13.). It was noteworthy that from the latter isolate two other genes (\( \text{aac}(3)-\text{Iia} \) and \( \text{armA} \)) were also missing. In this particular clone (H) \( \text{armA} \) was already successfully localized on a plasmid, suggesting that the loss of all these genes might have been the result of a single genetic event.
Indeed, we could localize $\textit{bla}_{\text{PER}}$ on the plasmid of the same size than the one previously $\textit{armA}$ was mapped on (FIGURES 4.6. and 4.8.). Meanwhile our coworkers in Edinburgh sequenced the $\textit{bla}_{\text{PER}}$ gene from NM 55. As it turned to be the $\textit{bla}_{\text{PER}-7}$ allele, its plasmid location received further importance. Originally this allele was described as mapping on the chromosome (Bonnin et al, 2011). As our strain was isolated two years earlier than theirs we speculate that the gene might have entered \textit{Acinetobacter} via a plasmid and stabilized in the chromosome. The actual mechanism of motility is still to be determined, as we could not conjugally transfer the plasmid.

We assume that the plasmid in NM 55 suffered a deletion of approximately 20 kb in size likely to contain not only the $\textit{bla}_{\text{PER}}$, but also the $\textit{armA}$ and $\textit{aac(3)-Iia}$ genes. We are planning to extend our investigations to map this entire region, which, provided the above assumption is correct, may contain several key resistance genes clustered.

During this cooperation with Prof. Sebastian Amyes's team some molecular details regarding the surroundings of the $\textit{bla}_{\text{PER-7}}$ gene were already determined. As, however, that part of the study was done in Edinburgh, these data and their implications will not be discussed here. For these details, please, refer to the original publication (see Appendix II).

5.4. $\textit{bla}_{\text{NDM-2}}$ in $\textit{Acinetobacter baumannii}$

The latest to spread and considered by many as the ultimate carbapenemase is the group of MBLs (Walsh, et al. 2005; Maltezou 2009; Walsh 2010; Cornaglia, et al. 2011). Within the MBL group New Delhi MBLs (NDM) have emerged very recently and have spread around the globe with remarkable speed (Nordmann et al, 2011; Poirel, et al. 2010; Bonomo 2011). Strains carrying the gene have spread to the environment and have been recovered from community-acquired infections, fecal flora of healthy people and from surface waters (Walsh et al, 2011). Early data suggested that the Indian subcontinent is a reservoir of the $\textit{bla}_{\text{NDM}}$ genes. In addition, several NDM-1-producing enterobacterial isolates have been reported in patients linked with the Balkan states or the Middle East, suggesting that those areas might
Acinetobacter baumannii in Abu Dhabi

Acinetobacter baumannii act as a secondary reservoirs of blaNDM-1 positive strains (Livermore et al, 2011; Poirel et al, 2010).

The blaNDM-1 gene has penetrated a broad variety of genera (Nordmann et al, 2011; Poirel, et al. 2010; Bonomo 2011), among others Acinetobacter (Karthikeyan et al. 2010, Chen et al 2011, Pfeifer et al. 2011). Therefore, when carrying out a local survey it was no surprise to find isolates, actually two from the same patient, carrying the gene. Cloning and sequencing the gene revealed it to be blaNDM-2, i.e. an allele recently described in an Israeli and in a German patient who had been hospitalized in Egypt (Espinal et al. 2011; Kaase et al. 2011). Very recently we had a chance to investigate the isolate originating from Egypt and found its plasmid and PFGE patterns indistinguishable from our strain (unpublished observation). Subsequently, we submitted our isolate to Prof. Jordi Vila (Barcelona), who confirmed that actually all three isolates exhibit the same patterns. Therefore we can conclude that as our patient had a clear connection with Egypt, it appears that NDM-2 expressing A. baumannii likely to have emerged and spread locally. We believe that the lack of the silent A → G substitution at position 468 found in the Israeli isolate and not affecting the activity of the enzyme produced (Espinal et al. 2011) is the sign of local microevolution.

The fact that while we could not transfer the blaNDM-2 gene into an E. coli recipient but easily into an A. baumannii strain was not surprising. However, it was not anticipated that the transfer would not be accompanied by any detectable transfer of plasmids (FIGURE 4.10.). The blaNDM-1 gene has been described before in A. baumannii both in the chromosome (Pfeifer, et al. 2011), as well on plasmids (Chen, et al. 2011). We believe, that in our strain the gene was located on the chromosome, which was recently confirmed, based on Southern hybridization data by Prof. J. Vila, Barcelona (personal communication). Consequently, the transfer was likely to have taken place by transformation between closely packed donor and recipient cells during the overnight incubation, even though we could not replicate it when using methods of transformation methods used in molecular biology. Nevertheless, transformation is a phenomenon well known in the genus (Juni 1972), and we
believe that its easiness, as we observed it could be instrumental in spreading the 
blanDM-2 gene.

Downstream of the blanDM-2 gene the same ble and ΔtrpF genes were identified as in the isolate recovered earlier in Germany carrying blanDM-1 (Pfeifer et al. 2011). More importantly, and similarly to other A. baumannii and Enterobacteriaceae isolates we found that an ISAbal25 element was located upstream of the blanDM-2 gene. Very recently it was shown that either the entire, intact ISAbal25, a version interrupted by insertions or fragments of ISAbal25 are always located upstream of the blanDM genes (Toleman, et al. 2012). Based on detailed sequence analyses of all upstream structures those authors concluded that it is very likely that the blanDM genes have actually been evolved in this species and have spread from here (Toleman, et al. 2012). Should this be the case, our isolate is a good example of a subsequent allele evolving in the Middle East, and spreading locally as well as getting transferred to Europe.
6. Conclusions

Based on our results we conclude that

1. *Acinetobacter baumannii* is well established in hospitals of Abu Dhabi both as sporadic infections as well as epidemics. The latter type represents the majority, almost 3 quarters, of the cases

2. The epidemic strains represent not only different clones, but also three different lineages (marked by \( \text{bla}_{\text{OXA69}}, \text{bla}_{\text{OXA64}}, \) and \( \text{bla}_{\text{OXA66}}, \) respectively)

3. In some cases multiple clones are simultaneously present in the same hospital (e.g. in Tawam clones D and H). On the other hand representatives of certain clones, while primarily characteristic to a "host" institution, are regularly found in other hospitals, as well

4. Epidemic strains are genetically much more uniform than their extremely heterogeneous sporadic counterparts

5. The epidemic strains contain more than twice as many resistance-related genes, as the ones isolated from sporadic cases

6. Unexpectedly in the region, the IS\(Ab1\)-linked \( \text{bla}_{\text{OXA23}} \) is present in the core genotype of all epidemic clones, while, also unexpectedly \( \text{bla}_{\text{OXA24}}, \text{bla}_{\text{OXA58}} \) are completely absent or rarely encountered suggesting the power of local factors affecting the type of the dominant strains

7. Aminoglycoside resistance genes are, with exceptions, more evenly distributed than most of those causing resistance to \( \beta \)-lactams (e.g. \( \text{bla}_{\text{OXA23}}, \text{bla}_{\text{PER}} \))

8. Nearly 100\% of the local epidemic strains are non-susceptible to all first-line drugs (ceftazidime, carbapenems, ciprofloxacin, gentamicin). Sporadic isolates, as an average, are much more sensitive. Nevertheless, non-susceptibility among them also reaches a level which makes initial treatment with first line drugs highly risky
9. With the exception of one isolate, strains were all susceptible to colistin, and tigecycline is likely to be effective, as well.

10. Based on these data we conclude that in the hospitals of the region it is primarily the lack of susceptibility to broad spectrum β-lactams, ciprofloxacin and gentamicin what may provide the advantage needed to secure survival

11. armA, a ribosomal methylase has penetrated several clones and some sporadic isolates in the region

12. The presence of armA was confirmed to be on a plasmid, at least in case of two clones

13. The armA-related unique pattern of amikacin susceptibility (also linked to false susceptibility reporting) is not due to the increased gene expression neither in case of armA nor efflux pump genes ade or abe

14. Unlike initially reported, blaPER-7 gene can also be localised on a plasmid outlining a possible path for its introduction into the species

15. Our data strongly suggest that the blaPER-7 is within a ca. 20 kb fragment of the plasmid together with armA and aac(3)-Iia

16. We identified two isogenic strains locally carrying blaNDM-2

17. We cloned and sequenced the gene and its surrounding revealing that both the upstream and downstream region is highly similar to what have been seen in strains carrying various alleles of the gene

18. Based on the almost complete identity of our strain (linked to Egypt) with two other isolates from the Middle East we surmise that this clone has evolved locally, and has spread to Europe providing a very recent example of pathogen tarfficing
7. REFERENCES


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Last and not least, my family, I would like to thank my husband Tarek for his love, patience and encouragement. My two cute little sons, Ahmed and Zeyad for their love and for giving me hard time.

This work is dedicated to the soul of my mother for her unlimited giving, love and support. Without her prayers, I will not be able to accomplish anything. May GOD bless her always with his mercy.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AAC</td>
<td>N-Acetyltransferases</td>
</tr>
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<td>ADC</td>
<td><em>Acinetobacter</em> Derived Cephalosporinases</td>
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<tr>
<td>ANT or AAD</td>
<td>O-Nucleotidyl Adenyl Transferases</td>
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<td>APH</td>
<td>O-Phosphotransferases</td>
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<td>Bla</td>
<td>Beta Lactamase</td>
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<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
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<td>DC</td>
<td>Dendritic Cell</td>
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<td>DNA</td>
<td>Desoxyribonucleic Acid</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<td>ERIC</td>
<td>Enterobacterial Repetitive Intergenic Consensus</td>
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<tr>
<td>Esbls</td>
<td>Extended Spectrum Beta Lactamases</td>
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<tr>
<td>EUCAST</td>
<td>European Committee On Antimicrobial Susceptibility Testing</td>
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<tr>
<td>ICU</td>
<td>Intensive Care Unit</td>
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<tr>
<td>Int</td>
<td>Integrase Gene</td>
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<tr>
<td>IS</td>
<td>Insertion Sequence</td>
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<td>Insertion Sequence Ab1</td>
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<tr>
<td>LPS</td>
<td>Lipopolysacchride</td>
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<tr>
<td>MATE</td>
<td>Multidrug and Toxic Compound Extrusion</td>
</tr>
<tr>
<td>Mbls</td>
<td>Metallo-Beta Lactamases</td>
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<tr>
<td>MDR</td>
<td>Multidrug Resistance</td>
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<tr>
<td>MIC</td>
<td>Minimal Inhibitory Concentration</td>
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<tr>
<td>MLST</td>
<td>Multilocus Sequence Typing</td>
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<tr>
<td>MMLV</td>
<td>Moloney Murine Leukemia Virus</td>
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<tr>
<td>MRSA</td>
<td>Methicillin-Resistant Staphylococcus Aureus</td>
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<tr>
<td>OMP</td>
<td>Outer Membrane Protein</td>
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<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
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<td>OXA</td>
<td>Oxacillinase</td>
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<tr>
<td>PBP</td>
<td>Penicillin Binding Protein</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PFGE</td>
<td>Pulse Field Gel Electrophoresis</td>
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<td>PMSF</td>
<td>Phenylmethylsulfonylefluoride</td>
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<tr>
<td>QS</td>
<td>Quorum Sensing</td>
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<tr>
<td>RND</td>
<td>Resistance Nodulation Division</td>
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<td>RT-PCR</td>
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<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
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<td>Spp</td>
<td>Species</td>
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<tr>
<td>TBE</td>
<td>Tris Boric EDTA</td>
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<td>Th</td>
<td>T Helper Cells</td>
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<tr>
<td>TSA</td>
<td>Tryptic Soy Agar</td>
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<tr>
<td>TSB</td>
<td>Tryptic Soy Broth</td>
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<tr>
<td>UAE</td>
<td>United Arab Emirates</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>UPGMA</td>
<td>Unweighted Pair Group Method with Arithmetic Mean</td>
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<tr>
<td>UTI</td>
<td>Urinary Tract Infection</td>
</tr>
<tr>
<td>VAP</td>
<td>Ventilator Associated Pneumonia</td>
</tr>
<tr>
<td>WW</td>
<td>Worldwide Lineages</td>
</tr>
<tr>
<td>Δtm</td>
<td>Melting Temperature</td>
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Plasmid-encoded PER-7 β-lactamase responsible for ceftazidime resistance in Acinetobacter baumannii isolated in the United Arab Emirates

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Objectives: To investigate the mechanism of ceftazidime resistance in two isogenic Acinetobacter baumannii strains from the United Arab Emirates.

Methods: Two A. baumannii strains, NM55 and NM128, were isolated 4 months apart from a 6-year-old patient in the United Arab Emirates. Genotypic characterization was performed by PFGE and the MIC of ceftazidime was determined by the agar dilution method. Detection of blaOXA and metallo-β-lactamase genes was performed by multiplex PCR. Analysis of blaPER-7, ISAbA1, blaADC and the ISCR1 element was carried out by standard PCR. Plasmid analysis was achieved by Southern blotting.

Results: Strain NM55 was resistant to ceftazidime, whereas strain NM128 was susceptible. Both isolates carried the blaOXA-23 and blaOXA-64 genes and were identical according to their PFGE patterns. ISAbA1 was present upstream of the blaOXA-23 gene, but absent upstream of blaADC-26, in both strains. Strain NM55 possessed a blaPER-7 gene with the presence of gst, a fragment of the abc transporter and a transposase gene downstream of it. The entire structure was part of an ISCR1 element and was located on an ~200 kb plasmid in strain NM55, while the ceftazidime-susceptible NM128 strain carried an ~180 kb plasmid without the blaPER-7 gene.

Conclusions: Ceftazidime resistance was mediated by a PER-7 β-lactamase encoded in an ISCR1 element located on a plasmid. This represents the first detection of a PER-7 β-lactamase encoded by a plasmid in A. baumannii.

Keywords: ISCR1 element, Middle East, plasmid-borne blaPER-7

Introduction

Acinetobacter baumannii is an opportunistic pathogen frequently causing outbreaks in intensive care units. The proportion of multidrug-resistant A. baumannii isolates has risen recently and is considered as a global sentinel event. Cephalosporin resistance in A. baumannii almost invariably results from the overexpression of a family of AmpC β-lactamases, called ADC (Acinetobacter-derived cephalosporinases), controlled by ISAbA1 upstream of the gene providing a strong promoter. Recently, a new variant of the Ambler class A PER-type β-lactamases, PER-7, with a broad activity against cephalosporins has been identified in A. baumannii. It was chromosomally encoded and associated with a mosaic class 1 integron structure, belonging to the ISCR1 family. Structurally, PER-7 has four amino acid substitutions compared with PER-1 and one compared with PER-6. The aim of this study was to identify the gene(s) responsible for ceftazidime resistance in the non-susceptible member of the pair of isogenic strains of A. baumannii isolated in the United Arab Emirates and to characterize its genetic environment.

Materials and methods

Bacterial strains and PFGE

Two strains of multidrug-resistant A. baumannii were isolated in May (NM55) and August (NM128) 2008 from tracheal aspirates of a 6-year-old patient in Tawam Hospital, Al Ain, United Arab Emirates. Species identification was confirmed by PCR detecting the blaOXA-51-like gene and by sequencing of a 455 bp section of the rpoB gene.
Genomic DNA was digested with ApaI and the fragments were separated using a CHEF-DRII system (Bio-Rad, Hercules, CA, USA).

Susceptibility testing
Antibiotic susceptibility tests were performed by the disc diffusion method according to BSAC guidelines. The antibiotics tested were imipenem, meropenem, cefotetan, rifampicin, aztreonam, chloramphenicol, rifampicin, cefoperazone, cefepime, cefotaxime and cefpodoxime. The ceftazidime and rifampicin MICs were determined according to BSAC recommendations.

Detection of $\beta$-lactamase genes and IS elements
The detection and sequencing of bla<sub>ADC</sub>-like genes were performed by PCR using the primers ADC-F (5′-GCGCCGTGAATCTTAAGTG-3′) and ADC-R (5′-CAGCTTATGCTGTGCTGGAT-3′), which align outside the bla<sub>ADC</sub>-like genes, detecting any genetic element upstream. The detection of OXA-type carbapenemases and metallo-$\beta$-lactamases was achieved using a multiplex PCR assay. The presence of the bla<sub>OXA</sub>-23-like gene and the detection of ISAba1 upstream were performed according to Evans et al. The characterization of the genetic environment of the bla<sub>PER-7</sub> gene. The genetic orientations are represented by horizontal arrows. Partial genes are represented by squares. nase, transposase.

Figure 1. Southern blot of the plasmids extracted from A. baumannii strains NM55 and NM128 hybridized with a bla<sub>PER-7</sub> probe. The gel on the left shows the plasmid profiles while the corresponding hybridization is shown on the right.

Figure 2. Schematic representation of the genetic environment of the bla<sub>PER-7</sub> gene. The genetic orientations are represented by horizontal arrows. Partial genes are represented by squares. nase, transposase.

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**Figure 2.** Schematic representation of the genetic environment of the bla<sub>PER-7</sub> gene. The genetic orientations are represented by horizontal arrows. Partial genes are represented by squares. nase, transposase.

Plasmid analysis
Plasmids were extracted using a standard protocol and transferred to Hybrid N+ membranes (GE Healthcare, UK). The hybridization and detection were carried out using the DIG DNA Labeling and Detection Kit (Roche Applied Sciences). Accurate sizing of the plasmid was carried out by PFGE against standard markers following S1 nuclease digestion.

Partial genes are represented by squares. nase, transposase.
Results

Both strains were identified as A. baumannii through the detection of the blaOXA-51-like and rpoB genes. The isolates were considered isogenic as they were isolated consecutively from the same patient 4 months apart and exhibited the same PFGE patterns. The strains were resistant to imipenem, meropenem, cefotaxim, aztreonam, cefoperazone, cefepime, cefotaxime and cefpodoxime. However, strain NM55 was also resistant to ceftazidime and rifampicin whereas strain NM128 was susceptible to both of these antibiotics. The MICs of ceftazidime were 64.0 mg/L for isolate NM55 and <0.2 mg/L for isolate NM128. Moreover, the MICs of rifampicin were 32.0 mg/L for isolate NM55 and 2.0 mg/L for isolate NM128.

Both strains harboured the blaADC-26 gene, but neither of them possessed any insertion elements upstream of the gene, suggesting that this gene was not overexpressed.

The blaOXA-23 gene was associated with ISAba1 upstream in both isolates, which can account for their resistance to carbapenems. The most notable difference between the strains was that only the ceftazidime-resistant NM55 gave a positive reaction when tested with the blaPER-specific PCR. Through sequencing, the blaPER-like gene was identified as blaPER-7 (GenBank accession no. AE154993.1).

Both strains harboured a large plasmid (Figure 1). The plasmid sizes were determined by S1 nuclease digestion, which showed ≏ sizes were determined by S1 nuclease digestion, which showed ≏ plasmid of NM128. No conjugal transfer of the plasmid was achieved, irrespective of the recipient used.

The blaPER-7 gene was located within a complex class 1 integron, also containing the arr-2 and cmlA7 genes in the variable zone of the classic integron class 1 element (Figure 2), which confer resistance to rifampicin and chloramphenicol, respectively. The 3′-CS region of the class 1 integron contained the qacE-D1 and sul1 genes, but the orf5 gene was absent. Downstream of blaPER-7 was located a gst gene. Interestingly, further downstream there was part of an abcd transporter gene (GenBank accession no. CP001172.1) and then a transposase gene (GenBank accession no. CP000863.1) (Figure 2).

Discussion

Here we report on the isolation of a blaPER-7-carrying, ceftazidime-resistant A. baumannii (NM55) from the Middle East, the first such isolate from the region. Unlike the French strain AP2,3 the gene in NM55 was located on an ≏200 kb plasmid, although also without an apparent insertion sequence (IS) element upstream to provide a surrogate promoter. The results of the hybridization analysis support the hypothesis of the plasmid location of blaPER-7. The blaADC-26 gene was detected in both strains; it is not an extended-spectrum AmpC cephalosporinase (ESAC) gene and lacked an IS element upstream providing a promoter, indicating ceftazidime resistance was not due to the activity of this enzyme. The genetic environment of the plasmid-borne blaPER-7 gene in strain NM55 differed (GenBank accession no. JQ639792), specifically in the downstream region, from the chromosomally located allele in the French isolate AP2: in the French isolate the chromosomal gene ABAYE3396 was located downstream, while we found both gat and a part of an abcd transporter gene. This genetic environment is similar to that described by Xia et al.10 in Aeromonas punctata isolated in China, which harboured a blaPER-1 gene inserted in an ISCR1 element. Further downstream, a transposase gene was located, interrupting the abcd transporter gene. This indicates a probable insertion of the ISCR1 element in this locus, which may represent a hot spot for the integration of mobile genetic elements. The strain NM55 was less susceptible to rifampicin in comparison with strain NM128, probably due to the presence of the arr-2 gene in the variable zone of the classic class 1 integron; however, both strains were resistant to chloramphenicol, suggesting that the mechanism of resistance was not caused only by the presence of the cmlA7 gene in the variable zone.

On the other hand, there are also similarities between the French isolate AP2 and NM55. Both had the same blaOXA-51-like gene, namely blaOXA-64, which is one of the most common genes in strains from the Middle East (A. Al Hasan, L. Al Hassan and S. G. B. Amyes, unpublished results). It is noteworthy that the French strain was isolated in 2010 whereas strain NM55 was isolated nearly 2 years earlier. This chronology may suggest that the PER-7 β-lactamase was originally carried into strains of A. baumannii on plasmids, such as the one described here, and some of the integrons migrated onto the chromosome. As in our study the plasmid was non-conjugative, the mechanism of its uptake is still to be determined.

This study shows the remarkable ability of A. baumannii to capture and/or lose genetic structures, which may be mediated by novel genetic structures, such as ISCR1, affecting their susceptibility to antibiotics that represent important therapeutic options. However, the strain had lost the blaPER-7 gene some 4 months after its first isolation, leaving the resultant strain, NM128, vulnerable to ceftazidime. The reasons for this loss are difficult to explain in an environment where cephalosporins are being used and it is a rare example of spontaneous resistance loss in A. baumannii.

The two isolates came from the same patient and had the same PFGE pattern, suggesting that the smaller plasmid of NM128 was a derivative of the plasmid of NM55, which had undergone a deletion, including blaPER-7. Further detailed analysis is underway to confirm the exact mechanism by which this may have occurred.

Acknowledgements

The cooperation of the staff of the Microbiology Laboratory, Tawam Hospital, Al Ain, United Arab Emirates in collecting these isolates is highly appreciated.

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transparency declarations

References


NDM-2 carbapenemase-producing
Acinetobacter baumannii in the United Arab Emirates

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Abstract

Screening 155 carbapenem non-susceptible Acinetobacter baumannii strains recovered in Abu Dhabi hospitals identified two metallo-β-lactamase blaNDM gene-carrying isolates. They were isolated 4 months apart from the urine of a cancer patient previously treated in Egypt, Lebanon and in the United Arab Emirates. They were clonally related and carried the blaNDM-2 gene recently identified in A. baumannii in Egypt and Israel. Sequences surrounding the blaNDM-2 gene showed significant similarities with those associated with blaNDM-1 in Enterobacteriaceae and A. baumannii. Repeated isolation of blaNDM-2-positive A. baumannii in the Middle East raises the possibility of the local emergence and spread of a unique clone.

Keywords: Acinetobacter, carbapenemase, Middle East, multidrug resistant, NDM-2

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Enteric bacteria carrying the gene encoding the carbapenemase NDM-1 have been increasingly reported worldwide. The blaNDM-1 gene is usually located on a plasmid and its expression confers resistance to all beta-lactams except aztreonam. Due to the frequent association with other resistance mechanisms, these strains are commonly multi-, or even pan-drug resistant [1,2]. A recent study showed that the blaNDM-1 gene could easily transfer beyond the boundaries of the Enterobacteriaceae family [3]. While the majority of species implicated were environmental microorganisms, reports regarding the presence of the blaNDM-1 gene in Acinetobacter baumannii, an opportunistic pathogen causing nosocomial outbreaks, are alarming [4]. Such strains have already been reported from India [5], China [6] and Germany [7]. Most of these isolates were multidrug resistant and some of them co-expressed other carbapenemases (i.e. blaOXA-23, blaOXA-181 and blampp). Recently, the NDM-2 variant (Pro to Ala substitution at position 28) was described [8]. This allele was first found in a multidrug-resistant A. baumannii strain isolated from a German patient having previously been hospitalized in Egypt [8], while a subsequent one was isolated in Israel [9]. These recent data regarding strains of Middle Eastern origin prompted us to carry out an investigation to identify blaNDM carrying A. baumannii in the Abu Dhabi Emirate.

One hundred and 55 carbapenem non-susceptible clinical A. baumannii isolates collected between 2008 and 2010 from hospitals of Abu Dhabi Emirate were included in the study. Strains were initially identified by the API20NE system (bioMérieux, Marcy l’Etoile, France) and this was confirmed by PCR specific to the blaOXA-51-like gene [10]. Boiled extracts of the isolates were screened by PCR for the presence of blaNDM-1 using primers NDM1-Fo (5¢-TGCCGAGCGACTTGGCCCTTG-3¢) and NDM1-Re (5¢-ACCGATGACCAGACCGACGCCCAGG-3¢), respectively. The reaction yielded a 379 bp-sized amplicon of the blaNDM1 gene. Among the 155 isolates, two blaNDM positive ones were identified. Both were recovered from a 55-year-old Egyptian female with a congenital single kidney. She had been treated for a metastatic colon carcinoma in 2004 in Egypt but due to the recurrence of the disease in 2006 she had received subsequent treatments at multiple centres in Cairo (Egypt), Beirut (Lebanon), Dubai and Al Ain (UAE). In March 2008, due to a urinary obstruction from the pelvic spread of the malignancy, she underwent surgery in Egypt with insertion of a metallic ureteric stent. This was followed by recurrent urinary tract infections caused by extended-spectrum β-lactamase (ESBL)-producing Escherichia coli and a multidrug-resistant Pseudomonas aeruginosa treated with ceftriaxone and meropenem. In April 2009, she was admitted to Tawam Hospital (Al Ain, UAE) because of an
BM4547 failed. followed by plating onto plates containing DNA with HindIII (New England Biolabs, Ipswich, MA, USA) containing fragments generated by partial digestion of genomic c.
A clone carrying the plasmid p132LigB with an insert of 10 kb containing the genes tested by PCR (i.e. Both isolates were negative for all other carbapenemase (studied were from May (AG132) and August 2009 (AG124). she remained afebrile and clinically stable. The two isolates from urine samples, but no antibiotics were given because following months, the same strain was repeatedly isolated upstream of the bla transfer the bla respectivly. Their plasmid profiles were identical, with mul-

<table>
<thead>
<tr>
<th>TABLE 1. Antibiotic susceptibility of the strains and derivatives</th>
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<tbody>
<tr>
<td><strong>MIC (mg/L)</strong></td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>AG132</td>
</tr>
<tr>
<td>AG124</td>
</tr>
<tr>
<td>DH5sig(pUC19)</td>
</tr>
<tr>
<td>DH5sig(p132LigB)</td>
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</tbody>
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Antibiotic susceptibility tests were carried out by CLSI guidelines [11]. IMI, imipenem; MER, meropenem; CAZ, ceftazidime; CIP, ciprofloxacin; GM, gentamicin; AM, amikacin; COL, colistin; TG, tigecycline; SAM, ampicillin/sulbactam; AZT, aztreo-

![FIG. 1. Genetic surrounding of blaNDM-2 in Acinetobacter baumannii AG132. P–promoter (GenBank accession No. JN112341).](image)

accidental dislodgement of the left solitary kidney nephrostomy. The first urine culture yielded A. baumannii. During the following months, the same strain was repeatedly isolated from urine samples, but no antibiotics were given because she remained afebrile and clinically stable. The two isolates studied were from May (AG132) and August 2009 (AG124).

Both isolates exhibited the same susceptibility profile (Table 1) when tested according to CLSI standards [11]. They harboured the naturally occurring blaOXA-70 gene and were positive for ISAbal, which was not, however, identified upstream of the blaOXA-70 nor of the blaAmpC genes [12]. Both isolates were negative for all other carbapenemase genes tested by PCR (i.e. blaOXA-23 [10], blaOXA-24, blaOXA-58-like [13], blaOXA-48/181 [14] blavim and blaVIM [15]. The strains exhibited identical pulsed-field gel electrophoresis patterns (data not shown) and irrespective of the methods used they had the same multilocus sequence types ST253 [16] or ST103 [17] according to the two typing methods, respectively. Their plasmid profiles were identical, with mul-

flanking regions of blaNDM (GenBank accession number JN112341) was generated by primer walking and subsequently confirmed by direct, bidirectional sequencing with primers ASndm1 (5′-GTCGCAAAGCCAGCTTGCAGCA-3′) and ASndm2 (5′-GCTTGGCTTGGCGTTTTTTT-3′). Detailed sequence analysis identified blaNDM-2 [8] that did not contain the silent A → G substitution at position 468 found in the blaNDM-2 allele described in the recent Israeli isolate [9]. The fact that insertion sequence ISAbal25 was present upstream of the blaNDM-2 gene in this isolate as found for the blaNDM-1 and blaNDM-2 genes in A. baumannii and in Enterobacteriaceae suggests that it may contribute to the dissemination of the blaNDM-like genes [7,9,18,19]. Downstream of blaNDM-2, the same sequences as those identified downstream of blaNDM-1 in A. baumannii 161/07 from Germany were detected [7]. Within this region, the presence of a putative bleomycin resistance gene and a phosphoribosyl anthranilate isomerase gene was confirmed (Fig. 1.).

After the recent identification of NDM-1-producing Klebsiella pneumoniae from Sultanate of Oman [20], this study constitutes another identification of that emerging threat in the Arabian Peninsula. Although the specific location where this patient had been infected with this strain cannot be clearly established, her prior hospitalization in the Middle East where two similar strains had been recently reported [8,9] raises the possibility of a link between all those isolates.

**Acknowledgements**

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**Transparency Declaration**

No competing financial interests exist.
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11. CLSI. Performance standards for antimicrobial susceptibility testing, m100-s20. Wayne, PA: Clinical and Laboratory Standards Institute, 2010.
Acinetobacter baumannii strain AG132 phosphorybosiol anthranilate isomerase (trpF), bleomycin resistance protein (BRP), and metallo-beta-lactamase NDM-2 (blaNDM-2) genes, complete cds; and insertion sequence ISAba125, complete sequence

GenBank: JN112341.1

FASTA

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DEFINITION    Acinetobacter baumannii strain AG132 phosphorybosiol anthranilate isomerase (trpF), bleomycin resistance protein (BRP), and metallo-beta-lactamase NDM-2 (blaNDM-2) genes, complete cds; and insertion sequence ISAba125, complete sequence.

ACCESSION    JN112341.1    GI:357595124

KEYWORDS    .

SOURCE    Acinetobacter baumannii

ORGANISM    Acinetobacter baumannii
Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Noraseceae; Acinetobacter; Anicetobacter calcoaceticus/baumannii complex.

REFERENCE    1 (bases 1 to 3684)


TITLE    NDM-2 carbapenemase-producing Acinetobacter baumannii in the United Arab Emirates


PUBLICATION    21252273

REFERENCE    2 (bases 1 to 3684)

AUTHORS    Sonneveld, A.M., Chassawi, A. and Pal,T.

TITLE    Direct Submission

JOURNAL    Submitted (12-JUN-2011) Microbiology and Immunology, United Arab Emirates University, Tawaz Street 1, Al Ain, Abu Dhabi Emirate 17666, United Arab Emirates

FEATURES

Location/Qualifiers

source

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```

PERSONAL DETAILS:
Date of Birth: 21/08/1976
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EDUCATION:
• M.Sc. United Arab Emirates University, Faculty of Medicine and Health Sciences, Department of Microbiology and Immunology; 2005.
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  Thesis Title: Fine Mapping of Sequences Important for FIV RNA Packaging and Their Mechanism of Function
• B.Sc. Alexandria University, Faculty of Science, Department of Environmental Sciences, Alexandria, Egypt; 1998
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ACADEMIC AND PROFESSIONAL APPOINTMENTS:
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4. Graduate Student
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   Department of Microbiology and Immunology
   Faculty of Medicine and Health Sciences
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5. Research Assistant
   06/98-08/02
   Remote Sensing and GIS Unit
   Department of Environmental Sciences
   Faculty of Science-Alexandria University
RESEARCH EXPERIENCE:

Following my undergraduate degree, I was offered an International Scholarship for pursuing Master’s program at the UAE University. For my thesis, I worked in Professor Rizvi’s laboratory towards characterizing the packaging determinants of Feline Immunodeficiency Virus (FIV) a lentivirus that causes AIDS in cats. FIV based vectors are being proposed as ideal candidates for human gene therapy trials due to several reasons. First, phylogenetically, FIV is distantly related to human retroviruses and therefore will minimize the chance of recombining with endogenous retroviruses, which may result in the generation of chimeric variants of unknown pathogenic potential. Also, FIV vectors can infect not only dividing but also non-dividing cells, the target cell population in human gene therapy including cells of the muscle, heart, brain, kidney, lungs, etc.

During the course of my thesis, I elaborated the previous work conducted in Professor Rizvi’s laboratory, which had identified two discontinuous packaging determinants in the 5’end of FIV genome. For my thesis work, I specifically addressed the question whether the sequences between the two discontinuous packaging determinants are dispensable for FIV RNA packaging. Towards this end, I conducted a detailed mutational analysis of the 5’ end of the viral genome. During the course of the study, two series of vectors were generated in which the intervening sequences were deleted or substituted with heterologous sequences to maintain the equal length of the region when compared to wild type virus. Using an in vivo packaging and transfection assay complemented with semi-quantitative RT-PCR, our analysis revealed that the intervening sequences could be dispensed for both FIV vector RNA packaging and propagation.

Although our deletion analysis suggests that the 5’ end UTR, is necessary for efficient FIV RNA packaging and propagation, we believed that in addition to these sequences, other sequences are also likely to contribute towards RNA packaging. This hypothesis is derived from the fact that, at least, in some viruses the 5’ and 3’ LTRs contain additional recognition sequences for packaging. With this goal in mind, we embarked on to dissect the role of FIV LTR elements towards packaging. Towards this end, we constructed another series of vectors that tested the effects of the U3, R, and U5 sequences on FIV RNA packaging in both the 5’ and 3’ contexts. Using our in vivo packaging assay, revealed that both the 5’ R/U5 region and the 3’ LTR contains weak packaging determinants in addition to the core packaging determinants present in the UTR and Gag sequences.

Since retroviral RNA packaging determinants are thought to fold into higher order structures, we folded the FIV genomic RNA regions found to be important for packaging using the RNA folding algorithm. Analysis of the predicted structures revealed that the 5’ end of the FIV genomic RNA folded into several stable stem loops, a situation similar to HIV, SIV, and MPMV. Based on the mutational analysis and the predicted higher order structure of this region, Professor Rizvi’s laboratory successfully secured extra mural grant from the Wellcome Trust, UK, in collaboration with Professor Andrew Lever of the University of Cambridge UK, to further investigate the secondary structure of the FIV packaging signal using genetic, biochemical, and X-ray crystallographic analyses. I am currently working on this project to validate the secondary RNA structure of FIV packaging determinant by introducing primary and “compensatory” mutations in the different part of the predicted structure. Introduction of the various destabilizing mutations within the structural elements should negatively impact packaging if these structures are important for packaging, while “compensatory” mutations should result in the restoration of packaging. Since the primary nucleotide sequence will be quite distinct from that of the original stem loop structure, restoration of packaging would confirm the existence of a stem loop and would imply that its role is primarily structural. The data we have so far generated looks very exciting and is being put together for publication.

When Professor Rizvi’s grant ran out in 2005, I briefly worked in the laboratory of Dr. Youssef Haik at the Faculty of Engineering. During this short stay, I worked on the concept of using magnetic hyperthermia induced by nanomagnetic particles to treat solid tumors. This project involved the formation of nanomagnetic particles, encapsulating them in biocompatible polymers, and testing them in vitro in tissue culture and finally in vivo in mice animal model systems.
Some of the techniques used to conduct these studies included:

1. Basic molecular biological techniques such as cloning (restriction enzyme digestion of DNA, ligation of the desired fragments, transformation of the DNA in E.coli, mini-prep analysis of transformants, etc.)
2. DNA, RNA, and protein purification
3. ELISAs
4. PCR and RT-PCR (including design of primers)
5. Northern, Southern, Slot and Western blots
6. Maintenance of cell lines
7. Transfection of tissue culture cell lines by various methods such as DEAE-Dextran, Lipofectamine, Calcium Phosphate
8. Day-to-day laboratory management including ordering supplies, maintaining inventories, keeping records of DNA clones, glycerol stocks, cell lines, antibodies, etc.

CURRENT PROFESSIONAL APPOINTMENT:

Currently I am working as a Medical Research Specialist I in the department of Microbiology and Immunology, in the bacteriology field.

Position summary, duties and responsibilities:

1. Provide comprehensive support to highly technical and sophisticated research and teaching activities of the department dealing with bacteriology and molecular biology.

2. Demonstrate laboratory practical classes to the undergraduate students of MSC and OSC, M.Sc. university research students and HCT Medical lab, and in addition organizes and assists their laboratory-based projects.

3. Some of the techniques used to conduct the research:
   • Extraction of bacterial DNA, processing of samples of different nature.
   • Bacteriological and molecular analysis.
   • PCR work up for different bacteria.
   • Electrophoresis, ligation, digestion, DNA isolation.
   • Identification of bacteria, yeast & anaerobes by API methods.
   • Immunosorbent assay for mycobacterium tuberculosis.
   • DNA mapping for mycobacterium tuberculosis.
   • Culture, isolation and preservation of different strains.
   • Perform different antibacterial procedures such as MICs, MBCs.
   • Perform E-test for quantitative assay.
   • Perform Enzyme linked Immunosorbent Assay (ELISA).
   • Pulse field gel electrophoresis (PFGE) to identify and differentiate between the different bacterial strains.

4. Prepare and present the practical class assignments.

5. Support the student research project and supervise them.

6. Coordinate the procurement (reagents, kits, disposables).

7. Organize equipment maintenance.

8. Proper disposal of infectious and radioactive.

9. Ensure proper functionality and calibration of the equipment.
10. Training, orientation, guidance and support to new and junior staff & students.

11. Keep updated with recent knowledge and procedures.

12. Other duties as assigned by immediate supervisors.

MANUSCRIPTS IN REFEREED JOURNALS:


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French: good

REFERENCES

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