Molecular Characterization of Multi-Drug Resistant Acinetobacter Baumannii in Kingdom Saudi Arabia

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A Thesis Submitted for the Fulfillment of PhD Degree in Medical Microbiology

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قالوا
سبحانك إلا علمنا
إلا ما علمتنا إنك أنت
العليم الكريم

صلاة الله العظيم
سورة البقرة الآية: 21
To My Parents
Acknowledgement

I am always indebted to ALLAH, Lord of Heavens and Lord of Earth, who is worthy of all praise.

I am heartily thankful to my supervisor, Prof. Hassan Abd-Aziz Musa, Professor of Microbiology, Faculty of Medicine, the National Ribat University. Whom I was away honored since he initially accepted to supervise my research. His encouragement, guidance & support from initial to final level enabled me to develop deep understanding of the project. Moreover, his continuing follow up supervision provided me with helpful ideas, which without them my research would not come to reality.

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Faten Mohamed Mohamed El-Abd
2015
المستخلص العربي

المستخلص

ميكروب الأسنينتيوباكتير باوماني يصنف على أنه أحد الميكروبات المكتسبة بالمستشفيات ويفقد أعراض وتلوث أثناء المعالجة بالمستشفيات. ويسبب هذا الميكروب الأمراض الإلتهاب الرئوي والحمى الشوكية والتهاب المجاري البولية وتلوث الحروق والجروح. بالإضافة إلى ذلك يصنف هذا الميكروب على أنه عديم المقاومة للمضادات الحيوية الخاصة بعلاج البكتيريا العصوية السالبة حرام وكذلك المضادات الحيوية الأكثر شيوعاً وستخداماً حالياً.

وُجِّهت力度 واستخداماً جليلاً للحتاج في تذكير وتتبع نسب الوفيات.

في هذه الدراسة تم فحص 80 عينة مستشفى عسير المركزية برحلة أُبَيْ في المملكة العربية السعودية خلال الفترة من شهر أكتوبر 2013 إلى مارس 2014.

تم عمل اختبار فحص تفاعل المضادات الحيوية وأوكزا كاربيناميز بطريقة تفاعل البلمرة المستسلم باستخدام أوكزا 51 أوكزا 24 أوكزا 08.

وأفادت النتائج التالية: 100% من ميكروب الأسنينتيوباكتير يمتلك عوكر 51 جينوهذه تعرف الأسنينتيوباكتير باوماني. 75% كانت مقاومة للمضادات الحيوية السيفالوسبيرون، الاميبوليجوزيد، الكينلولون. وكانت نسبة المقاومة للإمفينيم 43% والامي روحيئ 50% ونسبة أقل نسب مقاومة مع الكولستين 4.6 والتراميسوريم سلفاميثازول 6.7%.

أوضح الفحص أن الأسنينتيوباكتير باوماني جميعاً تحمل عوكر 51 جين. من بين 56 عينة مقاومة لجميع الكاربينام وجد أن 48 (85.6%) تحمل عوكر 42.20% زئب (6.4%) تحمل الأوكزا 24 (6.2%)

وقد تم رصد جين أوزبال في الأوكزا 24 و 24 عين بنسبة (71.3%) و (71.3%)

على التوالي ووجدت في الأوكزا 51 بنسبة (18).1%.

أوضح هذا البحث وجود زيادة ميكروب الأسنينتيوباكتير باوماني المقاوم لمجموعة الكاربينام في المملكة العربية السعودية (أبها) وأن وجود النوع (5) من جينات كاربينامز ويشكل رئيسي إيزبال/ أوكزا 24 و 24 تشكل خطوة متزايدة في المستشفيات.
Abstract

A. baumannii is ranked recently as a nosocomial microorganism and causes outbreaks of infection and health care-associated infections, including bacteremia, pneumonia, meningitis, urinary tract infection, and wound and burns infections.

Furthermore, A. baumannii is recognized to be among the most difficult antimicrobial-resistant Gram-negative bacilli to treat and control.

Currently, the regimen of therapy is considered by trials and errors, i.e. the use of one or more of widely known antimicrobial agents expected to be effective. Moreover, the therapeutic options are limited for multi-drug-resistant Acinetobacter infections. Thus, the development or discovery of therapies, well-controlled clinical trials of existing antimicrobial regimens and combinations, and greater emphasis on the prevention of health care-associated transmission of multi-drug-resistant Acinetobacter infection are essential.

In this study a total of 108 specimens were collected from Aseer central hospital, Saudi Arabia during the period from October 2013 to March 2014.

The current study was conducted to determine the antimicrobial susceptibility patterns and prevalence of OXA-type carbapenemases, among clinical isolates of A. Baumannii.

Isolates were identified as A. baumannii by PCR with specific primers for bla OXA-51-like gene. Their susceptibilities to different antibiotics were determined. Isolates were then subjected to multiplex-PCR targeting bla oxa-51, bla oxa-24, bla oxa-23 and bla oxa-58 genes.
Abstract

Results showed that 108 *Acinetobacter* species, possessed bla oxa-51-like gene and were identified as *A.baumannii*. Overall, 75% of all isolates were resistant to extended-spectrum cephalosporins, aminoglycosides and quinolones. Carbapenem resistance was 52.8% for imipenem and 50% for meropenem. The lowest rate of resistance was seen against colistin (4.6%) and trimethoprim-sulfamethoxazole (65.7%).

The *A. baumannii* isolates were investigated for the presence of OXA-type carbapenemases. All isolates harbored the naturally occurring bla\textsubscript{OXA-51-like} gene. Of 56 carbapenem-resistant isolates, 48 isolates (85.6%) carried bla\textsubscript{OXA-23-like}, 3 isolate (5.4%) carried bla\textsubscript{OXA-24-like} and two isolates (3.6%) had bla\textsubscript{OXA-58-like} genes. The ISAba1 element was consistently found upstream of bla\textsubscript{OXA-23} and bla\textsubscript{OXA-24} genes in 40 (71.3%) and 3(5.4%) isolates, respectively, while it was detected upstream bla\textsubscript{OXA-51} in only one (1.8%) isolate. Our findings illustrate further the challenge of increasing carbapenem-resistance in *A. baumannii* isolates in Saudi Arabia. The high distribution of class D carbapenemase-encoding genes, mainly ISAba1/OXA-23 and ISAba1/OXA-24 carbapenemases presents an emerging threat in our hospital.
# Contents

<table>
<thead>
<tr>
<th>Items</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedication</td>
<td></td>
</tr>
<tr>
<td>Acknowledgment</td>
<td></td>
</tr>
<tr>
<td>English Abstract</td>
<td></td>
</tr>
<tr>
<td>Arabic abstract</td>
<td></td>
</tr>
<tr>
<td>List of Tables</td>
<td>I</td>
</tr>
<tr>
<td>List of Figures</td>
<td>II</td>
</tr>
<tr>
<td>List of Abbreviation</td>
<td>III</td>
</tr>
</tbody>
</table>

- Chapter (1): Introduction & Literature Review

1.1 Introduction | 1 |
1.2 Review of literature | 2 |
  1.2.1.1 Historical Perspective of the Genus *Acinetobacter* | 2 |
  1.2.2 Pathogenesis of *Acinetobacter* infections | 5 |
  1.2.3 Clinical manifestations of *Acinetobacter* infection | 14 |
  1.2.4 Laboratory identification | 17 |
  1.2.5 Mechanisms of antibiotic resistance | 35 |
  1.2.6 Therapeutic strategies for Acinetobacter baumannii infection | 46 |
  1.2.7 Hospital outbreaks and control measures | 59 |
  1.2.8 Epidemiology of *Acinetobacter baumannii* | 64 |
1.3 Objectives | 74 |
  1.3.1 Study Justification | 74 |
  1.3.2 General objectives | 74 |
  1.3.3 Specific objectives | 74 |

- Chapter (2): Materials and Methods

2.1 Methods: | 75 |
  2.1.1 Study design: | 75 |
  2.1.2 Study area: | 75 |
  2.1.3 Study duration: | 75 |
# List of Contents

<table>
<thead>
<tr>
<th>Items</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.4 Study population</td>
<td>75</td>
</tr>
<tr>
<td>2.1.5 Inclusion criteria</td>
<td>76</td>
</tr>
<tr>
<td>2.1.6 Exclusion criteria</td>
<td>76</td>
</tr>
<tr>
<td>2.1.7 Ethical clearance</td>
<td>76</td>
</tr>
<tr>
<td>2.1.8 Specimen collection</td>
<td>76</td>
</tr>
<tr>
<td>2.1.9 Samples processing</td>
<td>77</td>
</tr>
<tr>
<td><strong>Chapter (3): Results</strong></td>
<td>81</td>
</tr>
<tr>
<td>3.1 Types of infections:</td>
<td>81</td>
</tr>
<tr>
<td>3.2 Isolation of Acinetobacter species:</td>
<td>81</td>
</tr>
<tr>
<td>3.3 Antibiotic resistance pattern:</td>
<td>82</td>
</tr>
<tr>
<td>3.4 PCR</td>
<td>82</td>
</tr>
<tr>
<td><strong>Chapter (4): Discussion, Conclusion and Recommendation</strong></td>
<td>94</td>
</tr>
<tr>
<td>References</td>
<td>99</td>
</tr>
<tr>
<td>Appendixes</td>
<td>145</td>
</tr>
<tr>
<td>Table No.</td>
<td>Table Title</td>
</tr>
<tr>
<td>----------</td>
<td>-------------</td>
</tr>
<tr>
<td>1.</td>
<td>Delineation of <em>Acinetobacter</em> genomic species</td>
</tr>
<tr>
<td>2.</td>
<td>Different organisms grow on media used for <em>Acinetobacter</em> isolation.</td>
</tr>
<tr>
<td>3.</td>
<td>Mechanisms of resistance in <em>Acinetobacter baumannii</em></td>
</tr>
<tr>
<td>4.</td>
<td>Sequences of primers used in this study for multiplex PCR for detection of genes encoding oxacillinases in <em>A. baumannii</em> isolates</td>
</tr>
<tr>
<td>5.</td>
<td>Distribution of the specimens according to gender</td>
</tr>
<tr>
<td>6.</td>
<td>Distribution of the specimens according to type of infection</td>
</tr>
<tr>
<td>7.</td>
<td>Distribution of the specimens according to type of specimen collected.</td>
</tr>
<tr>
<td>8.</td>
<td>Distribution of the specimens according to the ward of admission</td>
</tr>
<tr>
<td>9.</td>
<td>Number and percentages of <em>A. baumannii</em> isolates resistant to selected antimicrobial agents</td>
</tr>
<tr>
<td>10.</td>
<td>Distribution of OXA-type β-lactamase genes in 56 carbapenem-resistant <em>A. baumannii</em> nosocomial isolates</td>
</tr>
<tr>
<td>11</td>
<td>Antibiotic resistant profiles of multi-drug resistant <em>A. baumannii</em> isolates</td>
</tr>
<tr>
<td>12</td>
<td>Antibiotic resistant profiles of carbapenem-resistant <em>A.baumannii</em> isolates in relation to OXA-type β-lactamase genes.</td>
</tr>
</tbody>
</table>
# List of Figures

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Table Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Detection of oxa genes in <em>A.baumanii</em> by Multiplex PCR.</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Detection of oxa genes in <em>A.baumanii</em> by Multiplex PCR.</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Detection of oxa genes in <em>A.baumanii</em> by Multiplex PCR.</td>
<td></td>
</tr>
</tbody>
</table>
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acb complex</td>
<td>Acinetobacter calcoaceticus- baumannii complex</td>
</tr>
<tr>
<td>ADCs</td>
<td>Acinetobacter-derived cephalosporinases</td>
</tr>
<tr>
<td>AdeABC</td>
<td>Acinetobacter drug efflux</td>
</tr>
<tr>
<td>adk</td>
<td>Adenylate Kinase</td>
</tr>
<tr>
<td>AMK</td>
<td>Amikacine</td>
</tr>
<tr>
<td>APACHE score</td>
<td>Acute physiology and chronic health evaluation</td>
</tr>
<tr>
<td>API</td>
<td>Analytical profile index</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>bla</td>
<td>Beta-lactamase</td>
</tr>
<tr>
<td>CAZ</td>
<td>Ceftazidime</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFP</td>
<td>Cefepime</td>
</tr>
<tr>
<td>CFT</td>
<td>Cefotaxime</td>
</tr>
<tr>
<td>CHROM</td>
<td>Chromogenic</td>
</tr>
<tr>
<td>CIP</td>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute;</td>
</tr>
<tr>
<td>CMS</td>
<td>Colistimethate sodium</td>
</tr>
<tr>
<td>COL</td>
<td>Colistin</td>
</tr>
<tr>
<td>COS</td>
<td>Colistin-only-susceptible</td>
</tr>
<tr>
<td>FIC</td>
<td>Fractional inhibitory concentration index</td>
</tr>
<tr>
<td>GEN</td>
<td>Gentamicin</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IMP</td>
<td>Imipenem</td>
</tr>
<tr>
<td>IS</td>
<td>Insertion sequence</td>
</tr>
<tr>
<td>LAM</td>
<td>Leeds Acinetobacter Medium</td>
</tr>
<tr>
<td>LEV</td>
<td>Levofloxacin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipo-polysaccharide</td>
</tr>
<tr>
<td>MATE</td>
<td>Multidrug and toxic compound extrusion</td>
</tr>
<tr>
<td>MBLs</td>
<td>Metallo-β-lactamases</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug Resistant</td>
</tr>
<tr>
<td>MEP</td>
<td>Meropenem</td>
</tr>
<tr>
<td>MFP</td>
<td>Major Fusion Protein</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MLST</td>
<td>Multilocus Sequence Typing</td>
</tr>
<tr>
<td>OMF</td>
<td>Outer membrane factor</td>
</tr>
<tr>
<td>OMP</td>
<td>Outer membrane proteins</td>
</tr>
<tr>
<td>OXA</td>
<td>Oxacillinases</td>
</tr>
<tr>
<td>PBPs</td>
<td>Penicillin-binding proteins</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCR-ESI-MS</td>
<td>Multilocus PCR Electrospray ionization/Mass Spectrometry</td>
</tr>
<tr>
<td>PFE</td>
<td>Pulsed Field Gel Electrophoresis</td>
</tr>
<tr>
<td>PIP</td>
<td>Piperacillin</td>
</tr>
<tr>
<td>RAPD</td>
<td>Random amplified polymorphic DNA</td>
</tr>
<tr>
<td>REP</td>
<td>Repetitive Extragenic Palindromic REP</td>
</tr>
<tr>
<td>RND</td>
<td>Resistance Nodulation-cell Division</td>
</tr>
<tr>
<td>RR</td>
<td>Risk ratio</td>
</tr>
<tr>
<td>RTI</td>
<td>Respiratory tract infection</td>
</tr>
<tr>
<td>SBA</td>
<td>Sheep blood agar</td>
</tr>
<tr>
<td>SSI</td>
<td>Surgical site infection</td>
</tr>
<tr>
<td>SXT</td>
<td>Trimethoprim-sulfamethoxazole</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNAB</td>
<td>Tigecycline-nonsusceptible A. baumannii</td>
</tr>
<tr>
<td>TOB</td>
<td>Tobramycin</td>
</tr>
<tr>
<td>TSAB</td>
<td>Tigecycline-susceptible A. baumannii</td>
</tr>
<tr>
<td>VAP</td>
<td>Ventilator-associated pneumonia</td>
</tr>
<tr>
<td></td>
<td>Acinetobacter genomic species 13, described by Tjernberg and Ursing</td>
</tr>
<tr>
<td>---</td>
<td>-------------------------------------------------------------------</td>
</tr>
<tr>
<td>13TU</td>
<td>Acinetobacter genomic species 14, described by Bouvet and Jeanjean</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
</tr>
</tbody>
</table>
Chapter (1)

Introduction & Literature Review

1. Introduction:

*Acinetobacter baumannii* is an important opportunistic pathogen and is often involved in various nosocomial infections such as bacteremia, urinary tract infection, secondary meningitis, surgical site infection, and nosocomial and ventilator-associated pneumonia, especially in patients admitted to intensive care and burn units (*Peleg et al.*, 2008).

*Acinetobacter baumannii* is notorious for its remarkable innate and acquired resistance to multiple antimicrobial classes, including extended-spectrum cephalosporins and carbapenems. Resistance to carbapenems is most concerning, as carbapenems have a potent activity against *Acinetobacter* spp and are often used as a last resort for the treatment of infections due to multi-resistant *A. baumannii* isolates. The emergence of carbapenem-resistant *A. baumannii* has been described as the sentinel event of antimicrobial resistance (*Kuo et al.*, 2012).

Carbapenem resistance in *A. baumannii* can be mediated by various mechanisms, including impermeability due to loss of one of its major porins and, possibly, efflux, as shown recently for meropenem (*Peleg et al.*, 2008). Most frequently, though, it is mediated through enzymatic hydrolysis of the drug, particularly by carbapenem-hydrolysing class D β-lactamases (oxacillinases). The oxacillinases can be grouped into six subclasses: chromosomal OXA-51-like, acquired
OXA-23-like (OXA-23, OXA-27 and OXA-49), OXA-24/40-like (OXA-24, OXA-25, OXA-26, OXA-40 and OXA-72), OXA-58-like, OXA-143-like, and OXA-235-like (OXA-235, OXA-236 and OXA-237) β-lactamases (Higgins et al., 2013). Although they are weak carbapenem hydrolyser, they confer resistance when over-expressed, as a result of their association with mobile elements, such as ISAba1, which carries a strong promoter (Turton et al., 2006).

Carbapenem resistance due to OXA-carbapenemases has been reported from diverse geographical origins (Pawel et al., 2012).

1.2 Review of literature:

1.2.1.1 Historical Perspective of the Genus Acinetobacter

The history of the genus Acinetobacter dates back to the early 20th century, in 1911, when Beijerinck, a Dutch microbiologist, described an organism named Micrococcus calco-aceticus that was isolated from soil by enrichment in a calcium-acetate-containing minimal medium (Beijerinck, 1911). Over the following decades, similar organisms were described and assigned to at least 15 different genera and species, including Diplococcus mucosus (von Lingelsheim, 1908), Micrococcus calcoaceticus (Beijerinck, 1911), Alcaligenes haemolysans (Henriksen, 1973), Mima polymorpha (DeBord, 1939), Moraxella lwoffi (Audureau, 1940), Herellea vaginicola (DeBord, 1942), Bacterium anitratum (Schaub and Hauber, 1948), Moraxella lwoffi var. glucidolytica (Piechaud et al., 1951), Neisseria winogradskyi (Lemoigne et al., 1952), Achromo-bacter anitrus (Brisou, 1953), and Achromobacter mucosus (Mannheim et al., 1962).
The current genus designation, *Acinetobacter* (from the Greek ακινετοσ [akinetos], i.e., nonmotile), was initially proposed by Brisou and Prévot in 1954 to separate the nonmotile from the motile microorganisms within the genus *Achromobacter* (*Brisou and Prevot 1954*). It was not until 1968 that this genus designation became more widely accepted. Baumann *et al.* published a comprehensive survey and concluded that the different species listed above belonged to a single genus, for which the name *Acinetobacter* was proposed, and that further subclassification into different species based on phenotypic characteristics was not possible (*Baumann et al., 1968*). These findings resulted in the official acknowledgment of the genus *Acinetobacter* by the Subcommittee on the Taxonomy of Moraxella and Allied Bacteria in 1971 (*Lessel, 1971*). In the 1974 edition of Bergey's Manual of Systematic Bacteriology (*Lautrop, 1974*), the genus *Acinetobacter* was listed, with the description of a single species, *Acinetobacter calcoaceticus* (the type strain for both the genus and the species is *A. calcoaceticus* ATCC 23055) (*Beijerinck, 1911*). In the “Approved List of Bacterial Names,” in contrast, two different species, *A. calcoaceticus* and *A. lwoffii*, were included, based on the observation that some *acinetobacters* were able to acidify glucose whereas others were not (*Skerman et al., 1980*). In the literature, based on the same properties, the species *A. calcoaceticus* was subdivided into two subspecies or biovars, *A. calcoaceticus* bv. *anitratus* (formerly called *Herellea vaginicola*) and *A. calcoaceticus* bv. *lwoffii* (formerly called *Mima polymorpha*). These designations, however, were never officially approved by taxonomists (*Anton et al., 2008*).
1.2.1.2 Current Taxonomy

The genus *Acinetobacter*, as currently defined, comprises gram-negative, strictly aerobic, non fermenting, non fastidious, nonmotile, catalase-positive, oxidase-negative bacteria with a DNA G+C content of 39% to 47%. Based on more recent taxonomic data, it was proposed that members of the genus *Acinetobacter* should be classified in the new family *Moraxellaceae* within the order *Gammaproteobacteria*, which includes the genera *Moraxella, Acinetobacter, Psychrobacter*, and related organisms (*Rossau et al., 1991*). A major breakthrough in the long and complicated history of the genus was achieved in 1986 by Bouvet and Grimont, who based on DNA-DNA hybridization studies distinguished 12 DNA (hybridization) groups or genospecies, some of which were given formal species names, including *A. baumannii, A. calcoaceticus, A. haemolyticus, A. johnsonii, A. junii*, and *A. lwoffii* (*Bouvet et al., 1986*). Work done by Bouvet and Jeanjean, Tjernberg and Ursing, and Nishimura et al. (*Bouvet et al., 1989, Nishimura et al., 1988, Tjernberg et al., 1989*) resulted in the description of further *Acinetobacter* genomic species, including the named species *A. radioresistens*, which corresponds to *Acinetobacter* genomic species 12 described previously by Bouvet and Grimont (*Bouvet et al., 1986*). Some of the independently described (genomic) species turned out to be synonyms, e.g., *A. lwoffii* and *Acinetobacter* genomic species 9 or *Acinetobacter* genomic species 14, described by Bouvet and Jeanjean (14BJ), and *Acinetobacter* genomic species 13, described by Tjernberg and Ursing (13TU). More recently, 10 additional *Acinetobacter* species were described, including 3 species of human origin, *A. parvus, A. schindleri*, and *A. ursingii*.
(Nemec et al., 2003), and 7 species isolated from activated sludge (recovered from sewage plants), namely, A. baylyi, A. bouvetii, A. grimontii, A. tjernbergiae, A. towneri, A. tandoii, and A. gerneri (Carr et al., 2003), increasing the actual number of validly described (genomic) species to 31, of which 17 have been given valid species names (Table1) A. calcoaceticus, A. baumannii, Acinetobacter genomic species 3, and Acinetobacter genomic species 13TU, are very closely related and difficult to distinguish from each other by phenotypic properties. It has therefore been proposed to refer to these species as the A. calcoaceticus-A. baumannii complex (Gerner, 1992). However, this group of organisms comprises not only the three most clinically relevant species that have been implicated in the vast majority of both community-acquired and nosocomial infections, i.e., A. baumannii, Acinetobacter genomic species 3, and Acinetobacter genomic species 13TU (see below), but also an environmental species, A. calcoaceticus, that has frequently been recovered from soil and water but has never been implicated in serious clinical disease. Therefore, since it is the environmental species that has given its name to the complex, the designation A. calcoaceticus-A. baumannii complex may be misleading and not appropriate if used in a clinical context (Anton et al., 2008).

1.2.2 Pathogenesis of Acinetobacter Infection

1.2.2.1 Natural Habitats

Members of the genus Acinetobacter are considered ubiquitous organisms. This holds true for the genus Acinetobacter, since acinetobacters can be recovered after enrichment culture from virtually all samples obtained from soil or surface water (Baumann, 1968).
These earlier findings have contributed to the common misconception that A. baumannii is also ubiquitous in nature (Fournier, and Richet, 2006). In fact, not all species of the genus Acinetobacter have their natural habitat in the environment (Anton et al., 2008). Most Acinetobacter species that have been recovered from human clinical specimens have at least some significance as human pathogens (Seifert et al., 1994). Acinetobacters are part of the human skin flora. In an epidemiological survey performed to investigate the colonization of human skin and mucous membranes with Acinetobacter species, up to 43% of nonhospitalized individuals were found to be colonized with these organisms (Seifert et al., 1997). The most frequently isolated species were A. lwoffii (58%), A. johnsonii (20%), A. junii (10%), and Acinetobacter genomic species 3 (6%). In a similar study, a carrier rate of 44% was found for healthy volunteers, with A. lwoffii (61%), Acinetobacter genomic species 15BJ (12%), A. radioresistens (8%), and Acinetobacter genomic species 3 (5%) being the most prevalent species (Berlau et al., 1999). In patients hospitalized on a regular ward, the carriage rate of Acinetobacter species was even higher, at 75% (Seifert et al., 1997). Dijkshoorn et al. studied fecal carriage of Acinetobacter and found a carrier rate of 25% among healthy individuals, with A. johnsonii and Acinetobacter genomic species.
### Table (1): Delineation of Acinetobacter genomic species. (Anton et al., 2008)

<table>
<thead>
<tr>
<th>Species</th>
<th>Genomic species</th>
<th>Reference strain</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. baumannii</td>
<td>2</td>
<td>ATCC 19606T</td>
<td>Tjernberg, et al. 1989</td>
</tr>
<tr>
<td>A. baylyi</td>
<td></td>
<td>DSM 14961T</td>
<td>Carr, et al. 2003</td>
</tr>
<tr>
<td>A. bouvetii</td>
<td></td>
<td>DSM 14964T</td>
<td>Carr, et al. 2003</td>
</tr>
<tr>
<td>A. calcoaceticus</td>
<td>1</td>
<td>ATCC 23055T</td>
<td>Tjernberg, et al. 1989</td>
</tr>
<tr>
<td>A. gerneri</td>
<td></td>
<td>DSM 14967T</td>
<td>Carr, et al. 2003</td>
</tr>
<tr>
<td>A. grimonitii</td>
<td></td>
<td>DSM 14968T</td>
<td>Carr, et al. 2003</td>
</tr>
<tr>
<td>A. haemolyticus</td>
<td>4</td>
<td>ATCC 17906T</td>
<td>Tjernberg, et al. 1989</td>
</tr>
<tr>
<td>A. johnsonii</td>
<td>7</td>
<td>ATCC 17909T</td>
<td>Tjernberg, et al. 1989</td>
</tr>
<tr>
<td>A. junii</td>
<td>5</td>
<td>ATCC 17908T</td>
<td>Tjernberg, et al. 1989</td>
</tr>
<tr>
<td>A. parvus</td>
<td></td>
<td>NIPH384T</td>
<td>Nemec. et al., 2003</td>
</tr>
<tr>
<td>A. radioresistens</td>
<td>12</td>
<td>IAM 13186T</td>
<td>Tjernberg, et al. 1989</td>
</tr>
<tr>
<td>A. schindleri</td>
<td></td>
<td>NIPH1034T</td>
<td>Nemec. et al., 2001</td>
</tr>
<tr>
<td>A. tandoi</td>
<td></td>
<td>DSM 14970T</td>
<td>Carr, et al. 2003</td>
</tr>
<tr>
<td>A. tjernbergiae</td>
<td></td>
<td>DSM 14971T</td>
<td>Carr, et al. 2003</td>
</tr>
<tr>
<td>A. towneri</td>
<td></td>
<td>DSM 14962T</td>
<td>Carr, et al. 2003</td>
</tr>
<tr>
<td>A. ursingii</td>
<td></td>
<td>NIPH137T</td>
<td>Nemec. et al., 2001</td>
</tr>
<tr>
<td>“A. venetianus”</td>
<td></td>
<td>ATCC 31012</td>
<td>Vaneechoutte, et al. 1999</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>ATCC 19004</td>
<td>Tjernberg, et al. 1989</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>ATCC 17979</td>
<td>Tjernberg, et al. 1989</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>ATCC 17924</td>
<td>Tjernberg, et al. 1989</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>ATCC 11171</td>
<td>Tjernberg, et al. 1989</td>
</tr>
<tr>
<td></td>
<td>13TU</td>
<td>ATCC 17903</td>
<td>Tjernberg, et al. 1989</td>
</tr>
<tr>
<td></td>
<td>13BJ, 14TU</td>
<td>ATCC 17905</td>
<td>Tjernberg, et al. 1989</td>
</tr>
<tr>
<td></td>
<td>14BJ</td>
<td>CCUG 14816</td>
<td>Bouvet. et al., 1989</td>
</tr>
<tr>
<td></td>
<td>15BJ</td>
<td>SEIP 23.78</td>
<td>Bouvet. et al., 1989</td>
</tr>
<tr>
<td></td>
<td>15TU</td>
<td>M 151a</td>
<td>Tjernberg, et al. 1989</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>ATCC 17988</td>
<td>Bouvet. et al., 1989</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>SEIP Ac87.314</td>
<td>Bouvet. et al., 1989</td>
</tr>
<tr>
<td></td>
<td>Between 1 and 3</td>
<td>10095</td>
<td>Gerner, et al. 1993</td>
</tr>
<tr>
<td></td>
<td>Close to 13TU</td>
<td>10090</td>
<td>Gerner, et al. 1993</td>
</tr>
</tbody>
</table>
Introduction & Literature Review

11 predominating (Dijkshoorn et al., 2005). In contrast, A. baumannii, the most important nosocomial Acinetobacter species, was found only rarely on human skin (0.5% and 3% in references Berlau et al., 1999 and Seifert et al., 1997 respectively) and in human feces (0.8%) (Dijkshoorn et al., 2005), and Acinetobacter genomic species 13TU was not found at all (Dijkshoorn et al., 2005). More recently, Griffith et al. investigated the nares of healthy U.S. soldiers and did not find acinetobacters at all, but they did not use enrichment culture to increase the recovery rate (Murray et al. 2006). Notably, in tropical climates, the situation may be different. In Hong Kong, Chu et al. found 53% of medical students and new nurses to be colonized with acinetobacters in summer versus 32% in winter (Chu et al., 1999). Such a seasonal variability in skin colonization may contribute to the seasonal variation seen in the prevalence of A. baumannii in clinical samples (McDonald et al., 1999).

The inanimate environment has also been studied for the presence of acinetobacters. Berlau et al. investigated vegetables in the United Kingdom and found that 30 of 177 vegetables (17%) were culture positive for Acinetobacter (Berlau et al., 1999). Interestingly, A. baumannii and Acinetobacter genomic species 11 (each at 27%) were the predominant species, followed by A. calcoaceticus and Acinetobacter genomic species 3 (each at 13%), while Acinetobacter genomic species 13 was found only once. In Hong Kong, 51% of local vegetables were culture positive for Acinetobacter species, the majority of which were Acinetobacter genomic species 3 (75%), but one sample grew A. baumannii.
Houang et al. found *acinetobacters* in 22 of 60 soil samples in Hong Kong, and the most frequent species were *Acinetobacter* genomic species 3 (27%) and *A. baumannii* (23%), with only one sample yielding *A. calcoaceticus* (Houang et al., 2001). Some recently described *Acinetobacter* species, i.e., *A. baylyi, A. bouvetii, A. grimontii, A. tjernbergiae, A. towneri,* and *A. tandoii,* that were isolated from activated sludge are obviously environmental species and have, as yet, never been found in humans (Carr et al., 2003). In contrast, two other recently described species, *A. schindleri* and *A. ursingii,* have been recovered only from human specimens, while *A. parvus* was found in humans and was also cultured from a dog (Dortet et al., 2006).

In conclusion, although available data derived from only a few studies, some *Acinetobacter* species indeed seem to be distributed widely in nature, i.e., *A. calcoaceticus* is found in water and soil and on vegetables; *Acinetobacter* genomic species 3 is found in water and soil, on vegetables, and on human skin; *A. johnsonii* is found in water and soil, on human skin, and in human feces; *A. lwoffii* and *A. radioresistens* are found on human skin; and *Acinetobacter* genomic species 11 is found in water and soil, on vegetables, and in the human intestinal tract. At least in Europe, the carrier rate of *A. baumannii* in the community is rather low. Also, although it has been found in soil samples in Hong Kong and on vegetables in the United Kingdom, *A. baumannii* does not appear to be a typical environmental organism. Existing data are not sufficient to determine if the occurrence of severe community-acquired *A. baumannii* infections that have been observed in tropical climates (Anstey et al., 2002; Leung et al., 2006, Wang et al., 2002) may be associated with an environmental source (Anton et al., 2008).
1.2.2.2 Predisposing factors to infection

Various risk factors predisposing to severe infection with *Acinetobacter* spp. have been identified; some of these also apply to organisms causing other nosocomial infections. Susceptible patients include those who have recently undergone major surgery, those with severe underlying disease (e.g., malignancy, burns or immunosuppression), and particularly the elderly, although outbreaks of *Acinetobacter* infection have also been described in neonates (*Ng et al., 1989*). Use of antimicrobial agents, presence of invasive devices such as endotracheal and gastric tubes, and type of respiratory equipment (*Lortholary et al., 1995*).

A case-control analysis in which ICU patients with respiratory colonization or infection with *Acinetobacter* spp. were compared with matched ICU controls who had other gram negative bacilli in their sputum (*Peacock et al., 1988*) was used to investigate an outbreak of infection with aminoglycoside-resistant *Acinetobacter* spp. Involving 98 patients at a university hospital in North Carolina. The duration of the ICU stay before colonization or infection with *Acinetobacter* spp. was significantly longer for infected patients than for controls (14.7 versus 5.9 days). Although exposure to invasive devices and, procedures did not differ significantly between the two groups. Infected patients received respiratory therapy for significantly longer than did controls (14.7 versus 6.6 days).
Aminoglycoside usage in the two groups was comparable, but infected patients received an aminoglycoside for a longer duration before colonization or infection than did controls (9.0 versus 6.1 days) and had also received more cephalosporins than did controls (1.9 versus 1.2).

A similar study of 40 patients infected or colonized with *A. baumannii* (*Lortholary et al., 1995*), in which these patients were compared by logistic regression analysis with 348 non-infected and non-colonized patients who were present in the ICU at the same time, also demonstrated that the severity of the underlying disease as evaluated by the APACHE II score, and the presence of a previous infection that required antimicrobial treatment were independent factors for acquiring *A. baumannii* infection (*Knaus et al., 1985*).

In conclusion, extended ICU care as a result of severe underlying disease, prolonged respiratory therapy with mechanical ventilation, and previous antimicrobial therapy are all key factors in predisposing to *Acinetobacter* infection. Since the only factor amenable to control in the ICU setting is antimicrobial therapy, avoidance of unnecessary antibiotics should be a high priority in management of such patients. The use of antibiotics probably alters the normal flora and results in the selection of resistant microorganisms such as *Acinetobacter spp* (*Bergogne-Berezin and Towner, 1996*).
1.2.2.3 Virulence of Acinetobacter spp.

1.2.2.3.1 Siderophore-mediated iron acquisition systems

In order for *A. baumannii* to thrive in the iron-deficient environment of a human host, it secretes low-molecular-mass ferric binding compounds, or siderophores (*Dorsey et al.*, 2004).

1.2.2.3.2 Biofilm formation

The ability of *A. baumannii* to adhere to and form biofilms on inanimate objects and surfaces may explain its success in the hospital environment. Tomaras *et al.* demonstrated that biofilm formation in *A. baumannii* is phenotypically associated with exopolysaccharide production and pilus formation (*Tomaras et al.*, 2003). Adherence of *A. baumannii* to human bronchial epithelial cells and erythrocytes has also been demonstrated, with similar pilus-like structures appearing important for adherence. Interestingly, considerable variation in quantitative adherence was observed between strains, including greater adherence of strains from European clone II than from clone I (*Lee et al.*, 2006). However, no difference between outbreak and nonoutbreak strains was observed.

1.2.2.3.3 Adherence and OMP function

After adherence to human cells, it appears that *A. baumannii* can induce apoptosis via an OMP (Omp38). This protein appears to localize to the mitochondria, leading to both caspase-dependent and -independent pathways of apoptosis. However, it is not the only factor involved, as an Omp38 mutant caused incomplete attenuation of cell death (*Choi et al.*, 2005).
1.2.2.3.4 A. baumannii LPS

Several recent studies have described the innate immune response to A. baumannii and the importance of Toll-like receptor (TLR) signaling (Erridge et al., 2007). In a mouse pneumonia model, TLR4 gene-deficient mice had increased bacterial counts, increased bacteremia, impaired cytokine/chemokine responses, and delayed onset of lung inflammation compared to wild-type mice. A. baumannii LPS was identified as the major immuno-stimulatory factor. This was further illustrated by the attenuated effects of A. baumannii on mice deficient in CD14, an important molecule that enables LPS binding to TLR4 (Knapp et al., 2006). These findings were recently confirmed using human cells, but in contrast to the mouse model, TLR2 was also identified as an important signaling pathway. The latter study also demonstrated the potent endotoxic potential of A. baumannii LPS, which stimulated the proinflammatory cytokines interleukin-8 and tumor necrosis factor alpha equally to the stimulation by E. coli LPS at similar concentrations (Erridge et al., 2007). These studies suggest that A. baumannii endotoxin may incite a strong inflammatory response during infection (Anton et al., 2008).

Humoral immune responses have also been described for Acinetobacter infection, with antibodies being targeted toward iron-repressable OMPs and the O polysaccharide component of LPS (Smith et al., 1991). Of interest, a more recent study showed that mouse-derived monoclonal antibodies directed at A. baumannii OMPs expressed in an iron-depleted environment have bactericidal and opsonizing in vitro activity. These
antibodies were also able to block siderophore-mediated iron uptake (Goel and Kapil, 2001).

1.2.3 Clinical Manifestations of *Acinetobacter baumannii* Infections

1.2.3.1 Hospital-Acquired Pneumonia

In most institutions, the majority of *A. baumannii* isolates are from the respiratory tracts of hospitalized patients. In many circumstances, it is very difficult to distinguish upper airway colonization from true pneumonia. There is no doubt, however, that true ventilator-associated pneumonia (VAP) due to *A. baumannii* occurs. In large surveillance studies from the United States, between 5 and 10% of cases of ICU-acquired pneumonia were due to *A. baumannii* (Gaynes and Edwards, 2005). However, it is highly likely that in certain institutions, the proportion of ICU-acquired pneumonia due to *A. baumannii* is much higher. Typically, patients with *A. baumannii* infections have had prolonged ICU stays (Garnacho-Montero et al., 2005).

1.2.3.2 Community-Acquired Pneumonia

Community-acquired pneumonia due to *A. baumannii* has been described for tropical regions of Australia and Asia (Anstey et al., 2002, Leung et al., 2006). The disease most typically occurs during the rainy season among people with a history of alcohol abuse and may sometimes require admission to an ICU. It is characterized by a fulminant clinical course, secondary bloodstream infection, and mortality rate of 40 to 60% (325). The source of infection may be throat carriage, which occurs in up
to 10% of community residents with excessive alcohol consumption (Anstey et al., 2002).

1.2.3.3 Bloodstream Infection

In a large study of nosocomial bloodstream infection in the United States (1995-2002), A. baumannii was the 10th most common etiologic agent, being responsible for 1.3% of all monomicrobial nosocomial bloodstream infections (0.6 bloodstream infection per 10,000 admissions). A. baumannii was a more common cause of ICU-acquired bloodstream infection than of non-ICU-ward infection (1.6% versus 0.9% of bloodstream infections, respectively, in those locations). Crude mortality overall from A. baumannii bloodstream infection was 34.0% to 43.4% in the ICU and 16.3% outside the ICU. A. baumannii bloodstream infection had the third highest crude mortality rate in the ICU, exceeded only by P. aeruginosa and Candida spp. infections. A. baumannii infections were the latest of all bloodstream infections to occur during hospitalization, occurring a mean of 26 days from the time of hospital admission (Wisplinghoff et al., 2004).

It is therefore not certain if the high crude mortality rate represents its occurrence in patients with ongoing underlying critical illness or whether the organism does have significant attributable mortality. Sources of bloodstream infection were not described in the study mentioned above but are typically line related or attributed to underlying pneumonia, UTI, or wound infection (Seifert et al., 1995).

1.2.3.4 Traumatic Battlefield and Other Wounds

A. baumannii may occasionally cause skin/soft tissue infections outside of the military population. The organism caused 2.1% of ICU-
acquired skin/soft tissue infections in one assessment (Gaynes and Edwards, 2005). It is a well-known pathogen in burn units and may be difficult to eradicate from such patients (Trottier et al., 2007). However, its contribution to poor outcome in burn patients is debated (Albrecht et al., 2006). A. baumannii is commonly isolated from wounds of combat casualties from Iraq or Afghanistan. It was the most commonly isolated organism (32.5% of cases) in one assessment of combat victims with open tibial fractures. However, it appears to be of low pathogenicity at this site—after initial treatment, the organism was never isolated from follow-up cultures in any of the patients with open tibial fractures and did not appear to contribute directly to persistent nonunion or need for amputation (Johnson et al., 2007).

1.2.3.5 Urinary Tract Infection (UTI)

A. baumannii is an occasional cause of UTI, being responsible for just 1.6% of ICU-acquired UTIs in one study (Gaynes and Edwards, 2005). Typically, the organism is associated with catheter-associated infection or colonization. It is not usual for this organism to cause uncomplicated UTI in healthy outpatients.

1.2.3.6 Meningitis

Nosocomial, postneurosurgical A. baumannii meningitis is an increasingly important entity. The microbial epidemiology of nosocomial meningitis is evolving to include more gram-negative pathogens (Palabiyikoglu et al., 2006). It is not surprising that multidrug-resistant A. baumannii is among the pathogens implicated. Typical patients have undergone neurosurgery and have an external ventricular drain. Mortality
may be as high as 70%, although the cause of mortality is often difficult to discern (Metan et al., 2007).

1.2.3.7 Other Manifestations

A small number of case reports of *Acinetobacter* endocarditis exist (Rizos et al., 2007). Most, but not all, cases have involved prosthetic valves. *Acinetobacter* spp. may cause endophthalmitis or keratitis, sometimes related to contact lens use or following eye surgery (Levy et al., 2005). A single case report exists of a Shiga toxin-producing *A. haemolyticus* strain, which was associated with bloody diarrhea in a 3-month-old infant (Grotius et al., 2006).

1.2.4 Laboratory Identification

1.2.4.1 Isolation from Clinical Specimens

It is more useful to use a selective medium that suppresses the growth of other microorganisms. A selective and differential medium containing bile salts, sugars, and bromocresol purple is available commercially (Mandel et al., 1964).

For environmental screening in areas where *acinetobacters* may be present in only small numbers, liquid enrichment cultivation may also be useful (Baumann et al., 1968).

Specimens contaminated with a variety of microorganisms can be used to inoculate a liquid mineral medium containing a single carbon and energy source and ammonium or nitrate salt as the nitrogen source, with a final pH of 5.5 to 6.0. Vigorous shaking during incubation is needed so that any *acinetobacters* present can outgrow any pseudomonads. After
incubation for 24 to 48h, a loopful of the culture broth is inoculated on to
a selectivemedium and any presumptive *Acinetobacter* colonies are
identified further. This method has been used to recover *acinetobacters*
from fecal specimens (*Grehn and von Graevenitz, 1978*) and from
various clinical and environmental specimens (*Dijkshoorn et al., 1987*).

1.2.4.2 Morphological, Cultural, and Metabolic Characteristics

**Morphology:**

*Acinetobacters* are short, plump, gram-negative (but sometimes
difficult to destain) rods, typically 1.0 to 1.5 by 1.5 to 2.5mm in the
logarithmic phase of growth but often becoming more coccoid in the
stationary phase. Pairing or clustering of cells often occurs.

**Culture character:**

**General features**

*Acinetobacter* spp. normally form smooth, sometimes mucoid, pale
yellow to greyish-white colonies on solid media, although some
environmental strains that produce a diffusible brown pigment have been
described (*Pagel and Seyfried, 1976*).

The colonies are comparable in size to those of *enterobacteria*. *Acinetobacters* are strictly aerobic, non fastidious organisms that can
grow on common laboratory media. Most of *Acinetobacters* grow
between 20°C and 41°C with most strains having an optimum at
37°C. Growth at 44°C which is a characteristic feature of *A. baumannii*.
The pH range for growth of *Acinetobacters* is 5.5 to 6.0 (*Yavankar et al.,
2007*).
Chapter (1) Introduction & Literature Review

**Culture MEDIA:**

1- Ordinary Media

On nutrient agar media *Acinetobacters* grow forming smooth, mucoid, pale yellow to greyish white colonies about 1-2mm in diameter (*Bergogene and Towner, 1996*).

2- Enriched Media

On 5% sheep blood agar they produce smooth, opaque, raised, creamy colonies that are smaller than *enterobacteriaceae*, except for the colonies of *Acinetobacter calcoaceticus- baumannii complex* (Acb complex) which resemble those of *enterobacteriaceae*. Regarding hemolysis, *A.hemolyticus* and several other currently not well-defined species such as *Acinetobacter* genomic species 6,13BJ,14BJ,15BJ,16, and 17 show hemolysis on sheep blood agar, a property that is never present in *Acinetobacter* isolates belonging to the Acb complex (*Seifert and Dijkshoorn, 2008*).

3- Differential Media

On MacConky agar they grow forming non lactose fermenting colonies. However some colonies exhibit unexplained purplish hue that may cause the organism to be mistaken for a lactose fermenter (*Seifert and Dijkshoorn, 2008*).

4- Selective Media

Several selective media are available for the isolation of *Acinetobacters*

*a- Mandel s Media:*
Mandel et al. developed a media containing lactose and maltose as tested sugars, bile salts as selective agent and bromocresol purple as a Ph indicator which turns to yellow when there is acidification of the substrate. *Acinetobacters* does not ferment the two sugars and grows with colonies of the same color of the medium, sometimes with a slight color change to a more intense violet (*Mandel et al., 1964*).

**b- Herellea Agar Media:**

It is the modification of Mandel’s media by substituting sucrose for maltose (*MacFaddin, 1985*).

**c- Holton Media:**

Further more modification done by Holton on Herellea agar media by substituting desiccated ox bile for the bile salts and ampicillin, cefsulodin and vancomycin were added. Holton also substituted fructose, mannitol, phenylalanine and phenol red for lactose and bromocresol purple. Fructose, sucrose, and mannitol are fermentable carbohydrates and phenylalanine is added to further differentiate those organisms that produce phenylpyruvic acid. Phenol red is the pH indicator that turns yellow in the presence of acid and pink to mauve when high levels of alkalinity are produced in the medium by the growth of organisms. *Acinetobacters* does not ferment these sugars and grows giving pink colonies with mauve background (*Holton, 1983*).

It was observed that the previous existing media are either not sufficiently selective because of the absence of appropriate inhibitory agents for other organisms or too inhibitory because of the presence of a
high concentration of agents inhibitory for Acinetobacters particularly ampicillin (Jawad et al., 1994).

**d- Leeds Acinetobacter Medium (LAM):**

Leeds Acinetobacter Medium developed at the university of Leeds by Jawad et al. In this formulation, ampicillen was removed and the concentrations of the other antibiotics were adjusted to obtain optimum performance.

LAM medium, proved to be better than Herellea agar or Holtons agar in isolation of Acinetobacters as documented in a comparative study for the isolation of Acinetobacters from a local hospital environment at united kingdom. LAM recovered more Acinetobacters than Herellea agar or Holtons agar and can overcome the difficulty in differentiation of Acinetobacters from other bacteria by colonial morphology. As citrobacters produce the same pale lavender colonies which is characteristic for Acinetobacters on Herellea agar while on LAM citrobacters were morphologically different from Acinetobacters. Unfortunately Stenotrophomonas maltophilia and Burkholderia cepacia were found to grow on LAM with the same morphological characters of Acinetobacters (Jawad et al., 1994).

**e- Hardy Diagnostics Multidrug Resistant (MDR) Acinetobacter medium:**

Hardy Diagnostics (MDR) Acinetobacter medium contains further modifications of the Leeds formula to include some common antibiotics to act as selective agent for which most multi-drug resistant strains are resistant thus inhibiting non-multi-drug resistant strains of Acinetobacters.
Although the development of MDR Acinetobacter media is useful as it inhibit the growth of *citrobacters, Providencia alcalifaciens* and *Serratia marcescens* which grow on LAM media but the problem is that it allow the growth of *Stenotrophomonas maltophilia* and *Burkholderia cepacia* which have the same morphological feactures of *Acinetobacters* on MDR Acinetobacters Medium (table 2)

**f- Chromogenic agar(CHROMagar):**

CHROMagar *Acinetobacter* is a recently developed chromogenic media designed for detection and isolation of *Acinetobacter* species. This media inhibits the growth of most gram-positive cocci and yeast and employs a color-change identification method that allows *Acinetobacter* species to appear as red colonies.

In a study to evaluate the performance of CHROMagar *Acinetobacter* when compared to sheep blood agar, MacConkey agar and MacConkey agar with 6 µg/ml of imipenem for the detection of *A. baumannii* in surveillance cultures of hospitalized patients. Peri-anal swabs and sputum samples were utilized from patients admitted to the University of Maryland Medical Center ICUs from December 7 through December 21, 2009. Samples were plated onto four media in the following order: (1) 5% sheep blood agar (SBA), (2) MacConkey agar, (3) MacConkey agar with 6 µg/ml of imipenem, and (4) CHROMagar *Acinetobacter* (CHROMagar). SBA was the gold standard to which all media were compared. There were 165 samples collected during the study period. SBA and CHROMagar detected 18 of 18 (100%) *Acinetobacter* and 11 of 11 (100%) MDR-A. baumannii. MacConkey agar detected 16 of 18 (89%) *Acinetobacter* and 10 of 11
(91%) MDR- A. baumannii while MacConkey agar with 6 µg/ml imipenem detected 9 of 11 (82%) MDR-A. baumannii. CHROMagar did not differentiate MDR- A. baumannii from non-MDR-A. baumannii. CHROMagar may be useful for rapid detection of patients with MDR-A. baumannii if improved upon to better select for MDR-A. baumannii (Ajao et al., 2011).

**Metabolic Characteristics**

Oxidase negative, catalase positive, and nonfermentative. It is the negative oxidase test that serves as a rapid presumptive test to distinguish Acinetobacter spp. from otherwise similar nonfermentative bacteria. Most strains are unable to reduce nitrate to nitrite in the conventional nitrate reduction assay (Warskow and Juni, 1972).
Table (2): Detects different organisms that grow on media used for *Acinetobacter* isolation (Jawad et al., 1994).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Morphology after growth on</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Herellea agar</td>
</tr>
<tr>
<td><em>Acinetobacter SPP.</em></td>
<td>Pale lavender colonies with Yellow background</td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>Pale lavender colonies with Yellow background</td>
</tr>
<tr>
<td><em>Klebsiella pneumonia</em></td>
<td>Yellow colonies with Yellow background</td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em></td>
<td>Yellow colonies with Yellow backgrounds</td>
</tr>
<tr>
<td><em>Citrobacter spp.</em></td>
<td>Pale lavender colonies with Yellow</td>
</tr>
</tbody>
</table>
1.2.4.3. Molecular Epidemiologic Techniques

1.2.4.3.1 Phenotypic typing systems:

1.2.4.3.1.1 Biochemical profiles (biotyping)

The API 20NE system has been used to distinguish 31 different biotypes among 122 different Acinetobacter strains (Towner and Chopade, 1987), but this system sometimes has problems with sensitivity and reproducibility (Kropec et al., 1993). However, cluster analysis of carbon source growth assays has been used to identify a major grouping of isolates that was related to the epidemiological origin of the strains (Dijkshoorn et al., 1990).

A collection of 130 Acinetobacter strains identified by DNA hybridization to 18 different genomic species was used to assess the ability of the API 20NE system (bioMérieux, France) to identify Acinetobacter genomic species and to determine its accuracy. Fifty-eight (87%) of the 67 strains of genomic species defined in the database (version 5.1) were identified to the appropriate genomic species. The ACINETOBACTER BAUMANNII strains and the Acinetobacter haemolyticus strains were all identified correctly. Three of five Acinetobacter junii strains, three of eight Acinetobacter johnsonii strains, and 11 of 13 Acinetobacter lwoffii strains were also identified correctly. The 58 correctly identified strains represented 45% of the total 130 strains. Thirty-six of the 72 inappropriately identified strains were designated ACINETOBACTER BAUMANNII. Thirty-one of these 36 strains belonged to genomic species 1 (Acinetobacter calcoaceticus), 3, or 13TU. Analysis of the profiles showed that the API system does not discriminate between genomic species 1, 2, 3, and 13TU. Lumping of ...
these groups into the *Acinetobacter calcoaceticus-ACINETOBACTER BAUMANNII* complex in the API 20NE database would make the system considerably more accurate. Incorporation of these data into the database may improve identification of the remaining genomic species, including some that are not defined. However, the discriminative power of the tests in the API galleries is insufficient for correct identification of all *Acinetobacter* genomic species (*Bernards et al., 1996*).

A study was designed to compare phenotypic with molecular identification of nonfermenting gram-negative bacilli (non-*Pseudomonas aeruginosa*). Results of molecular analyses were compared with two commercially available identification systems (API 20 NE, VITEK 2 fluorescent card; bioMérieux, Marcy l'Étoile, France). By 16S rRNA gene sequence analyses, 92% of the isolates were assigned to species level and 8% to genus level. Using API 20 NE, 54% of the isolates were assigned to species and 7% to genus level, and 39% of the isolates could not be discriminated at any taxonomic level. The respective numbers for VITEK 2 were 53%, 1%, and 46%, respectively. Fifteen percent and 43% of the isolates corresponded to species not included in the API 20 NE and VITEK 2 databases, respectively. It was concluded that 16S rRNA gene sequencing is an effective means for the identification of clinically relevant nonfermenting gram-negative bacilli (*Bosshard et al. 2006*).

1.2.4.3.1.2 Antibiotic susceptibility patterns (Antibiograms)

Numerous studies have used antibiotic susceptibility patterns (antibiograms) to detect emerging resistance patterns and to group similar isolates, often on the basis of MICs, breakpoints, or zone diffusion sizes (*Struelens et al., 1993*).
Chapter (1) Introduction & Literature Review

Such results are often expressed as resistant, susceptible, or intermediate a more informative approach uses the actual diameters of inhibition zones in disc diffusion tests for cluster analysis and such groupings have been shown to correlate well with other typing and epidemiological data \cite{Dijkshoorn1993}. However, it must be emphasized that antibiogram typing results should be interpreted with caution, since unrelated strains may exhibit the same antibiogram \cite{Joly-Guillou1991} and changes in susceptibility may occur during episodes of infection \cite{Bergogne-Berezin1996}.

1.2.4.3.1.3 Serological reactions (serotyping)

There have been numerous attempts to type \textit{Acinetobacter} strains by serological reactions, but only limited success was obtained in early work \cite{Das1984}.

Most such schemes have been rendered obsolete by the taxonomic developments in the genus. More recent work involving checkerboard tube agglutinations and reciprocal cross-absorptions with polyclonal rabbit immune sera against heated cells has allowed the delineation of 34 serovars in \textit{A. nii} and 26 serovars in genomic species 3 \textit{A.baumannii} \cite{Traub1994}. However, antigenic differences between \textit{A. baumannii} and genomic species 3 serovars were not entirely satisfactory, and the relationship with strains belonging to genomic species13TU is unclear \cite{Bergogne-Berezin1996}.

Serotyping using a system based on monoclonal antibodies against certain O antigens developed in the 1990s \cite{Pantophlet2002} were not widely used because, since the 1990s, a variety of genotyping methods have become available for epidemiologic typing of \textit{Acinetobacter} strains \cite{Ferens2011}.
1.2.4.3.1.4 Phage typing

Two complementary sets of bacteriophages (comprising 25 phages, allowing the identification of 125 phage types, and 14 phages, allowing the identification of 25 phage types) have been used in a number of different epidemiological studies of Acinetobacter isolates from France and other European countries (Joly-Guillou et al., 1990). Predominant phage types (numbers 17 and 124) have been identified in some outbreaks (Santos et al., 1984). However, this system has been used only at the Phage Typing Center of the Institut Pasteur in Paris, and it seems that a substantial proportion of strains from other geographical locations may be nontypeable. Although some doubt has been cast upon the reproducibility of the results (Bouvet et al., 1990), phage typing may be useful, albeit time-consuming, when used in conjunction with other typing methods (Bergogne-Berezin and Towner, 1996).

1.2.4.3.1.5 Protein profiles

Both cell envelope and whole-cell protein patterns have been used in a series of epidemiological and taxonomic studies of Acinetobacter spp. Analysis of cell envelope protein patterns by sodium dodecyl sulfate-polyacrylamide gel electrophoresis has shown heterogeneity in unrelated strains, while multiple isolates from patients or outbreaks were indistinguishable (Dijkshoorn et al., 1987). This method has been used successfully to trace specific strains during endemic episodes and outbreaks in hospitals (Weernink et al., 1995). Electrophoretic analysis of whole-cell protein fractions has the advantage that sample preparation is simpler than preparation of cell envelopes. Similarities between Acinetobacter isolates from outbreaks and dissimilarities in unrelated control strains have been reported in several studies (Alexander et al., 1988).
1.2.4.4 Molecular typing systems

1.2.4.4.1 Plasmid profiling

The majority of Acinetobacter species contain indigenous plasmids. Plasmid analysis has been used successfully for epidemiological typing of A. baumannii strains (Nemec et al., 1999), and plasmid profiling is one of the few methods that have also been applied to study the epidemiology of Acinetobacter species outside the A. baumannii group (Seifert et al., 1997). Even though the method is fairly robust, interpretation of results must include the consideration that many plasmids are easily transferable and may be gained or lost, and this contributed to the replacement of plasmid profiling by more robust molecular methods for epidemiological studies of Acinetobacters (Anton et al., 2008). In a study to explore Acinetobacter spp. via antimicrobial susceptibility, plasmid profiles, and random amplified polymorphism DNA polymerase chain reaction (RAPD-PCR) typing, one hundred twelve Acinetobacter isolates (including 66 A. baumannii and 46 non-ACINETOBACTER BAUMANNII strains) were obtained from three university hospital. Their susceptibilities to 17 antibiotics were tested and then all Acinetobacter isolates were typed by plasmid analysis and RAPD-PCR method. A. baumannii isolates revealed nine different patterns of antibiotic resistance Of those, non A.baumannii, were associated with plasmid and RAPD-PCR typings (p <0.05). A.baumannii was more resistant to multiple antibiotics than non-A.baumannii (p <0.05). Seven different plasmid profiles were observed among 112 Acinetobacter isolates. Plasmids were found in 107 (95.5%) of the 112 isolates. Unlike in RAPD-PCR typing, there was no difference between
the type of Acinetobacter, A.baumannii or non-A.baumannii strains and plasmid profiles (p>0.05). By RAPD-PCR, six profiles were found for each A. baumannii and non-A.baumanni strains. The pattern 6 was the most common pattern among the isolates. Accordingly, RAPD typing was of particular significance in the epidemiological tracing and study of Acinetobacter more than plasmid profiling. This can be attributed to the nature of RAPD profiling, which depends on DNA analysis. Therefore, RAPD typing is more conservative and stable than that of plasmids (Nourkhoda et al., 2010).

1.2.4.4.2 Ribotyping

Ribotyping was developed primarily to identify acinetobacters, in particular strains of the A. calcoaceticus-A. baumannii complex, to the species level (Gerner et al., 1992). This method—using EcoRI, ClaI, and SalI for restriction of purified chromosomal DNA, followed by electrophoresis, blotting, and hybridization with a digoxigenin-11-UTP-labeled cDNA probe derived from E. coli rRNA—has also been used to type strains in several studies investigating the epidemiology of acinetobacters (Griffith et al., 2006). However, the discriminatory power of ribotyping is limited, and PFGE and other methods are less labor-intensive and more discriminatory (Silbert et al., 2004). More accurate typing results with a discriminatory power comparable to that of PFGE have been obtained using an automated ribotyping system (RiboPrinter; DuPont Qualicon, Wilmington, DE). Automated ribotyping generates typing results more rapidly than PFGE does, but it is expensive and requires specialized equipment that is available in only a few laboratories that perform high-throughput molecular epidemiology investigations (Anton et al., 2008).
1.2.4.4.3. Pulsed Field Gel Electrophoresis (PFGE)

Even in the face of sequence-based methods that are now available and are challenging PFGE as the gold standard for typing of many bacterial species, for *Acinetobacter* PFGE still remains the reference method of choice. It is a rather laborious method that requires several days before generating a typing result, but the necessary equipment is now standard not only in most reference laboratories but also in hospital-based laboratories. Generally, ApaI and/or SmaI is used for restriction of intact chromosomal DNA (*Bou et al., 2000*). The resulting chromosomal fragments are separated by electrophoresis, and fingerprint profiles are compared visually or using specialized computer programs that also allow the storage of profiles in a database. As with other so-called comparative typing systems that are based mainly on a side-by-side comparison of molecular fingerprint patterns of a limited number of strains, interlaboratory comparison has always been a problem with PFGE, but a recent study has shown that with sufficient standardization of protocols interlaboratory reproducibility can be achieved (*Seifert et al., 2005*). This approach would permit the recognition of epidemic strains and the early detection of multihospital or nationwide outbreaks, particularly if cases are geographically separated. As seen with other species, the discriminatory power of PFGE may be too high for large-scale epidemiologic and population studies, but the potentially greater value of newer methods, such as MLST or PCR-ESI-MS remains to be demonstrated for *Acinetobacter* (*Anton et al., 2008*).
1.2.4.4.4 PCR Based DNA Finger Printing Techniques

* Randomly amplified polymorphic DNA (RAPD) analysis

Randomly amplified polymorphic DNA PCR, involving amplification of random fragments of genomic DNA with single primers with an arbitrary sequence, has been used successfully to assess the strain relatedness of *Acinetobacter* isolates (*Graser et al.*, 1993).

* Repetitive Extragenic Palindromic (REP) PCR

An alternative approach, referred to as REP-PCR (*Bou et al.*, 2000), uses consensus primers for the highly conserved REP sequences to amplify intervening sequences located between these DNA motifs. Both RAPD and REP methods do not require specialized equipment and are fast, easy, and low-cost methods that allow grouping of *A. baumannii* strains with various degrees of genotypic relatedness. The discriminatory power of these methods, however, is inferior to that of PFGE. Inter-laboratory reproducibility of PCR-generated fingerprints was demonstrated in one study, using four different primers (DAF4, ERIC-2, M13, and REP1 plus REP2) and a highly standardized protocol (*Grundmann et al.*, 1997), *Huys et al.* (*Huys et al.*, 2005) recently used REP-PCR fingerprinting with a (GTG)5 primer to distinguish members of the pan-European multidrug-resistant *A. baumannii* clone III (*van Dessel et al.*, 2004) from the known clones I and II (*Dijkshoorn et al.*, 1996).

In general, PCR-based typing methods allow for a quick estimate of epidemiological relatedness in a defined setting (*Wroblewska et al.*, 2004), but they are not suited for large-scale comparative epidemiological studies. It remains to be shown if more rigorous standardization and automation of REP-PCR, such as by use of a
DiversiLab system (bioMérieux), which includes a microfluidics-based detection system, will allow bacterial strain typing with an increased interlaboratory reproducibility (*Healy et al.*, 2005).

**Amplified Fragment Length Polymorphism (AFLP)**

AFLP analysis was established in the 1990s. It is a highly sensitive DNA fingerprinting method by which DNA is digested with restriction enzymes, followed by selective amplification, electrophoretic separation of fragments, and visualization. It is a rather cumbersome and expensive method that is usually performed in a semiautomated procedure, with laser detection of fragments on a sequencing platform. The resulting complex profiles are digitized and usually analyzed with appropriate software. Apart from being a powerful tool in bacterial taxonomy (*Nemec et al.*, 2001), this high-resolution fingerprinting method has also been found to be useful for the characterization of *Acinetobacter* strains at the subspecies level and for outbreak investigation (*Dobrewski et al.*, 2006). Even though AFLP analysis is a relatively robust method, it requires a high level of standardization and extensive experience in interpretation of banding patterns even if sophisticated computer programs are available to aid in pattern analysis. Therefore, this method is restricted to reference laboratories and not suited for routine epidemiological analyses. In addition, data are not readily transportable between laboratories, mainly due to a lack of reproducibility when different sequencing platforms are used. Although clustering obtained with AFLP analysis compared well to PFGE-derived clustering in small-scale studies (*Silbert et al.*, 2004), a
Introduction & Literature Review

detailed and comprehensive side-by-side comparison of these two typing methods has never been performed (Anton et al., 2008).

1.2.4.4 Multilocus Sequence Typing (MLST).

MLST is a highly discriminative typing method that has been applied to a variety of bacterial pathogens, such as Neisseria meningitidis (Maiden et al., 1998), Streptococcus pneumoniae (Feil et al., 2000), and S. aureus (Enright et al., 2000). The MLST scheme that was recently developed for A. baumannii by Bartual and coworkers is based on 305- to 513-bp sequences of the conserved regions of the following seven housekeeping genes: gltA, gyrB, gdhB, recA, cpn60, gpi, and rpoD. The currently available MLST data are in good concordance with typing results generated by PFGE and AFLP analysis (Bartual et al., 2005).

The discriminatory power of the currently proposed MLST system is comparable to that of both PFGE and AFLP analysis. However, MLST is expensive and laborious and therefore not suited for routine outbreak analysis or other limited-scale analysis of the epidemiology of A. baumannii. It remains to be determined if this typing scheme is appropriate for the study of the population structure of A. baumannii and perhaps other Acinetobacter species, as shown successfully when this method was applied to other microorganisms. To date, MLST is one of the few so-called library typing systems used for the epidemiological study of A. baumannii, i.e., a typing system where typing data are translated into a numerical code that can be obtained in an identical manner at different laboratories by using the same protocol. It provides a portable method that may be suitable for global epidemiologic study and allow the recognition of epidemic, multiresistant, and virulent A.
baumannii clones and the monitoring of their national and international spread (Anton et al., 2008).

1.2.4.4. Multilocus PCR Electrospray ionization/Mass Spectrometry (PCR-ESI-MS).

PCR-ESI-MS is a form of high-throughput MLST that can be used for species identification of A. baumannii as well as Acinetobacter genomic species 3 and 13TU and, in addition, to determine clonality (Ecker et al., 2006). The conserved regions of six bacterial housekeeping genes (trpE, adk, efp, mutY, fumC, and ppa) are amplified from each isolate, amplification products are then desalted and purified, and the mass spectra are determined. A good correlation with PFGE typing was observed. As a major advantage, the PCR-ESI-MS genotyping method appears to be very fast (taking only 4 h), providing typing results on a time scale not achievable with most other systems (Anton et al., 2008).

1.2.5 Mechanisms of antibiotic resistance

Antibiotic resistance in Acinetobacter spp., particularly ACINETOBACTER BAUMANNII, is increasing rapidly. A. baumannii possesses two intrinsic b-lactamase genes, in addition to weak permeability and efflux systems, that together confer a natural reduced susceptibility to antibiotics. In addition, numerous acquired mechanisms of resistance have been identified in A. baumannii. The very high genetic plasticity of A. baumannii allows an accumulation of resistance determinants that give rise to multidrug resistance at an alarming rate. The role of novel genetic elements, such as resistance islands, in
concentrating antibiotic resistance genes in *A. baumannii* requires detailed investigation (*Laurent et al., 2011*).

### 1.2.5.1 β-Lactams

#### 1.2.5.1.1 Enzymatic mechanisms.

The most prevalent mechanism of β-lactam resistance in *A. baumannii* is enzymatic degradation by β-lactamases. However, in keeping with the complex nature of this organism, multiple mechanisms often work in concert to produce the same phenotype (*Fernandez et al., 2003*).

Inherent to all *A. baumannii* strains are chromosomally encoded AmpC cephalosporinases (*Ruiz et al., 2007*), also known as Acinetobacter-derived cephalosporinases (ADCs) (*Hujer et al., 2005*). Unlike that of AmpC enzymes found in other gram-negative organisms, inducible AmpC expression does not occur in *A. baumannii* (*Heritier et al., 2006*). The key determinant regulating overexpression of this enzyme in *A. baumannii* is the presence of an upstream IS element known as ISAba1. The presence of this element highly correlates with increased AmpC gene expression and resistance to extended-spectrum cephalosporins (*Ruiz et al., 2007*). Cefepime and carbapenems appear to be stable in response to these enzymes (*Hujer et al., 2005*).

The first identified OXA-type enzyme with carbapenem-hydrolyzing activity was from a clinical *A. baumannii* strain isolated in 1985 from Edinburgh, Scotland (*Paton et al., 1993*). This plasmid-encoded resistance determinant (initially named ARI-1) was found to be transferable, and the gene was later sequenced and named *blaOXA-23* (*Donald et al., 2000*).
Table (3): Mechanisms of resistance in *Acinetobacter Baumannii* to different antibiotics (Anton et al., 2008)

<table>
<thead>
<tr>
<th>Antimicrobial class and resistance Mechanism</th>
<th>Enzyme(s)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>β</em>-Lactams</td>
<td>TEM</td>
<td>Endimiani, <em>et al</em> 2007</td>
</tr>
<tr>
<td></td>
<td>SHV</td>
<td>Naiemi, <em>et al</em> 2005</td>
</tr>
<tr>
<td></td>
<td>ADCs</td>
<td>Ruiz, <em>et al</em> 2007</td>
</tr>
<tr>
<td></td>
<td>VEB</td>
<td>Naas, <em>et al</em> 2006</td>
</tr>
<tr>
<td></td>
<td>PER</td>
<td>Naas, <em>et al</em> 2006</td>
</tr>
<tr>
<td></td>
<td>CTX-M</td>
<td>Celenza, <em>et al</em> 2006</td>
</tr>
<tr>
<td></td>
<td>OXA</td>
<td>Anton <em>et al</em>, 2008</td>
</tr>
<tr>
<td></td>
<td>IMP</td>
<td>Koh, <em>et al</em> 2007</td>
</tr>
<tr>
<td></td>
<td>VIM</td>
<td>Lim, <em>et al</em> 2007</td>
</tr>
<tr>
<td></td>
<td>SIM</td>
<td>Lee, <em>et al</em> 2005</td>
</tr>
<tr>
<td><em>OMP</em></td>
<td>CarO (29 kDa)</td>
<td>Mussi, <em>et al</em> 2005</td>
</tr>
<tr>
<td></td>
<td>47-, 44-, and 37-kDa OMPs</td>
<td>Quale, <em>et al</em> 2003</td>
</tr>
<tr>
<td></td>
<td>22- and 33-kDa OMPs</td>
<td>Bou, <em>et al</em> 2000</td>
</tr>
<tr>
<td></td>
<td>33- to 36-kDa OMPs</td>
<td>del Mar Tomas, <em>et al</em> 2005</td>
</tr>
<tr>
<td></td>
<td>HMP-AB</td>
<td>Gribun, <em>et al</em> 2003</td>
</tr>
<tr>
<td></td>
<td>43-kDa OMP</td>
<td>Dupont, <em>et al</em> 2005</td>
</tr>
<tr>
<td></td>
<td>Omp W</td>
<td>Siroy, <em>et al</em> 2006</td>
</tr>
<tr>
<td>Efflux</td>
<td>AdeABC</td>
<td>Siroy, <em>et al</em> 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Siroy, <em>et al</em> 2006</td>
</tr>
<tr>
<td><em>Aminoglycosides</em></td>
<td>Acetyltransferases, nucleotidyltransferases, phosphotransferases</td>
<td>Hujer, <em>et al</em> 2006</td>
</tr>
<tr>
<td><em>Ribosomal (16S rRNA)</em> methylation</td>
<td></td>
<td>Doi, <em>et al</em> 2007</td>
</tr>
<tr>
<td>Efflux</td>
<td>AdeABC</td>
<td>Magnet, <em>et al</em> 2001</td>
</tr>
<tr>
<td></td>
<td>AdeM</td>
<td>Su, <em>et al</em> 2005</td>
</tr>
<tr>
<td><em>Quinolones</em></td>
<td>GyrA, ParC</td>
<td>Hamouda, <em>et al</em> 2004</td>
</tr>
<tr>
<td>Efflux</td>
<td>AdeABC AdeM</td>
<td>Higgins, <em>et al</em> 2004</td>
</tr>
<tr>
<td><em>Tetracyclines and glycylcyclines</em></td>
<td></td>
<td>Ribera, <em>et al</em> 2003</td>
</tr>
<tr>
<td>Tetracycline-specific efflux</td>
<td>Tet(A), Tet(B)</td>
<td>Ribera, <em>et al</em> 2003</td>
</tr>
</tbody>
</table>

*a* ADCs, *Acinetobacter*-derived cephalosporinases; HMP-AB, heat-modifiable protein in *ACINETOBACTER BAUMANNII*. 
This enzyme type now contributes to carbapenem resistance in *A. baumannii* globally (Corvec et al., 2007). OXA-27 and OXA-49 are closely related enzymes that make up the blaOXA-23 gene cluster in *A. baumannii* (Brown and Amyes, 2006). Two other acquired OXA-type gene clusters with carbapenemase activity have been described, including the blaOXA-24-like (encoding OXA-24, -25, -26, and -40) (Canduela et al., 2006) and the blaOXA-58-like (Giordano et al., 2007) carbapenemase genes. The crystal structure of OXA-24 was described and provides important insights for future drug development toward this emerging class of carbapenemases (Santillana et al., 2007). BlaOXA-58 was identified more recently and, similar to blaOXA-23, is often plasmid mediated (Poirel et al., 2005), which may explain its widespread distribution (Coelho et al., 2006). The final gene cluster, blaOXA-51-like genes (encoding OXA-51, -64, -65, -66, -68, -69, -70, -71, -78, -79, -80, and -82), is unique in that it is naturally occurring in *A. baumannii*, hence its chromosomal location and prevalence (Wroblewska et al., 2007). Similar to other class D enzymes, its product has a greater affinity for imipenem than for meropenem (Brown et al., 2005). Its role in carbapenem resistance appears to be related to the presence of ISAba1 (Turton et al., 2006). In the absence of this element, cloning studies suggest a minimal effect on carbapenem susceptibility, even in the presence of an overexpressed multidrug efflux pump (AdeABC) (Heritier et al., 2005). Given the multiplicity of β-lactam resistance mechanisms in *A. baumannii* (Poirel and Nordmann, 2006), the contributions of the acquired carbapenem-hydrolyzing oxacillinases to carbapenem resistance are often difficult to determine. This issue has been addressed by Heritier et al., who studied the changes in
susceptibility profiles of both natural and recombinant plasmids containing blaOXA-23, blaOXA-40 (only a recombinant plasmid, as no natural plasmid was identified), and blaOXA-58 in different host backgrounds. blaOXA-23 and blaOXA-40 appeared to produce higher MICs of imipenem than did blaOXA-58, and all blaOXA genes produced higher MICs of imipenem in the presence of an overexpressed AdeABC efflux pump. Inactivation of the blaOXA-40 gene led to susceptibility to carbapenems, and resistance was restored with complementation. Interestingly, the natural plasmids containing blaOXA-23 and blaOXA-58, extracted from clinical isolates, produced significantly greater levels of resistance to carbapenems than did their respective recombinant plasmids in similar host backgrounds (Heritier et al., 2005). This discrepancy is most likely due to the presence of IS elements in the natural plasmids (Anton et al., 2008).

The importance of IS elements for carbapenem resistance due to oxacilllnases in A. baumannii has only recently been appreciated (Corvec et al., 2007). These elements provide two main functions. First, they encode a transposase and therefore are mobile. Second, they can contain promoter regions that lead to overexpression of downstream resistance determinants. Most commonly, these elements have been described in association with blaOXA-23 (Corvec et al., 2007) and blaOXA-58 (Giordano et al., 2007), but they may also promote carbapenem resistance in association with blaOXA-51 (Turton et al., 2006). Interestingly, certain IS elements, especially ISAb1, appear relatively unique to A. baumannii (Segal et al., 2005). IS elements are also important for the expression of resistance to other antibiotics in A. baumannii (Ruiz et al., 2007).
Despite MBLs being less commonly identified in *A. baumannii* than the OXA-type carbapenemases, their hydrolytic activities toward carbapenems are significantly more potent (100- to 1,000-fold) (Poirel, and Nordmann, 2006). These enzymes have the capability of hydrolyzing all β-lactams (including carbapenems) except the mono-bactam aztreonam, which may assist in laboratory detection. Unlike the OXA-type enzymes, MBLs are most commonly found within integrons, which are specialized genetic structures that facilitate the acquisition and expression (via a common promoter) of resistance determinants. Most acquired MBL genes in *A. baumannii* have been found within class 1 integrons, often containing an array of resistance gene cassettes, especially those encoding aminoglycoside-modifying enzymes (Tsakris et al., 2006). Not surprisingly, *A. baumannii* strains carrying integrons have been found to be significantly more drug resistant than strains without integrons (Gu Tong et al., 2007). The clinical significance of this unique genetic structure is that overuse of one antimicrobial may lead to overexpression of multiple resistance determinants as a consequence of a common promoter. In isolation, integrons are not mobile and therefore are embedded within plasmids or transposons that act as the genetic vehicles for resistance dissemination (Anton et al., 2008).

1.2.5.1.2 Non enzymatic mechanisms.

Non-enzymatic Resistance to β-lactams and in particular carbapenems. Resistance to carbapenems in *A. baumannii* may be enhanced by interactions between broad-spectrum Blactamases and other resistance mechanisms, including porin(s) loss, active drug efflux, and (rarely) modification of penicillin-binding proteins (PBPs) (Poirel and Nordmann, 2006).
Several reports have associated decreased expression of certain porins with antimicrobial resistance in *A. baumannii*, including several outer membrane proteins (OMPs) that have some homology with the monomeric OmpA porin found in Enterobacteriaceae. Porins of this family have been characterized in several species of Acinetobacter, including *A. radioresistens*, *A. junii* and *A. baumannii*, and are known as slow porins that allow the penetration of β-lactams (*Gribun et al., 2003*). Three OMPs have been associated with resistance or decreased susceptibility to carbapenems, namely a 33–36 kDa protein, a 29kDa protein also known as CarO, and a 43 kDa protein, that show homologies with OprD from *P. aeruginosa*. An additional OMP (OmpW) has been identified in *A. baumannii* that shows significantly decreased expression in ceftriaxone-resistant clinical isolates. Efflux-mediated resistance is a common factor affecting antibiotic susceptibility in Gram-negative bacteria, and several efflux pumps have been described in *A. baumannii*. The AdeABC (*Acinetobacter* drug efflux) pump belongs to the Resistance Nodulation-cell Division (RND) family. The adeABC operon encodes the AdeA Major Fusion Protein (MFP), the inner membrane protein AdeB and the outer membrane factor (OMF) AdeC. In activation experiments in clinical isolates over-expressing AdeABC showed that cefepime, cefpirome, and cefotaxime were the most affected β-lactams (*Vila et al., 2007*). Over-expression of the naturally-occurring AdeABC efflux pump in association with carbapenem-hydrolyzing oxacillinases may confer a high-level of resistance to carbapenems (*Heritier et al., 2005*). A second RND efflux pump that contributes to β-lactam resistance in *A. baumannii* is AdeIJK, but no effect of this pump on carbapenem resistance has been observed (*Vila et al., 2007*).


1.2.5.2 Aminoglycosides

The presence of genes coding for aminoglycoside-modifying enzymes within class 1 integrons is highly prevalent in multidrug-resistant *A. baumannii* strains (*Tsakris et al., 2006*). All of the major enzyme classes have been described, including acetyltransferases, nucleotidyltransferases, and phosphotransferases (*Hujer et al., 2006*). More recently, 16S rRNA methylation has been described for *A. baumannii* (armA) strains from Japan, Korea, and the United States (*Doi et al., 2007; Lee et al., 2006; Yamane et al., 2005*). This emerging resistance mechanism impairs aminoglycoside binding to its target site and confers high-level resistance to all clinically useful aminoglycosides, including gentamicin, tobramycin, and amikacin (*Doi and Arakawa, 2007*). Interestingly, the genetic surroundings of armA appear very similar across gram-negative organisms, as it is plasmid borne and within a transposon (Tn1548) (*Doi et al., 2007*).

Apart from the AdeABC efflux pump, which less effectively transports amikacin and kanamycin due to their more hydrophilic nature (*Magnet et al., 2001*), aminoglycosides (gentamicin and kanamycin) are also substrates of the recently described AbeM pump, a member of the multidrug and toxic compound extrusion (MATE) family (*Su et al., 2005*).

1.2.5.3 Quinolones

Modifications to DNA gyrase or topoisomerase IV through mutations in the gyrA and parC genes have been well described for *A. baumannii* (*Hamouda and Amyes, 2004*). These mutations interfere with
Introduction & Literature Review

target site binding. Similar to aminoglycosides, many quinolones are also substrates for multidrug efflux pumps (Ribera et al., 2002), including the RND-type pump AdeABC (Higgins et al., 2004) and the MATE pump AdeM (Su et al., 2005). Thus far, plasmid-mediated quinolone resistance, mediated by qnr genes, has not been reported for A. baumannii (Anton, 2008).

1.2.5.4 Tetracyclines and Glycylcyclines

Resistance to tetracyclines and their derivatives can be mediated by efflux or ribosomal protection (Fluit et al., 2005). Tetracycline-specific efflux pumps include those encoded by the tet(A) to tet(E) determinants, most often found within gram-negative organisms, and the tet(K) determinant found in Staphylococcus aureus. Thus far, the tet(A) and tet(B) determinants have been described for A. baumannii. tet(A) was found within a transposon similar to Tn1721, in association with an IS element. tet(A) confers resistance to tetracycline but not minocycline, an agent with greater activity against A. baumannii. Ribosomal protection is mediated by the tet(M) and tet(O) determinants, with tet(M) being described rarely for A. baumannii (Ribera et al., 2003).

Apart from tetracycline-specific efflux pumps, this class of antimicrobials is also susceptible to efflux by the multidrug efflux systems, such as the AdeABC pump (Magnet et al., 2001). Importantly, tigecycline, which is the first of a new class of modified tetracycline antimicrobials known as glycylcyclines, is also a substrate for this emerging efflux system. By performing real-time PCR with the adeB gene in clinical and laboratory exposed isolates with increased MICs of tigecycline, increased adeB gene expression was identified (Peleg et al., 2007).
It was of concern that the rise in MIC of tigecycline occurred rapidly with in vitro passage, suggesting that the expression of this multidrug efflux pump can be upregulated swiftly in response to selective pressure. The role of the AdeABC efflux pump in reduced susceptibility to tigecycline was confirmed by insertional inactivation of the adeB gene, which led to a significant drop in the MIC of tigecycline (4 µg/ml to 0.5 µg/ml) (Ruzin et al., 2007). These data suggest that caution should be used in considering tigecycline treatment for A. baumannii infection in sites where drug levels may be suboptimal, such as the bloodstream (Peleg et al., 2007).

In study to assess the prevalence and characteristics of efflux-based tigecycline resistance in clinical isolates of A. baumannii collected from a hospital in China. A total of 74 A. baumannii isolates, including 64 tigecycline-nonsusceptible A. baumannii (TNAB) and 10 tigecycline-susceptible A. baumannii (TSAB) isolates, were analyzed. The majority of them were determined to be positive for adeABC, adeRS, adeIJK, and abeM, while the adeE gene was found in only one TSAB isolate. Compared with the levels in TSAB isolates, the mean expression levels of adeB, adeJ, adeG, and abeM in TNAB isolates were observed to increase 29-, 3-, 0.7-, and 1-fold, respectively. The efflux pump inhibitors (EPIs) phenyl-arginine-_-naphthylamide (PA_N) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) could partially reverse the resistance pattern of tigecycline. Moreover, the tetX1 gene was detected in 12 (18.8%) TNAB isolates, this is the first report of the tetX1 gene being detected in A. baumannii isolates. ST208 and ST191, which both clustered into clonal complex 92 (CC92), were the predominant sequence
types (STs). This study showed that the active efflux pump AdeABC appeared to play important roles in the tigecycline resistance of \textit{A. baumannii}. The dissemination of TNAB isolates in hospital of study is attributable mainly to the spread of CC92 (\textit{Mei Deng et al., 2014}).

### 1.2.5.5 Polymyxins

These compounds bind to and disrupt the negatively charged outer-membrane of Gram-negative bacteria (\textit{Landman et al., 2008}). The main mechanisms of resistance these antibiotics involve: (i) reduction of the net negative charge of the outer-membrane protein by modification of lipid A, an essential component of the bacterial lipopolysaccharide (LPS); and (ii) proteolytic cleavage of the antimicrobial compound and exclusion of peptides by a broadspectrum efflux-pump. In \textit{A. baumannii}, point mutations and frame shifts in the pmrA and pmrB genes have been shown to cause an increase in colistin MICs (\textit{Adams et al., 2009}).

### 1.2.5.6 Other Antibiotics

The prevalence of trimethoprim-sulfamethoxazole resistance in \textit{A. baumannii} is high in many geographic regions (\textit{Gu Tong et al., 2007}). As discussed above, integrons are very common among strains of \textit{A. baumannii} that have a multidrug resistance phenotype. The 3′-conserved region of an integron most commonly contains a qac gene fused to a sul gene, conferring resistance to antiseptics and sulfonamides, respectively (\textit{Walsh et al., 2005}). Consequently, sulfonamide resistance has been shown to be highly predictive of integron-carrying strains of \textit{A. baumannii}. Similarly, genes coding for trimethoprim (dhfr) and
chloramphenicol (cat) resistance have also been reported within integron structures in *A. baumannii* (*Gu et al., 2007*). Efflux may also contribute to resistance against these agents (*Su et al., 2005*).

### 1.2.6 Therapeutic strategies for acinetobacter baumannii infections

Prior to the 1970s, it was possible to treat *Acinetobacter* infections with a range of antibiotics, including aminoglycosides, β-lactams, and tetracyclines (*Bergogne et al., 1996*). However, resistance to all known antibiotics has now emerged in *A. baumannii* (*Falagas and Bliziotis, 2007*).

#### 1.2.6.1 Existing Antimicrobial Agents

Given the range and diversity of resistance determinants in *A. baumannii*, therapy should be based on the results of adequately performed antimicrobial susceptibility testing. Antibiotic selection for empirical therapy is challenging and must rely on recent institutional-level susceptibility data. Time to effective therapy clearly impacts patient outcomes (*Ibrahim et al., 2000*), and this may include patients with *A. baumannii* infection (*Kwon et al., 2007*). Thus far, carbapenems have been thought of as the agents of choice for serious *A. baumannii* infections. However, although these drugs are still active against the vast majority of *A. baumannii* strains worldwide, the clinical utility of this class of antimicrobial is increasingly being jeopardized by the emergence of both enzymatic and membrane-based mechanisms of resistance (*Quale et al., 2003*).
1.2.6.1 Sulbactam.

Of the β-lactamase inhibitors, sulbactam possesses the greatest intrinsic bactericidal activity against *A. baumannii* isolates (*Peleg et al.*, 2008). Results of clinical investigations have documented the efficacy of sulbactam (commercially available in the United States in combination with ampicillin) in mild-to-severe *A. baumannii* infections. Urban *et al.*, 1993 have reported one of the earliest experiences using ampicillin-sulbactam and observed that 9 of 10 patients who were seriously ill and receiving mechanical ventilation demonstrated clinical improvement using ampicillin-sulbactam at a dosage of 3 g of ampicillin and 1.5 g of sulbactam intravenously every 6 or 8 h. Sulbactam-containing regimens appeared to be comparable to regimens of other agents when the infecting organisms were susceptible to sulbactam in patients with *A. baumannii* pneumonia and bloodstream infections (*Wood et al.*, 2002). A study from Israel identified that treatment with ampicillin-sulbactam was the only statistically significant variable associated with reduced mortality in patients with multidrug-resistant *A. baumannii* bloodstream infection (*Smolyakov et al.*, 2003). Mixed results have been reported for use of sulbactam to treat *A. baumannii* meningitis, and this likely relates to impaired drug penetration. The optimal dosage of sulbactam to treat serious *A. baumannii* infections is unknown, but most authors recommend at least 6 g per day in divided doses for patients with normal renal function. Whether higher dosages are more efficacious or reduce the risk of resistance, or even whether ampicillin-sulbactam should be used in combination with other agents (as recommended by others), is yet to be determined (*Wood et al.*, 2002).
1.2.6.1.2 Polymyxins.

Unfortunately, familiarity with this older class of antibiotic is now increasing in many parts of the world. The polymyxins include colistin or polymyxin E and polymyxin B, and this class of drug has been a savior for the treatment of highly drug-resistant gram-negative bacteria. Colistin is most commonly used in the United States, and it is administered intravenously as a pro-drug known as colistimethate sodium (CMS). Colistin sulfate is used topically but, most importantly, is also the form that should be used in the laboratory for susceptibility testing. Current Clinical and Laboratory Standards Institute breakpoints for colistin are _2 mg/mL (susceptible) and _4 mg/mL (resistant). A wide range of observational studies have been published on the clinical efficacy and toxicity of colistin for treating modern day gram-negative bacteria \( (Peleg \text{ et al., } 2008) \). Efficacy ranges from~55% to180% depending on the study and appears to be equal to that of other antibiotics in similar populations. Nephrotoxicity and neurotoxicity remain as key concerns for increasing use in an era of multidrug-resistant pathogens \( (Falagas \text{ and Kasiakou, } 2005) \). Nephrotoxicity is a particular issue for those with preexisting renal impairment, the elderly population, and those who receive concomitant nephrotoxins. Many authors who have reported on the use of colistin have been with its tolerability. As has been discussed elsewhere, dosing remains confusing because formulations differ between countries \( (Li J \text{ et al., } 2006) \).

Current parenteral formulations in the United States are available as either Coly-Mycin M Parenteral (Parkdale Pharmaceuticals) or generically via a variety of manufacturers. The recommended dosages are
2.5–5.0 mg/kg per day of colistin base given in 2–4 divided doses (equivalent to 6.67–13.3 mg/kg per day of CMS) in those with normal renal function. Additional work is still required to determine the ideal dosing of intravenous colistin to maintain efficacy and minimize toxicity. Promising data are available for the use of inhaled CMS as adjunctive treatment for pneumonia caused by multidrug-resistant A. baumannii. However, more data are still required. Concerns about lung toxicity, drug distribution, alveolar penetration, emergence of resistance, and selection for organisms inherently resistant to colistin are all justified and still need clarification (Luyt et al., 2009). According to a recent FDA health alert, those prescribing nebulized CMS should use it immediately after preparation to prevent build up of the active colistin form, which can be toxic to the lungs. Although A. baumannii meningitis remains an uncommon health care–associated infection, its incidence is increasing, and it is often caused by multidrug-resistant organisms. Carbapenems should be used to treat these infections if the organism is susceptible, with or without an intrathecal or intraventricular aminoglycoside. For carbapenem-resistant cases, intravenous polymyxin (colistin or polymyxin B) plus an intrathecal or intraventricular polymyxin or aminoglycoside, with or without intravenous rifampin, would be recommended. Multiple case series and reports have now been published on the favorable efficacy and toxicity profile of intraventricular/intrathecal colistin (Kim et al., 2009). Chemical meningitis appears to be uncommon. The dosing recommended by the Infectious Diseases Society of America for adults is 10 mg daily of colistin or 5 mg daily of polymyxin B (Tunkel et al., 2004).
1.2.6.2 New Antimicrobials

1.2.6.2.1 Tigecycline

A concerning void of new therapeutic options exists for *A. baumannii* infections. Of the recently licensed antimicrobials, tigecycline, a 9-t-butylglycylamido semisynthetic derivative of minocycline, has provided some hope, but clinical data are still limited. As with other tetracycline derivatives, tigecycline inhibits the 30S ribosomal subunit, but its unique feature is its ability to evade the major determinants of tetracycline resistance, i.e., the tet(A) to tet(E) and tet(K) efflux pumps and the tet(M) and tet(O) determinants that provide ribosomal protection *(Fluit et al., 2005)*. Thus far, the in vitro activity of tigecycline against *A. baumannii* has been assessed largely by MIC testing. Most studies have reported a MIC50 of 1 μg/ml and an MIC90 of 2 μg/ml *(Sader et al., 2005)*, but studies from Germany *(Seifert et al., 2006)* and Spain *(Betriu et al., 2002)*, have reported MIC90s of 4 μg/ml and 8 μg/ml, respectively, with the last being determined by E test only. More recently, combination therapy with tigecycline has been studied using time-kill and E test synergy methodology. When it was tested against a non-carbapenem-susceptible *A. baumannii* strain, tigecycline alone allowed maximal killing at concentrations near the MIC, which was 1 μg/ml, with no benefit of using higher concentrations *(Scheetz et al., 2007)*. Importantly, concentrations just below the MIC (0.5 μg/ml to 0.7 μg/ml), which are consistent with the mean maximum serum steady-state concentration achieved with standard dosing (0.63 μg/ml after a 100-mg i.v. loading dose followed by 50 mg i.v. twice/day) *(Owen et al., 2004)*, caused significant regrowth at 24 h. No difference was observed for all tigecycline combinations, including combinations with amikacin,
meropenem, imipenem, ciprofloxacin, levofloxacine, ampicillin- sulbactam, rifampin, and polymyxin B \citep{Scheetz2007}. These data raise concerns about the use of tigecycline for bloodstream infections caused by organisms with MICs of >1 µg/ml. Such concerns have now been illustrated in several clinical reports involving \emph{A. baumannii}. Subinhibitory concentrations may promote the rapid emergence of resistance, leading to clinical failure \citep{Peleg2007}. At this point, it is not recommend using tigecycline for \emph{A. baumannii} bacteremia if another option is available. Conversely, the drug is concentrated in tissues, including the lung parenchyma \citep{Conte2005}, and thus its utility for treating \emph{A. baumannii} pneumonia or other tissue-based infections warrants further evaluation. A recent retrospective series including 22 patients with VAP caused by multidrug-resistant \emph{A. baumannii} showed that 18 patients had clinical resolution \citep{Schafer2007}. However, only three of these patients received tigecycline monotherapy, with the remainder also receiving therapy with imipenem and/or colistin. Further clinical data on tigecycline's efficacy in pneumonia are still awaited. The most common adverse effect of tigecycline is nausea \citep{Babinchak2005}.

1.2.6.2.2 Doripenem

Other agents on the licensing horizon with activity against gram-negative organisms include doripenem, a new parenteral carbapenem \citep{Mushtaq2004}.

In a study to determine the in vitro activities of doripenem, imipenem, and meropenem against clinical \emph{ACINETOBACTER BAUMANNII} isolates, 94 \emph{Acinetobacter} spp. strains from Intensive Care Units to Akdeniz University Microbiology laboratory isolated from
different clinical specimens were included in the study. Species are identified by conventional assays and BD Phoenix automated system (Becton Dickinson, USA) and susceptibility to imipenem, meropenem, doripenem was studied by E-test. Imipenem, meropenem, doripenem susceptibility of *ACINETOBACTER BAUMANNII* isolates were found to be 22.3%, 17.0%, 12.8%, respectively. As a result of the study, doripenem $M\text{MIC}_{50}/M\text{MIC}_{90}$ values of *ACINETOBACTER BAUMANNII* isolates are similar with of imipenem and meropenem (Yesim et al., 2013).

### 1.2.6.3 Other Combination Therapy

The use of combination therapy to treat multidrug- or pandrug-resistant gram-negative organisms has become an area of great interest (Rahal, 2006). This strategy aims to create an active combination out of two agents to which the organism tests nonsusceptible in the laboratory. Apart from trying to improve efficacy, combination therapy may also help to prevent the emergence of resistance when at least one agent is active in vitro (Chait et al., 2007).

The colistin/sulbactam combination therapy is promising in severe MDR *A. baumannii* VAP. Although, the difference was not statistically significant, clinical cure rates or bacteriological clearance rates were better in the combination group than colistin monotherapy (Kalin et al., 2014).

Four studies (1 prospective, 3 retrospective) were included in the meta-analysis. Sulbactam was given in combination with ampicillin, carbapenem, or cefoperazone ($n = 112$ participants). Comparator drugs included colistin, cephalosporins, anti-pseudomonas penicillins,
fluoroquinolones, minocycline/doxycycline, aminoglycosides, tigecycline, polymyxin, imipenem/cilastatin, and combination therapy ($n = 107$ participants). The combined clinical response rate odds ratio did not significantly favor sulbactam-based therapy over comparator therapy (odds ratio = 1.054, 95% confidence interval = 0.550–2.019, $p = 0.874$), nor did any of the individual study odds ratios.

On conclusion sulbactam-based therapy may be similarly efficacious to alternative antimicrobial therapies for the treatment of $A. baumannii$ infection (Haiqing et al., 2013).

In study to evaluate the outcomes of the patients who were infected with colistin-only-susceptible (COS) $ACINETOBACTER BAUMANNII$ and treated with either colistin monotherapy or colistin combined therapy. Rifampicin was the mostly used combined antibiotic with colistin. Colistin monotherapy was found effective as much as colistin combined therapy in terms of clinical and microbiological responses in patients with ventilator associated pneumonia (VAP) and also in patients with blood stream infections. Heteroresistant strains can emerge in patients who receive colistin monotherapy (Simsek et al., 2012).

A research was performed on rifampicin activity against selected MDR OXA-72 carbapenemase-producing $A. baumannii$ strains. Since it is widely accepted that rifampicin should not be used as monotherapy in order to avoid the rapid development of rifampicin resistance, evaluation of the efficacy of combination therapy with imipenem. Minimal inhibitory concentrations (MICs) of both rifampicin and imipenem were determined by use of the broth microdilution method. Evaluations of the interactions between rifampicin and imipenem were performed by
analysis of the fractional inhibitory concentration index ($\Sigma FIC$), determined using the checkerboard titration method. All tested isolates showed full susceptibility to rifampicin. The checkerboard method revealed synergism in 5 isolates (29%) and an additive effect in another 5 isolates (29%); no difference was reported in the remaining 7 isolates (41%). Strains moderately resistant to imipenem (MIC $\leq$ 64 mg/l) tended to show synergy or additive interaction. Conclusions that in vitro synergism or an additive interaction between rifampicin and imipenem most likely occurs in A. baumannii strains showing moderate resistance to imipenem (MIC $\leq$ 64 mg/l). Moreover, utilizing this combination in the therapy of infections caused by strains exhibiting higher levels of resistance (MIC $>$ 64 mg/l) is not recommended since in this setting imipenem could not prevent the development of rifampicin resistance (Piotr et al., 2014).

Study investigated the in vitro effect of various antibiotic combinations against carbapenem-resistant A. baumannii strains. Antibiotic susceptibility of A. baumannii strains was analysed. In vitro synergistic efficacy of colistin combined with tigecycline, cefoperazone/sulbactam or piperacillin/tazobactam was tested against carbapenem-resistant A. baumannii strains. Synergy studies were performed using an eplisometer test-strip method. Of the 50 carbapenem-resistant A. baumannii strains tested, 96% were susceptible to colistin and 64% were susceptible to tigecyclin E. coli–tigecycline, colistin–cefoperazone/sulbactam and colistin–piperacillin/tazobactam combinations were found to have synergistic effects against six (12%), two (4%), and one (2%), respectively, of the strains tested.
In conclusions Colistin combined with tigecycline, cefoperazone/sulbactam or piperacillin/tazobactam revealed synergistic effects in some carbapenem-resistant *A. baumannii* strains. These results, together with the shortage of treatment options and the risk of developing resistance to colistin, suggest that clinicians should use colistin combined with other antibiotics or β-lactamase inhibitors when treating carbapenem-resistant *A. baumannii* infection (*Ilkay et al., 2013*).

### 1.2.6.4 Future Therapeutic Considerations

Despite the absence of new therapeutic options for *A. baumannii* in clinical studies, the activity in the preclinical arena is notable. Such agents can be divided into those that inhibit a currently recognized mechanism of resistance or those that have a novel mechanism of action. With regard to the former, attention has been directed toward new β-lactamase inhibitors, especially those targeting the Ambler class B MBLs (*Toney et al., 2003*), as well as toward inhibitors of aminoglycoside-modifying enzymes and multidrug efflux pumps. As mentioned above, MBLs have played an important role in the emergence of carbapenem resistance in *A. baumannii*, although they are less prevalent than the OXA-type enzymes. Their structure and catalytic mechanism, being zinc dependent, contrast with those of other serine β-lactamases, hence their stability toward current β-lactamase inhibitors. Agents that chelate the active Zn2+ site appear to be the most promising; however, several challenges exist. As a consequence of the significant differences in the active site architecture between MBL types, the ability to develop a pan-MBL inhibitor is problematic (*Walsh et al. 2005*).
Of most concern, MBLs have homologous mammalian enzymes (Schilling et al., 2003) and therefore increase the potential for significant toxicity. Despite these hurdles, the development of compounds that target the metalloenzymes continues (Buynak et al., 2004). Thus far, inhibitors of both aminoglycoside-inactivating enzymes and multidrug efflux pumps have also been troubled by diverse targets, with bacteria often harboring multiple enzyme or pump types. More recently, cationic antimicrobial peptides that are capable of inhibiting both aminoglycoside phosphotransferases and acetyltransferases have been described (Boehr et al., 2003). The importance of multidrug efflux pumps in A. baumannii is increasingly being recognized, with tigecycline recently identified as a substrate of the RND-type pump AdeABC (Peleg et al. 2007). Through large-scale in vitro screening, a range of efflux pump inhibitors have been identified, with the majority being plant alkaloids (Piddock et al., 2006) but, more recently, also synthetic compounds (Bohnert and Kern, 2005). Unfortunately, progress has been slow, with agents such as phenyl-arginine-β-naphthylamide doing well when assessed in vitro but coming to demise due to toxicity concerns (Piddock et al., 2006).

With advances in genomics, proteomics, and chemical biology, new strategies for pathogen control are being devised. Justifiably, significant attention is being directed toward eukaryotic antimicrobial peptides, which are ubiquitous elements of the innate immune response in a variety of invertebrate, plant, and animal species (Brogden, 2005).

These cationic peptides act primarily by disturbing the cell membranes and share a similar structure and charge profile with the polymyxins, but the final steps in pathogen lethality have been shown to
be different. This mechanistic difference is clinically attractive and is well illustrated by the susceptibility of polymyxin-resistant *A. baumannii* strains to such peptides (*Saugar et al., 2006*). Bactericidal activity against *A. baumannii*, using both in vitro (*Radzishevsky et al., 2007*) and in vivo (*Braunstein et al., 2004*) models, has been reported. Combination studies, as determined by fractional inhibitory indexes, demonstrated that magainin II acted synergistically with β-lactams against multidrug-resistant *A. baumannii* but that four other peptides showed no synergy (*Giacometti et al., 2000*). More recently, modified peptides containing linear sequences of alternating acyl chains and cationic amino acids, known as oligo-AKs, were reported. In this study, the oligo-AK compound C12K-7α8 was compared to conventional antimicrobial peptides and standard antibiotics. It was found to have significant bactericidal activity, more so than imipenem and ciprofloxacin at 6× MIC, similar membrane binding properties to other peptides, no emergence of resistance after serial passages, and almost no toxicity toward human red blood cells. Furthermore, in a mouse peritoneal infection model using *E. coli*, C12K-7α8 prevented mortality similar to imipenem and ciprofloxacin, whereas conventional peptides did not (*Radzishevsky et al., 2007*). Overall, antimicrobial peptides have demonstrated great potential and may provide a feasible alternative for treatment of *A. baumannii* infections, including those caused by polymyxin-resistant *A. baumannii* (*Saugar et al., 2006*).

Other novel antimicrobial strategies for multidrug-resistant *A. baumannii* include the use of bacterial conjugation, resulting in antibacterial gene transfer. This highly innovative approach uses
attenuated *E. coli* as a vector for a conjugative plasmid carrying bactericidal genes that disrupt protein synthesis. While in *E. coli* the plasmid is tightly repressed, once it is transferred into the target pathogen, derepression occurs. In vitro, the donor *E. coli* cells led to killing of *A. baumannii* (*Shankar et al., 2007*). Novel topical agents that may be effective for environmental cleaning of *A. baumannii*, including highly charged copper-based biocides, have recently been reported (*Gant et al., 2007*). Other innovative therapeutic avenues, which have not yet been tested against *A. baumannii* but have the potential for efficacy, include the use of bacteriophage treatment (*Wang et al., 2006*), improvement in host response via passive or active immunization (*DiGiandomenico et al., 2007*), and modification of bacterial virulence by inhibition of quorum sensing (*Gonzalez and Keshavan, 2006*), other bacterial secretion systems (*Baron and Coombes, 2007*), or LPS biosynthesis (*De Leon et al., 2006*).

Recent preclinical studies have identified Th17 cells as critical mediators of broadly protective adaptive immunity, including protection against MDR infections. Studies of Th17 eliciting antigens, adjuvants and routes of immunization have identified potential vaccine strategies that may confer long-lived adaptive immunity against MDR Gram negative bacterial infections (*Campfield et al., 2014*).

Bactriophages causes the destruction of harmful bacteria in human as well as animal; they can be considered a good option to treat even antibiotic resistant microorganisms (*Kumar et al., 2014*).
1.2.7 Hospital Outbreaks and Control Measures

1.2.7.1 Infection control perspective

There are three major factors possibly contributing to the persistence of *A. baumannii* in the hospital environment, i.e., resistance to major antimicrobial drugs, resistance to desiccation, and resistance to disinfectants.

1.2.7.1.1 Resistance to major antimicrobial drugs

Resistance to antibiotics may provide certain *A. baumannii* strains with a selective advantage in an environment, such as the modern ICU, where microorganisms are confronted with extensive exposure to antimicrobials. Several researchers have observed that resistance rates in epidemic *A. baumannii* strains are significantly higher than those in sporadic *A. baumannii* strains (*Koeleman et al.*, 2001). Resistance to the fluoroquinolones in particular was associated with epidemic behavior (*Heinemann et al.*, 2000). Villers et al. identified previous therapy with a fluoroquinolone as an independent risk factor for infection with epidemic *A. baumannii*, and it appeared that the selection pressure caused by the indiscriminate use of fluoroquinolones was responsible for the persistence and epidemic spread of multidrug-resistant *A. baumannii* clones for at least 5 years (*Scheetz et al.*, 2007). The recently observed increase in carbapenem-resistant *A. baumannii* strains was associated almost exclusively with hospital outbreaks (*Coelho et al.*, 2006). It has been suggested that any clinical *A. baumannii* isolate with resistance to
multiple antibiotics indicates a potential nosocomial outbreak strain \cite{Koeleman2001}.

1.2.7.1.2 Resistance to desiccation

To assess the desiccation tolerance of \textit{A. baumannii}, Jawad \textit{et al.} compared the survival times on glass coverslips of 22 strains isolated from eight well-defined hospital outbreaks with the survival times of 17 sporadic strains. The overall mean survival time was 27 days, with a range of 21 to 33 days \cite{Jawad1998}. Of note, there were no differences in survival times between outbreak and sporadic strains; all investigated \textit{A. baumannii} strains had the ability of long-time survival on dry surfaces and therefore an increased potential for epidemic spread. It has also been shown that \textit{A. baumannii} strains survive desiccation far better than do other \textit{Acinetobacter} species, such as \textit{A. johnsonii}, \textit{A.junii}, and \textit{A.lwoffii} \cite{Jawad1996}.

\textit{A. baumannii} is able to survive long periods of desiccation through the presence of cells in a dormant state, via mechanisms affecting control of cell cycling, DNA coiling, transcriptional and translational regulation, protein stabilization, antimicrobial resistance, and toxin synthesis, and that a few surviving cells embedded in a biofilm matrix are able to resume growth and restore the original population in appropriate environmental conditions following a “bust-and-boom” strategy \cite{Gayoso2014}.

1.2.7.1.3 Resistance to disinfectants

Prolonged survival of \textit{A. baumannii} in a clinical setting, i.e., on patients' bed rails, has been found to be associated with an ongoing
outbreak in an ICU and illustrates that dry vectors can be secondary reservoirs where *A. baumannii* can survive (*Catalano et al., 1999*).

It has been speculated that resistance to disinfectants may contribute to the epidemicity of the organism in a clinical setting, but to our knowledge, the association of resistance to biocides and the propensity for epidemic spread has never been studied systematically. Wisplinghoff *et al.* compared the in vitro activities of various disinfectants, such as propanol, mecetronium ethylsulfate, polyvinylpyrrolidone-iodine, triclosan, and chlorhexidine, against sporadic and epidemic *A. baumannii* strains by using a broth macrodilution method (*Wisplinghoff et al., 2007*). They concluded that resistance to currently used disinfectants is probably not a major factor favoring the epidemic spread of *A. baumannii*, since all disinfectants inhibited growth of all *A. baumannii* isolates when concentrations and contact times recommended by the respective manufacturer were used. However, with most of the disinfectants tested, a substantial number of viable bacteria remained if contact times were <30 s or if diluted agents were used, as may occur in day-to-day clinical practice. No significant differences in susceptibility between outbreak-related and sporadic strains were observed under these conditions. Minor deviations from the recommended procedures leading to decreased concentrations or exposure times may play a role in nosocomial cross-transmission (*Anton et al., 2008*).

1.2.7.2 **Infection control interventions are appropriate with regard to A. baumannii outbreaks.**

(i) **Molecular epidemiologic investigations:** Should be conducted to determine if a clonal outbreak strain is present.
(ii) **Environmental cultures**: Should be used to determine if a common environmental source is present. If such a source is found, it should be removed from the patient care setting. Numerous potential sources have been identified in prior studies, including ventilator tubing, suction catheters, humidifiers, containers of distilled water, urine collection jugs, multidose vials of medication, intravenous nutrition, moist bedding articles, inadequately sterilized reusable arterial pressure transducers, and computer keyboards (*Fournier and Richet, 2006*). A high-profile outbreak arose from pulsatile lavage wound treatment, a high-pressure irrigation treatment used to debride wounds (*Maragakis et al., 2004*).

(iii) **Enhanced environmental cleaning** should be performed in order to eliminate the organism from the peripatient environment. In study to evaluate the impact of environmental cleaning and systematic sampling from environmental objects on the risk of infection by MDR *A. baumannii*. systematic sampling from environmental objects and molecular typing of all new MDR *A. baumannii* strains from patients and environmental isolates, where analyzed in the correlation (Pearson’s r) between new infected cases and positive environmental samples. The risk ratio (RR) of infection was estimated with Poisson regression. Results. The risk increased significantly with the number of positive samples in common areas (RR = 1.40; 95%CI = 0.99–1.94) and positive samples in boxes (RR = 1.19; 95%CI = 1.01–1.40). The number of cases also positively correlated with positive samples in boxes and common areas. It was concluded that once conventional measures have failed,
environmental cleaning, guided by systematic sampling from environmental objects, provided the objective risk reduction of new cases and enabled the full control of the outbreak (Jesús et al., 2013).

(iv) **Enhanced isolation procedures**, aimed at optimizing contact isolation (usage of gloves and gowns when dealing with colonized patients or their environment) and improving hand hygiene, should be implemented. In some circumstances, cohorts of patients or staff are used, but optimally patients should be nursed in single rooms with a dedicated nurse. This will be impractical in many settings.

(v) **Antibiotic management processes** should be used to ensure that “at-risk” antibiotics are not being used excessively. Optimally, a case-case control study should be performed to determine which antibiotics truly do increase the risk of multidrug-resistant *A. baumannii* strains (Kaye et al., 2005). Numerous studies have assessed antibiotic risk factors for infection with multidrug-resistant *A. baumannii*, although only a few have examined risk factors for emergence of pandrug resistance. Although exposure to any antibiotic active against gram-negative bacteria has been associated with the emergence of multidrug-resistant *A. baumannii* (Maslow et al., 2005), three classes of antibiotics have been implicated most frequently, including broad-spectrum cephalosporins (Carbonne et al., 2005), carbapenems (del Mar Tomas et al., 2005), and fluoroquinolones (Villers et al., 1998).
A number of investigators have demonstrated that interventions such as those described above can be effective in the control of *A. baumannii* infections (Pimentel et al., 2005). In some cases, despite these efforts, ongoing cases of multidrug-resistant *A. baumannii* infection continue to occur. Monitoring adherence to such infection control interventions is also important. Although health care worker hand carriage with *Acinetobacter* is typically transient, it may be more prolonged in individuals with damaged skin (Bayuga et al., 2002). In some scenarios, closure of wards to new admissions needs to be undertaken (Pimentel et al., 2005). Some authors have suggested that eradication of colonization be performed by techniques such as selective digestive tract decolonization or use of topical or aerosolized polymyxins (Urban et al., 2003). However, one will be hesitant to recommend these interventions due to the possible risks of polymyxin-resistant organisms. Rather, one will prefer greater assessment for colonized patients, greater attention to environmental decontamination, and improved hand hygiene as a means for prevention of patient-to-patient transfer (Anton et al., 2008).

### 1.2.8 Epidemiology of *acinetobacter baumannii*

#### 1.2.8.1 Europe

*A. baumannii* infections have been a substantial clinical issue in many parts of Europe (Van Looveren and Goossens, 2004). Since the early 1980s, hospital outbreaks of *A. baumannii* infections in Europe, mainly in England, France, Germany, Italy, Spain, and The Netherlands (Fournier and Richet, 2006), have been investigated using molecular
epidemiological typing methods. In the majority of cases, one or two epidemic strains were detected in a given epidemiological setting. Transmission of such strains has been observed between hospitals, most probably via transfer of colonized patients (Da Silva et al., 2007). Spread of multidrug-resistant A. baumannii is not confined to hospitals within a city but also occurs on a national scale. Examples are the spread of the so-called Southeast clone and the Oxa-23 clones 1 and 2 in Southeast England (Coelho et al., 2006), the dissemination of a multidrug-resistant A. baumannii clone in Portugal (Da Silva et al., 2007), the interhospital spread of a VEB-1 ESBL-producing A. baumannii clone from a total of 55 medical centers in northern and southeastern France (Naas et al., 2006), and the spread of an amikacin-resistant A. baumannii clone observed in nine hospitals in various regions in Spain (Vila et al., 1999). International transfer of colonized patients has led to the introduction and subsequent epidemic spread of multidrug-resistant A. baumannii strains from Southern into Northern European countries, such as Belgium and Germany (Bogaerts et al., 2006). Intercontinental spread of multidrug-resistant A. baumannii has also been described between Europe and other countries as a consequence of airline travel (Naas et al., 2007). These events highlight the importance of appropriate screening and possible isolation of patients transferred from countries with high rates of drug-resistant organisms (Anton et al., 2008).

In addition to these interinstitutional outbreaks, three international A. baumannii clones (the so-called European clones I, II, and III) have been reported from hospitals in Northern Europe (including hospitals in Belgium, Denmark, the Czech Republic, France, Spain, The Netherlands,
and the United Kingdom) as well as from hospitals in southern European countries, such as Italy, Spain, Greece, and Turkey (Dijkshoorn, et al., 1996; Nemec et al., 2004; van Dessel et al., 2004), and in Eastern Europe (Wroblewska et al., 2007). Initially detected by AFLP clustering at a similarity level of >80%, the epidemiological relationship of these clones was confirmed by ribotyping, pulsed-field gel electrophoresis (PFGE) (van Dessel et al., 2004), and most recently, multilocus sequence typing (MLST) (Bartual et al., 2005).

Carbapenem resistance in A. baumannii is now an issue in many European countries. Information on the prevalence of carbapenem resistance in various European countries is difficult to obtain, but it appears from the outbreak literature that carbapenem resistance rates are highest in Turkey, Greece, Italy, Spain, and England and are still rather low in Germany and The Netherlands. Carbapenem resistance in Eastern Europe appears to be increasing (Wroblewska et al., 2007). Rates appear to be lowest in Scandinavia, although sporadic isolates have been reported from patients transferred from elsewhere, including victims of the Indian Ocean tsunami (Kallman et al., 2006). In an industry-supported surveillance report (MYSTIC) from 48 European hospitals for the period 2002-2004, just 73.1% of isolates were susceptible to meropenem and 69.8% were susceptible to imipenem. Susceptibility to other antibiotics was also very low, with 32.4%, 34.0%, and 47.6% being susceptible to ceftazidime, ciprofloxacin, and gentamicin, respectively (Unal and Garcia, 2005). A. baumannii isolates resistant to the polymyxins have been detected in Europe, although at present these remain rare (Gales et al., 2006).
1.2.8.2 North America

There is a long history of multidrug-resistant \textit{A. baumannii} infections occurring in the United States. In 1991 and 1992, outbreaks of carbapenem-resistant \textit{A. baumannii} were observed in a hospital in New York City \textit{(Go et al., 1994)}. This followed an outbreak of infections due to ESBL-producing \textit{Klebsiella pneumoniae} during which use of imipenem increased substantially \textit{(Meyer et al., 1993)}. The organisms in this outbreak were multidrug resistant, retaining susceptibility only to polymyxins and ampicillin-sulbactam \textit{(Go et al., 1994)}. Numerous other hospitals in New York City also had clonal outbreaks of multidrug- or pandrug-resistant \textit{A. baumannii} \textit{(Landman et al., 2007)}, and similar outbreaks have frequently been reported from many other regions of the United States \textit{(Stephens et al., 2007)}. National surveillance studies have demonstrated significant trends in the emergence of multidrug-resistant \textit{Acinetobacter} strains \textit{(Gaynes and Edwards, 2005)}. For example, data from the National Nosocomial Infection Surveillance system collected from 1986 to 2003, involving many hospitals throughout the United States, showed significant increases in \textit{Acinetobacter} strains resistant to amikacin (5\% to 20\%; \textit{P} < 0.001), ceftazidime (25\% to 68\%; \textit{P} < 0.001), and imipenem (0\% to 20\%; \textit{P} < 0.001) \textit{(Gaynes and Edwards, 2005)}. In a more recent industry-supported surveillance study including isolates of \textit{Acinetobacter} spp. collected between 2004 and 2005 from 76 centers throughout the United States, only 60.2\% were susceptible to imipenem \textit{(Halstead et al., 2007)}. A further industry-supported surveillance study including isolates of \textit{Acinetobacter} spp. from 15 centers throughout the United States reported improved carbapenem and aminoglycoside
susceptibilities in 2005 compared with those in 2004. However, rates of nonsusceptibility were still substantial, as follows: 10% to 15% for carbapenems, 35% to 40% for ceftazidime/cefepime, 10% to 30% for aminoglycosides, and 35% to 40% for ciprofloxacin/levofloxacin (Rhomberg and Jones, 2007). The MIC$_{50}$ and MIC$_{90}$ of tigecycline for A. baumannii isolates collected from the United States between 2004 and 2005 were 0.5µg/ml and 1 µg/ml, respectively, with an MIC range of 0.03 µg/ml to 8 µg/ml (Waites et al., 2006). The MIC$_{90}$ for multidrug-resistant strains collected in the same time period was 2 µg/ml (Hoban et al., 2007), which is more consistent with that reported from individual centers. Global surveillance data for susceptibility to polymyxin B have reported an MIC$_{50}$ of $\leq$1 µg/ml and an MIC$_{90}$ of 2 µg/ml against Acinetobacter sp. isolates from four major geographic regions (Asia-Pacific, Europe, Latin America, and North America). The rate of polymyxin B-nonsusceptible Acinetobacter spp. in North America was reported to be 1.7%. This compares with 1.9%, 2.7%, and 1.7% in the Asia-Pacific region, Europe, and Latin America, respectively. Overall, 2.8% and 3.2% of carbapenem-resistant and multidrug-resistant Acinetobacter spp., respectively, were resistant to polymyxin B (Gales et al., 2006).

It is clear that more attention is being paid to A. baumannii infections in the United States now than at any time in the past. This may reflect increased recognition of pandrug-resistant strains. There are some data to suggest that the proportion of intensive care unit (ICU)-acquired pneumonia cases being found to be due to A. baumannii is actually increasing. In a review from the CDC, 7% of ICU-acquired pneumonias
were due to *Acinetobacter* in 2003, compared to 4% in 1986 (P < 0.001). The proportion of urinary tract infections (UTIs) and skin/soft tissue infections due to *Acinetobacter* also significantly increased during this period (*Gaynes and Edwards, 2005*). There is some evidence that nosocomial *Acinetobacter* infections have some seasonal variation in the United States, with an unexplained upswing in late summer months (*McDonald, et al., 1999*).

An important contribution to the epidemiology of infections with *A. baumannii* in the United States is the return of military personnel who have fought in Iraq or Afghanistan. An increase in infections with *A. baumannii* was first observed in U.S. military personnel in March 2003, soon after combat operations commenced in Iraq. Most injured military personnel were first treated at field hospitals before being evacuated to the Landstuhl Regional Medical Center (Germany) or the Walter Reed Army Medical Center (United States). Most of these infections were detected at or soon after admission to these institutions. In a careful outbreak investigation, it was determined that neither preinjury skin colonization nor introduction of the organism from soil at the time of traumatic injury was the source of infection. Rather, multiple *A. baumannii* isolates were cultured from a range of inanimate surfaces in field hospitals and were genotypically linked to patient isolates. Typically, these isolates were multidrug resistant, being resistant to fluoroquinolones, cephalosporins, and piperacillin-tazobactam. Just 10% were nonsusceptible to carbapenems (*Scott et al., 2007*). However, in a paper by Hawley *et al.*, the rate of non-imipenem-susceptible *A. baumannii* was 37% for injured deployed military personnel. Such rates
are more consistent with those seen in Europe than in the United States. The MIC$_{90}$ of tigecycline for these strains was 8 µg/ml (Hawley et al., 2007). Hujer and colleagues found that carbapenem-resistant isolates from patients at Walter Reed Army Medical Center typically produced OXA-23 or OXA-58 carbapenemase (Hujer et al., 2006). Interestingly, in a study comparing the clonal relatedness of A. baumannii strains from injured military personnel from the United States with that of strains from the United Kingdom, the main outbreak strains were indistinguishable (Turton et al., 2006). This provides further support that A. baumannii acquisition is occurring in the field hospitals.

A comparable situation with Canadian soldiers injured in Afghanistan and British soldiers injured in Iraq has recently been reported (Tien et al., 2007). Outbreaks of multidrug-resistant A. baumannii in Canadian civilian hospitals appear to be less common than those in comparable institutions in the United States but have certainly still been reported (Simor et al., 2002).

1.2.8.3 Latin America

Rates of nonsusceptibility to meropenem, imipenem, ceftazidime, piperacillin-tazobactam, ciprofloxacin, and gentamicin in Latin America appear to be among the highest in the world. For example, just 71% of isolates were susceptible to meropenem or imipenem in an assessment from a surveillance program in the period 2002-2004 (Unal et al., 2005). In a surveillance study involving Argentina, Brazil, Chile, and Colombia from 1997 to 2001, resistance rates were highest in Argentina, but no countries were spared multidrug-resistant isolates (Tognim et al., 2004).
Chapter (1) Introduction & Literature Review

A variety of carbapenemases have been identified in *A. baumannii* isolates in Latin America, including IMP-1 and IMP-6 in Brazil (*Sader, et al., 2005*), OXA-23 in Brazil and Colombia (*Dalla et al., 2003, Villegas et al., 2007*), and OXA-58 in Argentina (*Coelho et al., 2006*). Interestingly, the SPM- and VIM-type MBLs, which are widespread in Brazil (SPM) and other parts of Latin America (VIM) in *P. aeruginosa* strains, have not yet been reported for *A. baumannii* strains in these regions (*Anton et al., 2008*).

1.2.8.4 Africa

Data on the extent of antibiotic resistance in *A. baumannii* in Africa are largely limited to South Africa at the present time, although there are scattered reports from other countries (*Samuel et al., 2006*). Brink and colleagues have shown that about 30% of *A. baumannii* bloodstream isolates in South Africa are carbapenem resistant, >40% are resistant to cefepime and piperacillin-tazobactam, and >30% are resistant to ciprofloxacin and levofloxacin (*Brink, et al., 2007*). Such resistant strains are endemic in some units (for example, burns and ICUs) and have been spread from institution to institution (*Marais et al., 2004*).

1.2.8.5 Asia and the Middle East

Numerous outbreaks of pandrug-resistant *A. baumannii* have been documented in Asian and Middle Eastern hospitals, and a variety of carbapenemases have been described to originate there (*Chan et al., 2007*). Rates of nonsusceptibility in SENTRY isolates (2001-2004) exceeded 25% for imipenem and meropenem, 40% for cefepime and ceftazidime, 40% for ampicillin-sulbactam, 35% for amikacin, and 45%
for ciprofloxacin (Gales et al., 2006). Unfortunately, resistance to tigecycline (Navon et al., 2007) and polymyxin B (Ko et al., 2007) already exists in this region.

In study in North of Jordan between March 2005 and December 2006) have demonstrated Emergence of extensive drug-resistant Acinetobacter Baumannii. The antimicrobial susceptibility profile showed high resistance profile to β-lactam antibiotics (cefotaxime 92.2%, cefuroxime 98.4%, ceftazidime 89.1%) and ciprofloxacin 89.1%. Imipenem and meropenem showed increased resistant rates (70.1 and 71.6%, respectively) with nearly half of the isolates being resistant to amikacin (52.5%). All isolates were susceptible to colistin (Ghulam et al., 2011)

1.2.8.6 Australia and Pacific Islands

Initial reports of A. baumannii from Australia came from the Northern Territory, where community-acquired infections are well described (Anstey et al., 2002). Such infections have a vastly different epidemiology from that seen in hospital-acquired infections, with male gender, age of >45 years, Aboriginal ethnic background, cigarette smoking, alcoholism, diabetes mellitus, and chronic obstructive airway disease being important risk factors. Also, these community-acquired strains are significantly more susceptible to antimicrobials (Anstey et al., 1992). Throat carriage and microaspiration may be involved in the pathogenesis of these infections (Anstey et al., 2002).
The first described Australian outbreak of hospital-acquired *A. baumannii* was in Western Australia. These isolates were resistant to gentamicin, cephalosporins, and ticarcillin, with some isolates also being resistant to ciprofloxacin. Molecular epidemiological analysis identified that 11% of staff hand samples were positive for the same strain of *A. baumannii* as that causing patient infection (*Riley et al., 1996*). More recently, outbreaks of *A. baumannii* have affected other major cities along the eastern seaboard of Australia, including Brisbane, Sydney, and Melbourne (*Playford et al., 2007*). Unfortunately, these outbreaks have involved carbapenem-resistant strains of *A. baumannii*, with OXA-23 contributing to this phenotype (*Valenzuela et al., 2007*). As seen in other countries, strains within institutions are often clonally related (*Peleg et al. 2006*). Also, interhospital spread of multidrug-resistant *A. baumannii* strains has likely occurred in certain cities (*Pimentel et al., 2005*). Outbreaks of carbapenem-resistant *A. baumannii* have also occurred in French Polynesia (*Naas et al., 2005*). Most recently, reduced susceptibility to tigecycline of multidrug-resistant *A. baumannii* strains has been described in Australia (*Iredell et al., 2007*).
1.2.9 Objectives

Study justification:

1. *A. baumannii* is becoming resistant to the most potent antibiotics existing including the new carbapenem antimicrobial agents and other common antibiotics used today.

2. It is our intention that this data could help in better understanding the spread of carbapenem-resistant *A. baumannii* and taking effective measures for controlling nosocomial infections by this pathogen.

3. The genetic background for *A. baumannii* in Asser Region, Abha, KSA are not studied before.

1.2.9.2 General objectives:

Isolation, antibiotic sensitivity and genetic study of *Acinetobacter baumannii* from different specimens of patients in Asser Region, KSA

1.2.9.3 Specific objectives:

1. To isolate *Acinetobacter baumannii* from different specimens

2. To evaluate the antimicrobial susceptibility of *A. baumannii* nosocomial isolates from Aseer Central hospital, Abha.

3. To evaluate the occurrence, distribution and dissemination of acquired class D carbapenemase-encoding genes among these isolates.

4. To find out the commonest resistant genes in the isolate.

5. To assess the distribution and dissemination of the resistant strain in different sites of the hospital.
Chapter (2) Materials and Methods

2.1 Methods:

2.1.1 Study design:

This study was a cross sectional hospital based descriptive study for the detection of the \textit{blaOXA} and \textit{ISAba1} genes by multiplex PCR method.

2.1.2 Study area:

This study was carried out in collaboration between Aseer central hospital and microbiology department, college of medicine, Najran University in southern Saudi Arabia.

2.1.3 Study duration:

Nosocomial \textit{A. baumannii} isolates were included during the study period from October 2013 to March 2014.

2.1.4 Study population:

The number of patients included in the study was 108. All of them were from Aseer central hospital, Saudi Arabia. All ages and gender were included. The study subjects were selected from inpatient. Subjects were selected from both new and currently admitted patient.
2.1.5 Inclusion criteria:

Random sampling of Acinetobacter that fulfill the definition of multidrug resistance

2.1.6 Exclusion criteria:

Random sampling of Acinetobacter that not fulfill the definition of multidrug resistance

2.1.7 Ethical clearance:

The research content, objectives and sampling procedures were explained to all participants included in this research. All human rights were preserved, there was no violation according to the requirement adopted in Aseer central hospital, Saudi Arabia. All candidates were requested to sign the ethical clearance form (Appendix 1).

2.1.8 Specimen collection

Non-duplicating A. baumannii strains were isolated from blood, urine, respiratory secretions (sputum and tracheal aspirate), stool, pus, throat swabs, ascetic fluid and tips of central venous catheters. The study included different patients (one isolate/patient) who were hospitalized for ≥48 h and classified according to the Centers for Disease Control and Prevention/National Healthcare Safety Network (CDC/NHSN) criteria (Horan et al., 2008). The isolated nosocomial strains were identified using conventional biochemical tests, API 20NE (Biomerieux), Micro-Scan Walkaway automated systems (Dade Behring, CA), according to the
Materials and Methods

manufacturer’s instructions and Detection of the \textit{blaOXA} & \textit{ISAba1} genes by multiplex PCR method.

\textbf{2.1.9 Samples processing:}

\textbf{2.1.9.1 Culture media}

The cultures were carried out on 5\% sheep blood, MacConkey and chocolate agars. 5\% sheep blood and chocolate agar were incubated at 35\degree C in carbon dioxide or ambient air for a minimum of 24h. MacConkey agar was incubated only in ambient air \textit{(Baily and Scott's, 2014)}.

\textbf{2.1.9.2 Identification of the organism}

\textbf{2.1.9.2.1 Colonial features:}

Each plate was examined for the colony morphology: on sheep blood agar, colonies were smooth, opaque, raised, creamy and smaller than Enterobacteriaceae. On MacConkey agar colonies are non lactose fermenter, but exhibit a purplish hue that may cause the organism to be mistaken for a lactose fermenter.

\textbf{2.1.9.2.2 Confirmation of Acinetobacter species:}

Gram stain, API 20NE (Biomerieux) (appendix 2), MicroScan Walkaway automated systems (Dade Behring, CA)

\textbf{2.1.9.3 Multidrug resistance Definition}

Multidrug resistance was defined in this analysis as resistance to Three or more representatives of the following classes of antibiotics: quinolones (ciprofloxacin), extended-spectrum cephalosporins (cefta-
Materials and Methods (Magiorakos et al., 2012).

2.1.9.4 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the breakpoint method, using MicroScan Walkaway automated systems following the guidelines of the Clinical and Laboratory Standards Institute; CLSI (Murray, et al., 2003). The antibiotics tested included amikacin, ceftazidime, cefotaxime, cefepime, gentamicin, tobramycin, imipenem, meropenem, piperacillin, ciprofloxacin, levofloxacin, trimethoprim-sulfamethoxazole and colistin.

Quality control was performed by using *E. coli* ATCC 2592 all isolates were sent to the microbiology department of the Najran University College of Medicine for molecular analysis.

2.1.9.5 Bacterial strains preservation:

Long-term preservation was obtained by freezing the bacteria in Luria-Bertani (LB) broth with 10% dimethyl sulfoxide (Hsien et al., 2005).

2.1.9.6 Polymerase chain reaction (PCR):

2.1.9.6.1 DNA extraction.

*A. baumannii* strains (n = 108) were grown overnight in 5 ml broth medium at 37°C and the cell biomass were collected by centrifugation at 7000 x g for 10 min, and washed twice using sterile distilled water (Al-Arfaj et al., 2011). Genomic DNA was extracted using QIAamp DNA Kits (Qiagen; USA) following the manufacturer’s instructions (Appendix 3).
2.1.9.6.2 Identification of the oxacillinases genes

All isolates were subjected to the multiplex PCR to detect *bla*_{OXA-51-like}, *bla*_{OXA-23-like}, *bla*_{OXA-24-like} and *bla*_{OXA-58-like} genes, as previously reported (Woodford et al., 2006). All primers used in this study are listed in Table (4). The PCR was carried out in thermocycler (Cyclogene, Techne, UK). A single reaction mixture contained: 30 ng of genomic DNA, 20 pM of each primer, 10 µl reaction buffer, 3 µl 25 mM MgCl2, 1 µl dNTPs and 0,25 µl go Taq Polymerase (Promega, USA) in a final volume of 50 µl. Initial denaturation (94°C for 3 min) was followed by 30 cycles of amplification. Each cycle consisted of 94°C for 25 s, 52°C for 40 s, 72°C for 50 s. A final extension step (72°C for 5 min) completed the amplification.

2.1.9.6.3 Screening for the presence of ISAba1

*A. baumannii* strains were assayed for ISAba1 sequence by PCR with primers ISAba1-F and ISAba1-R (Table 4) giving rise to a 549 bp fragment. A single reaction mixture contained: 30 ng of genomic DNA, 10 pM of each primer, 5 µl reaction buffer, 1,5 µl 25 mM MgCl2, 0,5 µl dNTPs and 0,125 µl go Taq Polymerase (Promega) in a final volume of 25 µl. The amplification conditions were following: Initial denaturation (95°C for 5 min) was followed by 35 cycles of amplification. Each cycle consisted of 95°C for 45 s, 56°C for 45 s, 72°C for 3 min and a final extension step at 72°C for 5 min. Whether ISAba1 preceded the OXA carbapenemase genes was determined using PCR experiments by combinations of the ISAba1-F and reverse primers designed for the *bla*_{OXA-23-like}, *bla*_{OXA-24-like}, *bla*_{OXA-51-like} and *bla*_{OXA-58-like} genes (Turton et al., 2006).
Materials and Methods

2.1.9.7 Statistical analysis:

Results were analyzed by using Statistical Package for Social Sciences (SPSS; Version 10).

Table (4): Sequences of primers used in this study for multiplex PCR for detection of genes encoding oxacillinases in *A. baumannii* isolates.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXA51-F</td>
<td>TAA TGC TTT GAT CGG CCT TG</td>
<td>(Woodford et al., 2006)</td>
</tr>
<tr>
<td>OXA51-R</td>
<td>TGG ATT GCA CTT CAT CTT GG</td>
<td></td>
</tr>
<tr>
<td>OXA23-F</td>
<td>GAT CGG ATT GGA GAA CCA GA</td>
<td>(Woodford et al., 2006)</td>
</tr>
<tr>
<td>OXA23-R</td>
<td>ATT TCT GAC CGC ATT TCC AT</td>
<td></td>
</tr>
<tr>
<td>OXA24-F</td>
<td>GGT TAG TTG GCC CCC TTA AA</td>
<td>(Woodford et al., 2006)</td>
</tr>
<tr>
<td>OXA24-R</td>
<td>AGT TGA GCG AAA AGG GGA TT</td>
<td></td>
</tr>
<tr>
<td>OXA58-F</td>
<td>AAG TAT TGG GGC TTG TGC TG</td>
<td>(Woodford et al., 2006)</td>
</tr>
<tr>
<td>OXA58-R</td>
<td>CCC CTC TGC GCT CTA CAT AC</td>
<td></td>
</tr>
<tr>
<td>ISAb1-F</td>
<td>CAC GAA TGC AGA AGT TG</td>
<td>(Turton et al., 2006)</td>
</tr>
<tr>
<td>ISAb1-R</td>
<td>CGA CGA ATA CTA TGA CAC</td>
<td></td>
</tr>
</tbody>
</table>
Chapter (3)

Results

A total of 108 non-duplicating *A. baumannii* isolates were collected during the study period. The age of the patients from whom the organism were isolated ranged from 4 to 85 years (median, 45 years). Most of the patient 72 (66.7%) were males (Table 5).

3.1 Types of infections:

The studied isolates were recovered from respiratory (52.8%), wound (29.6%) and urinary tract (11.1%) infections (Table 6). The primary clinical specimens included tracheal aspirate (31.5%), sputum (24.1%), wound swab (22.2%) and urine (11.1%) (Table 7). The majority (66; 61.1%) of isolates were recovered from patients in ICU, followed by surgical (19; 17.6%) and medical (13; 12%) wards (Table 8).

3.2 Isolation of *Acinetobacter* species:

*Acinetobacter species identified by:*

- Oxidase negative

- Colonal appearance:

  * API 20NE (Biomerieux)

  * MicroScan Walkaway automated systems (Dade Behring, CA).
3.3 Antibiotic resistance pattern:

The antimicrobials tested and the percentages of isolates determined to be resistant are listed in Overall, greater than 75% of all isolates were resistant to extended-spectrum cephalosporins, aminoglycosides and quinolones. Carbapenem resistance was 52.8% for imipenem and 50% for meropenem. The lowest rate of resistance was seen against colistin (4.6%) and trimethoprim-sulfamethoxazole (65.7%)(Table 9,11).

3.4 PCR

The A. baumannii isolates were investigated for the presence of OXA-type carbapenemases. All isolates harbored the naturally occurring \( \text{bla}_{\text{OXA-51-like}} \) gene. Of 56 carbapenem-resistant isolates, 48 isolates (85.6%) carried \( \text{bla}_{\text{OXA-23-like}} \), 3 isolate (5.4%) carried \( \text{bla}_{\text{OXA-40-like}} \) and two isolates (3.6%) had \( \text{bla}_{\text{OXA-58-like}} \) genes. The \( \text{ISAb1} \) element was consistently found upstream of \( \text{bla}_{\text{OXA-23}} \) and \( \text{bla}_{\text{OXA-24}} \) genes in 40 (71.3%) and 3 (5.4%) isolates, respectively, while it was detected upstream \( \text{bla}_{\text{OXA-51}} \) in only one (1.8%) isolate(Table10,12).
Table (5): Distribution of the specimens according to gender.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NO.</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femal gender</td>
<td>36</td>
<td>33.3%</td>
</tr>
<tr>
<td>Male gender</td>
<td>72</td>
<td>66.7%</td>
</tr>
</tbody>
</table>

Table (6): Distribution of the specimens according to type of infection.

<table>
<thead>
<tr>
<th>Type of infection</th>
<th>NO.</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTI</td>
<td>57</td>
<td>52.8%</td>
</tr>
<tr>
<td>SSI</td>
<td>32</td>
<td>29.6%</td>
</tr>
<tr>
<td>UTI</td>
<td>12</td>
<td>11.1%</td>
</tr>
<tr>
<td>Primary bacteraemia</td>
<td>7</td>
<td>6.5%</td>
</tr>
</tbody>
</table>

Table (7): Distribution of the specimens according to type of specimen collected.

<table>
<thead>
<tr>
<th>Type of specimen</th>
<th>No.</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tracheal aspirate</td>
<td>34</td>
<td>31.5%</td>
</tr>
<tr>
<td>Sputum</td>
<td>26</td>
<td>24.1%</td>
</tr>
<tr>
<td>Wound swab</td>
<td>24</td>
<td>22.2%</td>
</tr>
<tr>
<td>Blood</td>
<td>6</td>
<td>5.6%</td>
</tr>
<tr>
<td>Urine</td>
<td>12</td>
<td>11.1%</td>
</tr>
<tr>
<td>Others*</td>
<td>6</td>
<td>5.6%</td>
</tr>
</tbody>
</table>

*Others; 3 ascitic fluid, 2 stool and one CSF samples.
Results

Table (8): Distribution of the specimens according to the ward of admission.

<table>
<thead>
<tr>
<th>Ward admission</th>
<th>NO.</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medical</td>
<td>13</td>
<td>12%</td>
</tr>
<tr>
<td>Surgical</td>
<td>19</td>
<td>17.6%</td>
</tr>
<tr>
<td>Pediatrics</td>
<td>10</td>
<td>9.3%</td>
</tr>
<tr>
<td>ICU</td>
<td>66</td>
<td>61.1%</td>
</tr>
</tbody>
</table>

Table (9): Number and percentages of *A. baumannii* isolates resistant to selected antimicrobial agents.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th><em>A. baumannii</em> isolates (n=108)</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftazidime</td>
<td>92</td>
<td>85.2%</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>103</td>
<td>95.4%</td>
</tr>
<tr>
<td>Cefepime</td>
<td>97</td>
<td>89.8%</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>88</td>
<td>81.5%</td>
</tr>
<tr>
<td>Imipenem</td>
<td>56</td>
<td>52.8%</td>
</tr>
<tr>
<td>Meropenem</td>
<td>54</td>
<td>50%</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>89</td>
<td>82.4%</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>93</td>
<td>86.1%</td>
</tr>
<tr>
<td>Amikacin</td>
<td>81</td>
<td>75%</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>86</td>
<td>79.6%</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>88</td>
<td>81.5%</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>71</td>
<td>65.7%</td>
</tr>
<tr>
<td>Colistin</td>
<td>5</td>
<td>4.6%</td>
</tr>
</tbody>
</table>
Table (10): Distribution of OXA-type β-lactamase genes in 56 carbapenem-resistant *A.baumannii* nosocomial isolates.

<table>
<thead>
<tr>
<th><em>bla</em>&lt;sub&gt;oxa&lt;/sub&gt; allele</th>
<th>No.</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>bla</em>&lt;sub&gt;oxa&lt;/sub&gt;-51 only</td>
<td>2</td>
<td>3.6%</td>
</tr>
<tr>
<td><em>ISAba1</em>- <em>bla</em>&lt;sub&gt;oxa&lt;/sub&gt;-51</td>
<td>1</td>
<td>1.8%</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;oxa&lt;/sub&gt;-51/<em>bla</em>&lt;sub&gt;oxa&lt;/sub&gt;-23</td>
<td>8</td>
<td>14.3%</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;oxa&lt;/sub&gt;-51/<em>ISAba1</em>- <em>bla</em>&lt;sub&gt;oxa&lt;/sub&gt;-23</td>
<td>40</td>
<td>71.3%</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;oxa&lt;/sub&gt;-51/<em>ISAba1</em>- <em>bla</em>&lt;sub&gt;oxa&lt;/sub&gt;-24</td>
<td>3</td>
<td>5.4%</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;oxa&lt;/sub&gt;-51/<em>bla</em>&lt;sub&gt;oxa&lt;/sub&gt;-58</td>
<td>2</td>
<td>3.6%</td>
</tr>
</tbody>
</table>
Table (11) : Antibiotic resistant profiles of multi-drug resistant *A. baumannii* isolates

<table>
<thead>
<tr>
<th>NO.of isolates</th>
<th>Antibiotic resistant pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, TOB, GEN, SXT</td>
</tr>
<tr>
<td>13</td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, TOB, GEN</td>
</tr>
<tr>
<td>9</td>
<td>CAZ, CFT, CFP, PIP, CIP, LEV, AMK, TOB, GEN</td>
</tr>
<tr>
<td>8</td>
<td>CAZ, CFT, CFP, PIP, AMK, TOB, GEN, SXT</td>
</tr>
<tr>
<td>7</td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, TOB, GEN, SXT</td>
</tr>
<tr>
<td>6</td>
<td>CAZ, CFT, CFP, PIP, CIP, LEV, AMK, TOB, GEN, SXT</td>
</tr>
<tr>
<td>5</td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, GEN, SXT</td>
</tr>
<tr>
<td>5</td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, TOB, GEN, SXT, COL</td>
</tr>
<tr>
<td>3</td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, TOB, SXT</td>
</tr>
<tr>
<td>2</td>
<td>CAZ, CFT, CFP, PIP, CIP, LEV, AMK, TOB, SXT</td>
</tr>
<tr>
<td>2</td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, TOB, GEN</td>
</tr>
<tr>
<td>1</td>
<td>CAZ, CFT, CFP, LEV, AMK, SXT</td>
</tr>
<tr>
<td>1</td>
<td>CAZ, CFT, CFP, PIP, CIP, LEV, SXT</td>
</tr>
<tr>
<td>1</td>
<td>CAZ, CFT, CFP, PIP, CIP, LEV, AMK, SXT</td>
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</tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>1</td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, GEN</td>
</tr>
<tr>
<td>1</td>
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</tr>
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</tr>
<tr>
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<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, GEN</td>
</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>1</td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, GEN</td>
</tr>
<tr>
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<td>CAZ, CFT, CFP, PIP, LEV, AMK, TOB, GEN, SXT</td>
</tr>
<tr>
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<tr>
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<td>CAZ, CFT, PIP, CIP, LEV, AMK, TOB, GEN</td>
</tr>
<tr>
<td>1</td>
<td>CAZ, CFT, PIP, TOB</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>1</td>
<td>CAZ, CIP, LEV</td>
</tr>
<tr>
<td>1</td>
<td>CAZ, PIP, CIP, LEV, SXT</td>
</tr>
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<tr>
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</tr>
<tr>
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<td>CFT, IMP, MEP, CIP, LEV, AMK, TOB, SXT</td>
</tr>
<tr>
<td>1</td>
<td>CIP, LEV, SXT</td>
</tr>
</tbody>
</table>
Table (12) : Antibiotic resistant profiles of carbapenem-resistant *A.baumannii* isolates in relation to OXA-type $\beta$-lactamase genes.

<table>
<thead>
<tr>
<th>$bla_{oxa}$</th>
<th>sample NO.</th>
<th>Total no</th>
<th>Resistant pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>$bla_{oxa-51}$ only</td>
<td>1,5</td>
<td>2</td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, AMK, TOB, GEN, SXT, AZ, CFT, CFP, PIP, IMP, AMK, TOB, EN, SXT</td>
</tr>
<tr>
<td><em>ISAbal</em>- $bla_{oxa-51}$</td>
<td>11</td>
<td>1</td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, TOB, SXT</td>
</tr>
<tr>
<td>$bla_{oxa-51}/bla_{oxa-23}$</td>
<td>2,6,7,12, 13,14,15, 16</td>
<td>8</td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, TOB, GEN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, TOB, GEN, SXT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CAZ, CFT, CFP, PIP, IMP, CIP, LEV, AMK, TOB, GEN, SXT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, TOB, SXT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CAZ, CFT, CFP, PIP, IMP, CIP, LEV, TOB, GEN, SXT</td>
</tr>
<tr>
<td></td>
<td>17to 56</td>
<td>40</td>
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<td></td>
<td></td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, GEN, SXT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, GEN, COL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, TOB, GEN</td>
</tr>
<tr>
<td></td>
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<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, TOB, GEN, SXT</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, TOB, GEN, SXT, COL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, TOB, GEN</td>
</tr>
<tr>
<td><strong>bla</strong>&lt;sub&gt;oxa-51/ISAbaI-&lt;/sub&gt;</td>
<td><strong>3,4</strong></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>-------</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td><strong>bla</strong>&lt;sub&gt;oxa-51/bla&lt;/sub&gt;</td>
<td><strong>8,9, 10</strong></td>
<td><strong>3</strong></td>
<td></td>
</tr>
</tbody>
</table>

| CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, TOB, GEN, SXT |
| CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, AMK, TOB, SXT |
| CAZ, CFT, CFP, PIP, IMP, MEP, CIP, LEV, TOB, GEN, SXT |
| CFT, CFP, PIP, IMP, MEP, CIP, LEV, TOB, GEN |

Chapter (3) Results
Fig. (1): 2% W/V agarose gel electrophoresis for detection of oxa genes in *A. baumannii* by Multiplex PCR.

Lane 1 and 5 were positive for oxa-51-like gene (353 bp)

Lane 2, 6 and 7 were positive for oxa-51-like gene (353 bp) and oxa-23-like gene (501 bp)

Lane 3 and 4 were positive for oxa-51-like gene (353 bp) and oxa-58-like gene (599 bp)

Lane 8, 9 and 10 were positive for oxa-51-like gene (353 bp), oxa-24-like gene (246 bp) and ISAba1 (549 bp)

Lane N negative control (no template).

Lane M shows 100 bp DNA ladder (promega, USA)
Fig. (2): 2% W/V agarose gel electrophoresis for detection of oxa genes in *A. baumannii* by PCR.

Lane 11 was positive for oxa-51-like gene (353 bp) and ISAb1 (549bp)

Lane 12 to 16 were positive for oxa-51-like gene (353 bp), oxa-23-like gene (501 bp)

Lane 17 to 24 were positive for oxa-51-like gene (353 bp), oxa-23-like gene (501 bp) and ISAb1 (549bp)

Lane N negative control (no template).

Lane M shows 100 bp DNA ladder (promega, USA)
Fig. (3): 2% W/V agarose gel electrophoresis for detection of oxa genes in *A. baumanii* by PCR.

Lane 25 to 38 were positive for oxa-51-like gene (353 bp), oxa-23-like gene (501 bp) and ISAba1 (549 bp).

Lane N negative control (no template).

Lane M shows 100 bp DNA ladder (Promega, USA).
Fig. (4): 2% W/V agarose gel electrophoresis for detection of oxa genes in *A.baumanii* by PCR.

Lane 39 to 56 were positive for oxa-51-like gene (353 bp), oxa-23-like gene (501 bp) and ISAba1 (549bp)

Lane N negative control (no template).

Lane M shows 100 bp DNA ladder (promega, USA)


Discussion

Chapter (4)

Discussion, Conclusion and Recommendation

A. baumannii has successfully become a significant nosocomial pathogen because of its remarkable ability to acquire antibiotic resistance and to survive in nosocomial environments. In this study, half of the isolates were of respiratory origin. Other sources were wound, urine and blood. This can be partly explained by the fact that A. baumannii, as the second most frequent pathogen causing respiratory tract infection like pneumonia (Xia et al., 2006), was thus more frequently detected in respiratory sample. The finding of this study showed that >60% of A. baumannii isolates were obtained from hospitalized patients in ICU wards. This finding is in line with previous reports about the role of A. baumannii in nosocomial infections among high-risk ICU patients (Peleg et al., 2008; Feizabadi et al., 2008).

Overall, the resistance rates of A. baumannii to cephalosporins, aminoglycosides and quinolones obtained in this study were higher than those reported in previous studies in Saudi Arabia. In a Saudi study the resistance rates of A. baumannii to Ceftazidime was 75.1%, Amikacin was 76.9%, Gentamicin was 77.8% and was Ciprofloxacin 64.0% (Memish et al., 2012). In another Saudi studies the resistance rates of A. baumannii to Ceftazidime was 91%, Amikacin was 56%, Gentamicin was 71% (Asaad et al., 2013)

\Carpabenem resistance in this study is of considerable concern, since this class of antimicrobial agent was, until recently, considered to be among the most potent against many microorganisms including A. baumannii. In 2 recent Saudi studies, the resistance rates of A. baumannii
to IMP and MEM were 62% and 67%, respectively (Al-Arfaj et al., 2011; Al-Agamy et al., 2013).

In a 4-year Chinese study, carbapenem resistance increased from 15% for IMP and 23% for MEM in 2008 to 90% and 92% in 2011, respectively (Ting et al., 2013). In Taiwan, the prevalence of imipenem-resistance over 10 years was from 3% in 2002 to 59% in 2010 (Kuo et al., 2012). According to the SENTRY program, the resistance to imipenem ranged from 32.8% in North America to 51.7% in Latin America (Gales et al., 2011).

Polymyxins have emerged as alternatives against A. baumannii. In this study, colistin resistance was 4.6%. The SENTRY study reported that the resistance rate to polymyxin B ranged from 2% in North America to 0.9% in Europe (Gales et al., 2011).

The emergence of resistance to polymyxin will pose a serious therapeutic problem because no new antimicrobial agents are currently available for treatment of infections caused by this pathogen.

In this study, all 108 A. baumannii isolates carried the chromosomally-encoded blaoXA-51-like gene. These findings support those of other studies demonstrating that the detection of blaoXA-51-like gene can be used as a supplementary tool to identify the organism to the species level, confirmed by additional methods (Howard et al., 2012; Lee et al., 2012).

The most frequent enzymatic mechanism of carbapenem resistance in A. baumannii is the production of oxacillinases and several studies
have identified a variety of oxacillinases in carbapenem-resistant *A. baumannii* isolates.

**Conclusion:**

In this study, half of the *A. baumannii* isolates were of respiratory origin. The majority of *A. baumannii* isolates were recovered from hospitalized patients in ICU wards. Carbapenem resistance was 52.8% for imipenem and 50% for meropenem.

Our findings illustrate further the challenge of increasing carbapenem-resistance in *A. baumannii* isolates in Saudi Arabia. The high distribution of class D carbapenemase-encoding genes, mainly ISAbal/OXA-23 and ISAbal/OXA-24 carbapenemases presents an emerging threat in our hospital. The diversity of resistance genes is particularly worrisome due to the difficult choice of empirical antibiotic therapy in seriously ill patients and the possible contribution to increased hospital stay and associated costs.

**Recommendations:**

coli
tin combined with other antibiotics or β-lactamase inhibitors when treating carbapenem-resistant *A. baumannii* infection (*Ilkay et al., 2013*).

Local molecular surveillance is essential to help control carbapenem-resistant *A. baumannii* nosocomial infections and prevent DNA exchange among endemic nosocomial pathogens.
The \( \text{bla}_{\text{OXA-23}} \) carbapenemase producing \( A.\text{baumannii} \) are becoming globally widespread in Europe (Gur et al., 2008; Bogaerts et al., 2008), South America (Carvalho et al., 2009; Merkier et al., 2008), and Asia (Feizabadi et al., 2008; Niumsup et al., 2009).

In this study, \( \text{bla}_{\text{OXA-23}} \) carbapenemase was detected in 48 (85.6%) of the 56 carbapenem-resistant isolates and the \( \text{IS}_{\text{Aba1}} \) element was located upstream of 40 (71.3%) \( \text{bla}_{\text{OXA-23}} \) producing strains. It is well established that the promoting sequence \( \text{IS}_{\text{Aba1}} \) has to be present to ensure oxacillinase expression and, consequently, the development of resistance to many antimicrobials, creating a serious problem for choice of therapy (Peleg et al., 2008; Howard et al., 2012).

Our data agreed with the findings of previous studies that the acquisition of \( \text{IS}_{\text{Aba1}}/\text{bla}_{\text{OXA-23}} \) is the main mechanism for carbapenem resistance among \( A.\text{baumannii} \) isolates in Saudi Arabia (Al-Arfaj et al., 2011; Al-Agamy et al., 2013).

The \( \text{bla}_{\text{OXA-24-like}} \) gene has been reported in Portugal, Spain (Villalón et al., 2013), Poland (Paweł et al., 2012), Iran (Feizabadi et al., 2008), the United States (QI C et al., 2008) and Asia (Ben et al., 2011). In Saudi Arabia, \( \text{bla}_{\text{OXA-24-like}} \) gene was detected in a rate of 4–45% of \( \text{Acinetobacter} \) isolates (Al-Arfaj et al., 2011; Al-Agamy et al., 2013).

It is noteworthy that 3 (5.4%) \( A.\text{baumannii} \) isolates in our study were positive for \( \text{IS}_{\text{Aba1}}/\text{bla}_{\text{OXA-24-like}} \) genes. Our findings indicate that there are different mechanisms for carbapenem resistance among \( A.\text{baumannii} \) isolates recovered from different Saudi regions. The spread of these genes among isolates deserve further attention.
In this study, the mechanism of carbapenem resistance is not clear in the 12 carbapenem-resistant isolates encoding the $bla_{OXA-51}$ gene alone (2 isolates), $bla_{OXA-51}/bla_{OXA-58}$ combination (2 isolates) and $bla_{OXA-51}/bla_{OXA-23}$ combination (8 isolates) as the sole carbapenemase gene determinants which are not associated with the ISAbal element. Further investigations are required to delineate the resistance mechanism in these isolates.


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Ethical clearance statement:

Name of the research: Molecular characterization of Multi-drug Resistant *Acinetobacter baumannii* in kingdom Saudi Arabia.

This proposal for PhD degree in Medical Microbiology.

Thereby declaring that all participants included in this research have been explained to them the research content, objectives and sampling procedures. All human rights were preserved there is no violation according to the requirements adopted in Aseer Central Hospital, Abha, Saudi Arabia

Thanks;

Name of principle investigator: Faten Mohamed Mohamed El-Abd.
API 20NE:

Principle:

The API 20NE system facilitates the identification of non-fastidious Gram-negative rods not belonging to the *Enterobacteriaceae* within 48 hours.

The API 20NE strip consists of microtubes containing dehydrated media and substrates. The media microtubes containing conventional tests are inoculated with a bacterial suspension which reconstitutes the media. After incubation, the metabolic end products are detected by indicator systems or the addition of reagents. The substrate microtubes contain assimilation tests and are inoculated with a minimal medium. If the bacteria are capable of utilizing the corresponding substrate, then they will grow.

Materials:

- API 20NE strips - store at 2-8°C
- 0.85% sterile saline
- Mineral oil
- Zinc dust
- AUX Medium
- James Reagent
- Nitrate 1 - store at 2-80 C
Appendix (II)

- Store at 2-8°C
- Nitrate 2 - store at 2-8°C
- Oxidase Reagent

Procedure:

1. Preparation of Inoculum

   Procedure manual Toronto Medical Laboratories/mount Sinai Hospital Microbiology Department.

   a) Add 2 ml. of 0.85% saline to a sterile test tube.

   b) Using a sterile inoculating loop, carefully touch the centre of a well isolated colony (2-3 mm. Diameter) and thoroughly emulsify in the saline. The suspension turbidity should be equal to a 0.5 McFarland standard.

2. Preparation of the Strip

   a) An incubation tray and lid are supplied for each strip.

   b) Dispense 5 ml of distilled water in to the tray.

3. Inoculation of the Strip

   a) Remove the cap from the tube containing the bacterial suspension and insert a sterile pipette.

   b) Tilt the API 20NE incubation tray and fill the TUBE section of the NO to PNPG microtubes by placing the pipette tip against the side of the cupule.
Appendix (II)

c) Open an ampule of AUX Medium and add 200 uL of the bacterial suspension to the ampule. Mix well with a pipette while avoiding the formation of air bubbles.

d) Using the AUX Medium bacterial suspension, fill both the TUBE and CUPULE section of [GLU] to [PAC]. Do not overfill the cupules. Fill to a flat or slightly convex meniscus.

e) After inoculation, completely fill the CUPULE section of the 3 underlined tests, GLU, ADH and URE tubes with mineral oil.

f) Using the excess bacterial suspension, inoculate an agar slant or plate (non-selective media such as nutrient agar, blood agar or tryptic (trypticase) soy agar is suggested) as a purity check and for oxidase testing, and/or additional biochemical testing. Incubate the slant or plate with the API 20NE strip.

4. Incubation of the Strip

a) After inoculation, place the plastic lid on the tray and incubate the strip for 24 hours at 30°C in a non-CO incubator.

5. Reading the Strip

a) After 24 hours incubation, record all reactions not requiring the addition of reagents.

b) Perform the oxidase test. A portion of the growth from the agar slate or plate, inoculated from the 20NE bacterial suspension, should be rubbed onto filter paper to which a drop of oxidase reagent (1% tetramethyl-p-phenylenediamine dihydrochloride) has been added. The area where the growth has been added will turn dark purple within 10 seconds if the reaction is positive and will be colourless or light purple if negative.
Appendix (II)

Note:

(a) Nichrome wire loops should NOT be used in performing the oxidase test. Nichrome wire can cause a false positive reaction.

(b) The oxidase test should NOT be performed using bacterial growth from selective media such as MacConkey, EMB, etc

c) Assimilation tests are observed for bacterial growth. An opaque cupule indicates a positive reaction.

d) Protect the assimilation tests with the incubation tray lid during the reading of the Nitrate and TRP tests.

e) Perform the Nitrate test.

   i. Add one drop of Nitrate 1 and one drop of Nitrate 2 reagents to NO cupule.

   ii. After 5 minutes a red color indicates a positive reaction.

   iii. A negative reaction may be due to the production of nitrogen.

       Add Zinc dust to the NO cupule. After 5 minutes a colorless cupule indicates a positive reaction. A pink-red cupule indicates a negative reaction.

f) Perform the TRP test.

   i. Add one drop of JAMES Reagent.

   ii. The reaction takes place immediately, producing a pink color in the entire cupule if the reaction is positive.

Interpretation

1. Use the API 20NE analytical profile index.

2. The tests are separated into groups of three. The following numerical value is assigned to each positive reaction recorded:
1. Positive reaction in the first test of the group.

2. Positive reaction in the second test of the group.

3. Positive reaction in the third test of the group.

By adding the values corresponding to positive reactions in each group, a seven digit number is obtained.

3. The strip must be reincubated in the following cases:

   i. If the profile cannot be found in the Analytical Profile Index.

   ii. If the following note is indicated for the profile obtained:

      * Identification not valid

      * Before 48-hr incubation

   iii. If the strip is to be reincubated, remove the reagents from the NO and TRP cupules and then cover these tests with mineral oil.

   iv. Reincubate the strip for another 24 hours at 30°C in a non-CO incubator.

   v. Read all the tests again, except for NO TRP and GLU.
## Reading Table:

<table>
<thead>
<tr>
<th>Tests</th>
<th>Substrates</th>
<th>Reactons/ enzymes</th>
<th>Negative Results</th>
<th>Positive Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₃</td>
<td>Potassium nitrate</td>
<td>NITrate reduction to Nitrites</td>
<td>NIT¹+ NIT₂/Coulourless</td>
<td>5 min pink-red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIT rates to nitrogen</td>
<td></td>
<td>Colourless</td>
</tr>
<tr>
<td>TRP</td>
<td>Tryptophane</td>
<td>Indole production</td>
<td>JAMES / immediate colourless /pink pale green/ yellow</td>
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<td>GLU</td>
<td>Glucose</td>
<td>Acidification</td>
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</tr>
<tr>
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<td>Arginine</td>
<td>Arginine dihydrolase</td>
<td>Yellow</td>
<td>Orange/pink/red</td>
</tr>
<tr>
<td>URE</td>
<td>Urea</td>
<td>Urease</td>
<td>Yellow</td>
<td>Orange/pink/red</td>
</tr>
<tr>
<td>ESC</td>
<td>Esculin</td>
<td>Hydrolysis (gluco-sidase)</td>
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<td>Grey/brown/black</td>
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<tr>
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<td>Gelatine (with India ink)</td>
<td>Hydrolysis (protease)</td>
<td>No pigment diffusion</td>
<td>Diffusion of Black pigment</td>
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<td>Galactosidase</td>
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<td>Yellow</td>
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<td>Opaque</td>
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<td>[ARA]</td>
<td>Arabinose</td>
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<td>Opaque</td>
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</tr>
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<td>Opaque</td>
</tr>
<tr>
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<td>Opaque</td>
</tr>
<tr>
<td>[CIT]</td>
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<td>Transparent</td>
<td>Opaque</td>
</tr>
<tr>
<td>[PAC]</td>
<td>Phenyl-acetate</td>
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<td>Opaque</td>
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<tr>
<td>OX</td>
<td>See oxidase test</td>
<td>Cytochrome oxidase</td>
<td>Colorless/ light purple</td>
<td>Dark purple</td>
</tr>
</tbody>
</table>
DNA extraction:

Genomic DNA was extracted using QIAamp DNA Kits (Qiagen; USA).

**Isolation of genomic DNA from bacterial plate cultures**

1. Remove bacteria from culture plate with an inoculation loop and suspend in 180 µl of Buffer ATL (supplied in the QIAamp DNA Mini Kit) by vigorous stirring.

2. Add 20 µl proteinase K, mix by vortexing, and incubate at 56°C until the cells is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.

**Note:** Proteinase K must be used. QIAGEN Protease has reduced activity in the presence of Buffer ATL. Lysis is usually complete in 1–3 h. Lysis overnight is possible and does not influence the preparation. In order to ensure efficient lysis, a shaking water bath or a rocking platform should be used. If not available, vortexing 2–3 times per hour during incubation is recommended.

3. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

4. Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
Appendix (III)

It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of Buffer AL, which in most cases will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

5. Add 200 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

It is essential that the sample, Buffer AL, and the ethanol are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the QIAamp Mini spin column. This precipitate does not interfere with the QIAamp procedure or with any subsequent application.

Do not use alcohols other than ethanol since this may result in reduced yields.

6. Carefully apply the mixture from step 5 (including the precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 xg (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.*
Close each spin column to avoid aerosol formation during centrifugation. It is essential to apply all of the precipitate to the QIAamp Mini spin column. Centrifugation is performed at 6000 xg (8000 rpm) in order to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all the solution has passed through.

7. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 xg (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.*

8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

9. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

This step helps to eliminate the chance of possible Buffer AW2 carryover.

10. Place the QIAamp Mini spin column in a clean 1.5 ml micro-centrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 xg (8000 rpm) for 1 min.

A 5 min incubation of the QIAamp Mini spin column loaded with Buffer AE or water, before centrifugation, generally increases DNA yield. A third elution step with a further 200 µl Buffer AE will increase yields by up to 15%. Volumes of more than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation.

Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield. Eluting with 4 x 100 µl instead of 2 x 200 µl does not increase elution efficiency.

For long-term storage of DNA, eluting in Buffer AE and placing at −20°C is recommended, since DNA stored in water is subject to acid hydrolysis.