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Prevalence of Carbapenemases in *Acinetobacter baumannii*

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1. Introduction

Acinetobacter baumannii (A. baumannii) is an important opportunistic pathogen and causes a variety of nosocominal infections especially in Intensive Care Units (ICU's) (Bergogne-Berezin & Towner, 1996; Villegas & Harstein, 2003). These infections include bacteraemia, surgical-site infections, secondary meningitis, urinary tract infections and ventilator associated pneumonia (Bergogne-Berezin & Towner, 1996; Villegas & Harstein, 2003). *Acinetobacter baumannii* has multiresistant phenotypes, including resistance to broad-spectrum β-lactams, fluoroquinolones, aminoglycosides and carbapenems and therefore treatment of this pathogen is complicated (Coelho et al., 2004; Dalla-Costa et al., 2003; Jeon et al., 2005; Landman et al., 2002; Naas et al., 2005; Vahaboglu et al., 2006; Zarrilli et al., 2004). The multiresistant phenotypes of *A. baumannii* also contributed to the emergence of multi drug resistant *Acinetobacter baumannii* (MDRAB), which have become more prevalent within the past decade (Coelho et al., 2004) and has also caused an increase in the number of nosocomial infections in the past decade (Joly-Guillou, 2005).

Over the last 20 years, there has been an increase in the interest of the *Acinetobacter* species (Giamarellou et al., 2008). The increase in interest is due to i) worldwide expansion of ICU's, leading to a change in the types of infections caused by *Acinetobacter spp* and ii) due to the emergence of MDRAB and cases of pan-drug resistant *A. baumannii* (PDRAB) have also been reported (Giamarellou et al., 2008).

The acquired carbapenem resistance in *A. baumannii* is often associated with carbapenemase production; IMP, VIM and SIM-type metallo-β-lactamase production or the OXA-24, OXA-23 and OXA-58 type class D carbapenemases (Brown & Amyes, 2006; Poirel & Nordmann, 2006). Also associated with acquired carbapenem resistance in *A. baumannii* is the over production of natural oxacillinase (OXA-51) (Poirel & Nordmann, 2006).

Carbapenemases are the most versatile of all β-lactamases and many of them recognize almost all hydrolysable β-lactams (Livermore & Woodford, 2006; Nordmann & Poirel 2002; Walther-Rasmussen & Hoiby, 2006). Most carbapenemases are resistant to commercial β-lactamase inhibitors (Livermore & Woodford, 2006; Nordmann & Poirel, 2002; Walther-Rasmussen & Hoiby, 2006). Carbapenemases are divided into three subclasses on the basis of their hydrolysis characteristics (Frere et al., 2005). The first carbapenemases described

were from Gram-positive bacilli and were inhibited by EDTA (Frere et al., 2005). These carbapenemases were described as metalloenzymes and have one zinc atom in the active site (Frere et al., 2005). This zinc atom facilitates hydrolysis of a bicyclic β-lactam ring (Frere et al., 2005). The second form of carbapenemases use serine at the active sites and are inactivated by clavulanic acid and tazobactam (β-lactamase inhibitors) (Rasmussen et al., 1996; Yang et al., 1990). Molecular classes A, C and D have serine in the active site and form part of the β-lactamases (Bush, 1988). The molecular class B of the β-lactamases are metalloenzymes and have zinc in the active site (Bush, 1988). The enzymes from the molecular classes A, B and D have the ability to hydrolyse carbapenems, which results in an elevated carbapenem minimum inhibitory concentration (Bush, 1988).

The aim of this study was to optimise and evaluate multiplex polymerase chain reaction (PCR) assays to rapidly differentiate the four subgroups of the oxacillinase (OXA) genes and the five subgroups of the metallo-β-lactamase (MBL) antibiotic resistant genes. The PCR assays results were compared to the phenotypic tests i) Hodge test and ii) Double disk synergy test. Antibiotic resistance testing is important to decrease the spread of antibiotic resistant strains of *A. baumannii* in clinical settings.

2. History of Acinetobacter baumannii

Acinetobacter baumannii (Figure 1) was first isolated in 1911 from a soil sample by MW Beijerink (Kuo et al., 2004). *Acinetobacter spp* were first thought to be non-virulent saprophytes (Bergogne-Berezin & Towner, 1996). In the 1970s the widespread use and misuse of antibiotics started (Kuo et al., 2004). In 1986 *Acinetobacter baumannii* was taxonomically classified (Bouvet et al., 1986). The first carbapenem resistant *A. baumannii* (CRAB) isolates were discovered in 1991 (Kuo et al., 2004). The first carbapenem hydrolyzing oxacillinase (CHDL's) was identified in 1995 (Scaife et al., 1995). It was initially named ARI-1 and was later renamed OXA-23 (Scaife et al., 1995).

Fig. 1. *Acinetobacter baumannii* isolates (www.acinetobacter.org)

The first reported outbreak of CRAB occurred in the USA in 1991 (Go et al., 1994). Carbapenem Resistant *A. baumannii* isolates were isolated from a leukaemia patient in the oncology ward of a Taiwanese hospital in May 1998 (Hsueh et al., 2002). These isolates were observed to be resistant to almost all antibiotics e.g. cephalosporins, aztreonam, aminoglycosides and ciprofloxacin and were therefore named pan-drug resistant *A. baumannii* (PDRAB) (Hsueh et al., 2002). The rise in the number of multi drug resistant *A. baumannii* (MDRAB) strains has been due to the extensive use of antimicrobial chemotherapy against bacterial infections (Hsueh et al., 2002).

3. Classification of *Acinetobacter baumannii*

In 1986 *Acinetobacter baumannii* was taxonomically classified (Bouvet et al., 1986). *Acinetobacter* are grouped into three main complexes: i) *Acinetobacter calcoaceticus-baumannii* complex, which is glucose oxidizing and non-haemolytic; ii) *Acinetobacter lwoffii,* which are glucose negative and non-haemolytic and iii) *Acinetobacter haemolyticus*, which is haemolytic (Euzeby, 2008). The full classification of *A. baumannii* is listed in Table 1.

Domain	*Bacteria*
Phylum	*Proteobacteria*
Class	*Gammaproteobacteria*
Order	*Pseudomonadales*
Family	*Moraxellaceae*
Genus	*Acinetobacter*
Species	*A. baumannii* *A. baylyi* *A. beijerinckii* *A. bouvetii* *A. calcoaceticus* *A. gerneri* *A. grimontii* *A. gyllenbergii* *A. haemolyticus* *A. johnsonnii* *A. junnii* *A. lwoffii* *A. parvus* *A. radioresistens* *A. schindleri* *A. soli* *A. tandoii* *A. tjernbergiae* *A. towneri* *A. ursingii* *A. venetianus* 14 species are still unnamed

Table 1. Nomenclature of *Acinetobacter baumannii* (Euzeby, 2008)

There are 21 recognized genomic species of the genus *Acinetobacter* and 14 unnamed genomic species (Euzeby, 2008).

4. General characteristics of *Acinetobacter baumannii*

Acinetobacter baumannii are Gram-negative, non-fermentative, non-motile, oxidase-negative, aerobic coccobacilli that are ubiquitous in nature and commonly found within the hospital environment causing a variety of opportunistic nosocomial infections (Bergogne-Berezin et al., 1996). The bacteria can be isolated from water, soil and the environment and from human skin (Bergogne-Berezin et al., 1996). The morphology of *Acinetobacter* spp is variable in Gram-stained human clinical specimens and thus cannot be used to differentiate *Acinetobacter* from other causes of common nosocomial infections (http://microbewiki.kenyon.edu/index.php/Acinetobacter_baumannii). *Acinetobacter baumannii* are non-lactose fermenting bacteria, however they partially ferment lactose on MacConkey agar (http://microbewiki.kenyon.edu/index.php/Acinetobacter_baumannii). All the species of the *Acinetobacter* genus grow well on MacConkey agar (except for *A. lwoffii*), when salt is absent (http://microbewiki.kenyon.edu/index.php/Acinetobacter_baumannii). *Acinetobacter baumannii* are strict aerobes and grow well on nutrient agar (http://microbewiki.kenyon.edu/index.php/Acinetobacter_baumannii). Infection by *A. baumannii* is difficult to combat due to the Gram-negative nature of the cell wall as the outer wall provides a barrier so that the antimicrobial agent is unable to enter the bacterial cell (Projan, 2004).

Acinetobacter baumannii is an opportunistic pathogen that is successful in colonizing and persisting in the hospital environment and is able to resist desiccation (Getchell-White et al., 1989; Jawad et al., 1996). The bacterium is also able to survive on inanimate surfaces for months (Kramer et al., 2006). *Acinetobacter baumannii* is among the most common causes of device related nosocomial infections (Dima et al., 2007; Thongpiyapoom et al., 2004), resulting when the bacterium is able to resist both physical and chemical disinfection, by forming a biofilm (Cappelli et al., 2003; Loukili et al., 2006; Pajkos et al., 2004). Biofilm associated proteins (BAP's) were first characterized in *S. aureus* (Cucarella et al., 2001) and have been found in a number of other Gram-positive and Gram-negative pathogenic bacteria.

4.1 Optimal Growth conditions for *Acinetobacter baumannii*

Acinetobacter baumannii form part of the natural flora of the skin and mucous membranes of humans (Seifert et al., 1997). *Acinetobacter baumannii* are ubiquitous in clinical and natural environments and commonly colonises the skin, oropharynx secretions, respiratory secretions, urine, irrigating and intravenous solutions (Seifert et al., 1997). *Acinetobacter baumannii* can be cultured from sputum or respiratory secretions, wound and urine (Go & Cuhna, 1999). The pathogen colonises the gastro-intestinal tract and is associated with nosocomial meningitis, nosocomial pneumonia and bacteraemia (Go & Cuhna, 1999).

Acinetobacter baumannii grown on trypticase soy agar produce circular, convex, smooth and slightly opaque colonies, which are 1.5 to 2.0 mm in diameter after 24 hours at 30°C or 3.0 to 4.0 mm after 48 hours (Garrity et al., 2005). *Acinetobacter baumannii* do neither haemolyse horse blood nor sheep blood when grown on blood agar plates (Garrity et al., 2005). Seasonal variations have been reported for nosocomial *A. baumannii* infections and

bacteraemia, with increased incidences occurring in the summer months (McDonald et al., 1999).

5. Risk factors for *Acinetobacter baumannii* infections

Acinetobacter baumannii can survive on various surfaces within hospitals, including catheters and other medical equipment (http://microbewiki.Kenyon.edu/index.php/Acinetobacter_baumannii). Thus environmental contamination is an important source of infection as pathogens are spread directly from surfaces or through the hands of healthcare workers to patients (Corbella et al., 1996). Infected or colonized patients are important reservoirs of *A. baumannii*. *Acinetobacter baumannii* is passed from patient to patient via direct and indirect contact (D'Agata et al., 2000). The main risk factors of *Acinetobacter baumannii* bacteraemia are invasive procedures e.g. central venous catheterization, mechanical ventilation and surgery (Seifert et al., 1995b). Another major risk factor for *A. baumannii* infections is the widespread use of broad-spectrum antibiotics (Cisneros & Rodriguez-Bano, 2002). Other risk factors include prolonged hospital stay, ICU stay, enteral feeding, previous administration to another unit and previous use of third generation cephalosporins (Mulin et al., 1995; Scerpella et al., 1995).

The risk factors within the ICU's concern the immunosuppressed patients, patients previously exposed to antimicrobial therapy, patients who underwent high invasive procedures and patients who suffered from previous sepsis (Garcia-Garmendia et al., 2001). Other risk factors include pneumonia as a source of infection, inappropriate empirical treatment and prior treatment with carbapenems (Robenshtok et al., 2006). Surgical procedures performed within the emergency operating theatre is another major risk factor contributing to the spread of epidemic cases of *A. baumannii*, however the main risk factor was the previous use of fluoroquinolones (Villers et al., 1998).

5.1 Pathogenesis of Acinetobacter baumannii

Acinetobacter baumannii infections are associated with systems of high fluid content e.g. lungs, cerebrospinal fluid, peritoneal fluid and the urinary tract and usually only occur in the immunocompromised patients (Cuhna, 2007). Patients with *A. baumannii* bacteraemia usually have signs and symptoms that are related to the organ system involved (Cuhna, 2007). Symptoms include wound infections, outbreaks of nosocomial pneumonia, catheter associated bacteriuria, urethritis and continuous ambulatory peritoneal dialysis (CAPD) associated peritonitis (Cuhna, 2007). Bacteraemia results in septic shock in 25-30% of all cases and disseminated intravascular coagulation frequently occurs (Cisneros et al., 1996: Seifert et al., 1997). Colonisation may occur after an invasive infection (Corbella et al., 1996), especially in burn patients (Wisplinghoff et al., 2004). Problems rarely associated with *A. baumannii* infections include meningitis, endocarditis, urinary tract infections, pneumonia and cholangitis (Cuhna, 2007). Other problems that rarely occur are soft tissue infections and complicated skin, abdominal infections and central nervous system (CNS) infections (Fournier & Richet, 2006). Allen and Green documented the first report of airborne spread of *A. baumannii* in 1987. *Acinetobacter baumannii* survives much better on fingertips or on dry surfaces when tested under stimulated hospital environmental conditions (Jawad et al., 1996). The skin of patients and medical personnel is involved in the transmission of *A. baumannii* strains and in some outbreaks; molecular typing has identified the epidemic

strain on the skin of the patients (Gerner-Smidt, 1987; Patterson et al., 1991). Contaminated reusable medical equipment e.g. ventilator tubing, respirometers and arterial pressure monitoring devices are used for the management of severely ill patients serve as another route of transmission to patients (Beck-Sague et al., 1990; Cefai et al., 1990). Fomites e.g. bed mattresses (Sheretz & Sullivan., 1985), pillows (Weernink et al., 1995), a tape recorder, television set and a fan (Jawad et al., 1994) were found to be contaminated with *Acinetobacter* and served as reservoirs during nosocomial outbreaks.

The mortality rate within the hospitals is high, with a 23% mortality recorded for hospitalized patients and a 43% mortality rate among patients in intensive care (Falagas et al., 2006). The Antimicrobial Availability Task Force (AATF) of the Infectious Disease Society of America identified *Acinetobacter baumannii, Aspergillus* spp, extended spectrum β-lactamase producing *Enterobacteriaceae*, *Pseudomonas aeruginosa, Staphylococcus aureus* as "particularly problematic pathogens" and there is a desperate need for new drug development (Talbot et al., 2006).

It is difficult to distinguish between colonization and infection regarding *A. baumannii* (Joly-Guillou, 2005). There is controversy over whether infections caused by *A. baumannii* result in unfavourable outcomes (Blot et al., 2003; Falagas et al., 2006). The isolation of *A. baumannii* in hospitalized patients is an indicator of severe illness with an associated mortality of approximately 30% (Wilson et al., 2004).

Community acquired *A. baumannii* (CAAB) occurs within an individual with one or more cultures of blood, collected within 48 hours of admission, that is positive for *A. baumannii* complex and is identified by a biochemical method (API 20NE system) (bioMerieux, France) (Schreckenberger & Von Graevenitz, 2000). Patients with CAAB associated pneumonia had an increased mortality rate and presented with a more severe disease than the patients without pneumonia (Wang et al., 2002). The development of CAAB is associated with underlying malignancies e.g. lung cancer, lymphoma and thymic carcinoma (Wang et al., 2002). *Acinetobacter baumannii* genomic species were responsible for CAAB; however there is no evidence of clonal spread of *A. baumannii* in the community (Wang et al., 2002). Carbapenems, cefopirome, cefepime, ceftazidime, aminoglycosides and fluoroquinolones are the antimicrobials of choice for treating CAAB (Wang et al., 2002).

5.2 Virulence factors of *Acinetobacter baumannii* strains

Acinetobacter baumannii have very few virulence factors (Cisneros & Rodriguez-Bano, 2002), however some strains have virulence factors associated with invasiveness, transmissibility or the enhanced ability to colonise immunocompromised patients (Dijkshoorn et al., 1996). Ethanol stimulates the virulence of *A. baumannii* (Smith et al., 2004), which led to the identification of a number of genes, affecting virulence towards *Caenorhabditis elegans* and *Dictyostelium discoideum* (Smith et al., 2007).

A new strain (OXA-23 clone II) was identified in a military hospital and was found to be a particularly virulent strain, which is very difficult to eliminate from medical facilities (promedmail). There are three major European clones of *A. baumannii* (Giamarellou et al., 2008) Clone I, found in South Africa, Czech Republic, Poland, Italy and Spain; Clone II, found in South Africa, Spain, Turkey, Greece and France and clone III is found in the Netherlands, Italy, France and Spain (Van Dessel et al., 2004). Clones I and II are responsible for the outbreaks of

A. baumannii bacteraemia in South Africa and Northern Europe (Van Dessel et al., 2004). *Acinetobacter* can efficiently transfer genes horizontally (only observed and analysed in *A. baylyi*), especially the genes encoding antibiotic resistance (Gerischer, 2008).

A large portion of the *A. baumannii* genome is dedicated to pathogenesis, with a large number of genes occurring within virulence islands (Perez et al., 2007). *Acinetobacter baumannii* together with *Acinetobacter* DNA group13TU is involved in the majority of *Acinetobacter* hospital outbreaks (Bergogne-Berezin & Towner, 1996). Strains of the *Acinetobacter* DNA group 3 and *A. junii* have only occasionally been implicated in outbreaks of nosocomial infections (Bernards et al., 1997). *Acinetobacter baumannii* has environmental resilience and a wide range of resistance determinants, therefore making it a successful nosocomial pathogen (Nordmann, 2004). *Acinetobacter baumannii* has caused numerous global outbreaks and displayed ever increasing rates of resistance (Villegas & Harstein, 2003). In hospital outbreaks the emergence of imipenem-resistant strains has been documented (Brown et al., 1996; Go et al, 1994).

6. Clinical manifestations of *Acinetobacter baumannii* infections

The clinical manifestations of *A. baumannii* are non-specific and present as a trans-maculopapular rash affecting the palms of the hands and the soles of the feet of endocarditis patients, or necrotic lesions of the skin and soft tissue (Seifert et al., 1995). *Acinetobacter baumannii* bacteraemia is polymicrobial, and is often associated with *Klebsiella pneumoniae* (Seifert et al., 1995).

6.1 Treatment of *Acinetobacter baumannii* infections

Acinetobacter baumannii are Gram-negative bacteria and therefore are particularly difficult to treat due to the presence of an outer membrane (Projan, 2004). The recommended treatment therefore is a limited spectrum active β-lactam e.g. ceftazidime or imipenem the most active agent against *A. baumannii* and an aminoglycoside (Cisneros & Rodriguez-Bano, 2002).

There is incomplete current knowledge of the clinical response and bacterial mechanisms of resistance to antimicrobials (Kahlmeter et al., 2006). The reliability and comparability of different methods of susceptibility testing e.g. disc diffusion and broth microdilution have not been consistent for *A. baumannii* (Swenson et al., 2004). The persistence of subtle growth beyond an obvious end point by broth microdilution is of great concern in the case of β-lactams, which therefore explains its poor reaction with the disc diffusion method (Swenson et al., 2004). Doripenem, a novel carbapenem is active against susceptible *A. baumannii* (Fritsche et al., 2005; Jones et al., 2004; Jones et al., 2005; Mushtaq et al., 2004). Doripenem was not effective against *A. baumanni* isolates producing bla_{OXA-23} or bla_{IMP-4} or metallo-β-lactamases (Mushtaq et al., 2004).

6.2 Carbapenems as treatment for *Acinetobacter baumannii* infections

Carbapenems are structurally related to the penicillins ("penams"), differing only by the substitution of carbon ("carba") for the sulfur atom at position 1 and the presence of a double bond between C2 and C3 (Bradley, 1997; Wise, 1986). A hydroxyethyl side chain instead of the acylamino group found in penicillins and cephalosporins is present and provides resistance to most β-lactamases (Bradley, 1997; Wise, 1986).

Carbapenems were introduced into clinical practice in the 1970's and the 1980's marked the emergence of Gram-negative bacterial resistance to carbapenems (Nordmann & Poirel, 2002; Walsh, 2005). Carbapenems were derived from the naturally occurring antibiotic, thienamycin, which is produced by the soil microorganism *Streptomyces cattleya* (Jacobs, 1986). The first carbapenemases described were from Gram-positive bacilli and were inhibited by EDTA (Frere et al., 2005). Carbapenems are recognised as the gold standard for treating infections caused by resistant Gram-negative bacteria (Rahal, 2006).

6.3 Combination therapy as a strategy for the treatment of multiple drug resistant *Acinetobacter baumannii*

Sulbactam is an inhibitor of β-lactamases and has *in vitro* bactericidal activity against *Acinetobacter spp* (Cisneros & Rodriguez-Bano, 2002). The efficacy of sulbactam against susceptible *A. baumannii* is similar to imipenem (Rodriguez-Hernandez, 2000). Sulbactam exhibits bacteriostatic action against *A. baumannii* (Corbella et al., 1998). Sulbactam is also used to treat meningitis caused by multiple drug resistant *Acinetobacter baumannii* (MDRAB) (Cisneros & Rodriguez-Bano, 2002). Combinations of sulbactam with aminoglycosides, rifampin and azithromycin have demonstrated synergy against imipenem susceptible strains (Appleman et al., 2000; Savov et al., 2002). There is little or no advantage to the combination sulbactams with cephalosporins (Appleman et al., 2000; Savov et al., 2002).

Polymyxins (colistimethate and polymyxin B) are the only alternative treatment for sulbactam resistant *A. baumannii* strains (Wood & Reboli, 1993). Colistin was used in the 1960's and the 1970's, but had many adverse side effects, including nephrotoxicity, neuro-muscular blockage (Cisneros & Rodriguez-Bano, 2002). Colistin disrupts the outer cell membranes of many Gram-negative bacilli by changing the permeability of the membrane and causing a bactericidal effect (Cisneros & Rodriguez-Bano, 2002). Colistin is only recommended for patients who have no other treatment alternatives (Cisneros & Rodriguez-Bano, 2002). Rifampicin combined with colistin or sulbactam acts synergistically against MDRAB (Hogg et al., 1998).

7. Mechanisms of antibiotic resistance in *Acinetobacter baumannii*

The general mechanisms of resistance are enzyme-mediated resistance, genetic adaption, efflux pumps and changes in the structure of outer membrane components (Cloete, 2003). Enzyme mediated resistance is the ability of the bacteria to produce enzymes that transform the antibiotics into non-toxic or inactivated forms (Ma et al., 1998). Efflux pumps involve a large number of seemingly unrelated (structurally) compounds, pumped out of the cell, which lowers the concentration of the drug within the cell and therefore prohibits the drug to take proper effect (Nikaido, 1996). Changes in the structure of the outer membrane and its components e.g. porins and alterations in the penicillin binding proteins (PBP's), allows for the cells to develop resistance to antimicrobials on the basis of exclusion because the drugs are no longer able to penetrate the cells and therefore the drugs can't reach their intended site of action in the cell (Cloete, 2003).

Acinetobacter baumannii has become resistant to many classes of antibiotics and is well suited for genetic exchange (Lorenz & Wackernagel, 1994; Metzgar et al., 2004). *Acinetobacter baumannii* are among a unique class of Gram-negative bacteria that are described as "naturally transformable" (Lorenz & Wackernagel, 1994; Metzgar et al., 2004). *Acinetobacter*

strains lack the *mut*S gene, which is part of the mismatch repair system that preserves genetic stability and exhibits increased mutation rates (Young & Ornston, 2001). It is unknown whether *A. baumannii* are naturally competent or whether through the alteration of environmental conditions facilitates pathogenicity or antibiotic resistance gene acquisition (Fournier et al., 2006). The key resistance genes identified were those coding for VEB-1, AmpC, and OXA-10 beta-lactamases, various amino glycoside-modifying enzymes (AME) and those genes encoding for the tetracycline efflux pumps (Fournier *et al*., 2006).

Plasmids, transposons and the bacterial chromosome are involved in antibiotic resistance within *A. baumannii* (Bergogne-Berezin & Towner, 1996). Carbapenemases occurring within *A. baumannii* belong to the class D family of serine-β-lactamases or the imipenemase (IMP)/Verona integrase (VIM) class B family of metallo-β-lactamases (Brown & Amyes, 2006). Imipenem is the most active drug against *A. baumannii* (Cisneros & Rodriguez-Bano, 2002). Resistance to carbapenems is associated with reduced drug uptake due to porin deficiency and reduced affinity for the drug due to modification of the PBP's by mutations (Clark, 1996).

Acinetobacter baumannii's largest virulence island contains genetic elements, which are homologous to the type IV secretion systems of *Legionella* and *Coxiella burnetti* (Goldstein et al., 1983). Over 25 years ago *A. baumannii* was observed to acquire antimicrobial resistance factors through conjugation of plasmids (Goldstein et al., 1983). Transposons are important in the dissemination of genetic determinants of resistance in *Acinetobacter* spp (Devaud et al., 1982; Palmen & Hellingwerf, 1997) and many of the transposons contain integrons, predominantly from class I. Integrons contain an *int* gene and gene cassettes that can be mobilized to other integrons or to secondary sites in the bacterial genome (Poirel et al., 2005).

A multi drug resistant (MDR) phenotype in *A. baumannii* occurs when integron-born resistance determinants acting against different classes of antibiotics co-exist, giving rise to MDR gene cassettes (Seward, 1999; Yum et al., 2002). Insertion sequences (IS), which promote gene expression, have played an important role in explaining the regulation of resistance (Segal et al., 2005). The IS_{Aba1} element found in *A. baumannii* but not in *Enterobacteriaceae* or in *Pseudomonas aeruginosa* (Segal et al., 2005), results in the over expression of Amp C and OXA-51/OXA-69-like beta–lactamases and in decreased levels of susceptibility to ceftazidime and carbapenems (Heritier et al., 2006).

7.1 Oxacillinase (OXA) genes in *Acinetobacter baumannii*

Carbapenemases are classified into four major functional groups (groups 1 to 4) with multiple subgroups of group 2 that are differentiated according to a group specific inhibitor or substrate profiles (Bush et al., 1995). According to this classification scheme carbapenemases are found primarily in group's 2f and 3 (Nordmann et al., 1993; Yang et al., 1990).

Class D carbapenemases are classified into four subgroups (Vahaboglu et al., 2006). Subgroup 1, the OXA-23 group (including OXA-27 and OXA-49), are the plasmid encoded genes (Vahaboglu et al., 2006). The OXA extended spectrum beta-lactamases are able to hydrolyze extended spectrum cephalosporins (Aubert, 2001; Walther-Rasmussen & Hoiby, 2006). The OXA-23 was the first OXA carbapenemase (OXA β-lactamases that inactivate

carbapenems) within *A. baumannii* obtained from a clinical isolate (Aubert, 2001; Walther-Rasmussen & Hoiby, 2006). The OXA-23 genes originated in *A. radioresistens* (Turton et al., 2005). This plasmid-encoded enzyme was found in 1985 in Scotland before the introduction of carbapenems (Paton et al., 1993). It was initially named "*Acinetobacter* resistant to imipenem" (ARI-1) and has been discovered in Brazil, England, Polynesia, Singapore, Korea and China (Brown & Amyes, 2006; Jeon et al., 2005). Subgroup 2 is the OXA-24 group (including OXA-25, OXA-26 and OXA-40), which is chromosomally encoded (Vahaboglu et al., 2006). The OXA-24 carbapenemase has a crystal structure and therefore suggests a novel catalytic role for Tyr112 and Met223 side chains (Santillana et al., 2007). Subgroup 1 and 2 share 60% identity (Heritier et al., 2005b).

Subgroup 3 consists of OXA-51 and its variants, which are chromosomally encoded (Vahaboglu et al., 2006). The OXA-51/69 expression varies according to the presence of IS_{Aba1} (Poirel & Nordmann, 2006). The OXA-51 gene was first detected in Argentina in 2005, within genetically distinct *A. baumannii* isolates (Brown & Amyes, 2005). Subgroup 3 has 56% identity with subgroup 1 and 61% to 62% identity with subgroup 2 (Brown et al., 2005). Subgroup 4 contains OXA-58, which is a plasmid-encoded gene (Vahaboglu et al., 2006) and was first detected in Toulouse (France) in 2003 (Heritier et al., 2005a; Poirel et al., 2005). Subgroup 4 shares less than 50% homology with the other three groups (Poirel et al., 2005). The OXA-58 gene is rapidly disseminating and those isolates, which contain both OXA-51-type and OXA-58 genes, are pandrug-resistant *A. baumannii* (PDRAB) (Coelho et al., 2006). The plasmid borne carbapenemase, OXA-58, was found in France, England, Argentina, Spain, Turkey, Romania, Austria, Greece, Scotland and Kuwait (Coelho et al., 2006; Marque et al., 2005; Pournaras et al., 2006). It is uncertain whether these genes are acquired or occur naturally in *A. baumannii* (Brown & Amyes, 2006). In *A. baumannii* isolates with OXA-51 as the sole carbapenemase, carbapenem resistance was associated with an insertion sequence IS_{AbaI} and it is thought that this might be the promoter for the hyper-production of β-lactamase genes (Turton et al., 2006).

Bacteria producing carbapenemase enzymes have a reduced susceptibility to imipenem (Ambler et al., 1991). However, the minimum inhibitory concentration (MIC) of imipenem can range from mildly elevated to fully resistant (Ambler et al., 1991). Therefore, these β-lactamases may not be recognised following routine susceptibility testing (Ambler et al., 1991). Beta-lactamases have the ability to hydrolyse carbapenems, resist commercially available β-lactamase inhibitors and are susceptible to inhibition by metal ion chelators (Lim et al., 1988). The widespread presence of oxacillinases and their division into distinct subgroups, indicates that these enzymes are an essential component of the genetic makeup of *Acinetobacter* spp (Walther-Rasmussen & Hoiby, 2006). The OXA enzymes as emerging carbapenemases are increasingly associated with outbreaks of *A. baumannii containing OXA-40 and OXA-58* in the United States (Hujer et al., 2006; Lolans et al., 2006). The OXA-51/69-like beta-lactamase is a "naturally occurring" chromosomal enzyme in *Acinetobacter baumannii* and has been found in isolates from four continents (Heritier et al., 2005a).

7.2 Metallo-β-lactamase (MBL) genes in Acinetobacter baumannii

Metallo-β-lactamases form part of the class B β-lactamases, capable of hydrolyzing carbapenems and other β-lactam antibiotics except for aztreonam (Walsh et al., 2005; Walsh,

2005). Class B β-lactamases differ from class A and class D carbapenemases by having a metal ion, zinc, in their active site, which participates in catalysis (Walsh et al., 2005; Walsh, 2005). There are five types of metallo-β-lactamases (MBL's) that have been identified in *A. baumannii* (Brown & Amyes, 2006). The most common metallo-β-lactamases include "Verona integron-encoded metallo-β-lactamases" (VIM), "Imipenem hydrolyzing β-lactamase"(IMP), "German Imipenemase" (GIM), Seoul imipenemase (SIM) and Sao Paulo metallo-β-lactamases (SPM-1) enzymes, which are located on a variety of integron structures and are incorporated as gene cassettes (Brown & Amyes, 2006). The integration of the integron on the plasmids or transposons allows for facilitated transfer between bacteria (Watanabe et al., 1991).

Imipenem (IMP) metallo-β-lactamases were first described in a *P. aeruginosa* strain found in Japan in 1988 (Watanabe et al., 1991). Metallo-β-lactamases is not the predominant carbapenemases found within *A. baumannii* however the following carbapenemases have been described: IMP-1, IMP-2, IMP-4, IMP-5, IMP-6, and IMP-11 (Walsh et al., 2005; Walsh, 2005). The IMP type MBL's have stronger carbapenem-hydrolysing activity than the OXA-type-β-lactamases (Laraki et al., 1999). The VIM, IMP, SPM, and GIM genes are found on cassettes in class 1 integrons, although IMP genes have also been found on class 3 integrons (Collis et al., 2002). Watanabe et al. (1991) reported the detection of IMP-1, located on an integron situated on a conjugative plasmid, in *Serratia marcescens* and other *Enterobacteriaceae* in Japan. The imipenem-hydrolyzing β-lactamase has been detected in rare clinical isolates of *Enterobacter cloacae* in Argentina, the USA and France (Nordmann et al., 1993; Pottumarthy et al., 2003; Radice et al., 2004; Rasmussen et al., 1996).

Imipenem hydrolyzing β-lactamase contains the conserved active site motifs S-X-X-K, S-D-N and K-T-G of the class A β-lactamases (Aubron, 2005; Yu et al., 2006). The carbapenemases have conserved cysteine residues at positions 238 and 69 that form a disulfide bridge (Aubron, 2005; Yu et al., 2006). Genes encoding IMP-2 β-lactamases were found on plasmids in *Enterobacter asburiae* isolated from river water in the US and on plasmids from an *E. cloacae* isolated from China (Aubron, 2005; Yu et al., 2006). The disulfide bond is necessary for the hydrolytic activity and is used to stabilize the enzyme structurally (Majiduddin & Palzkill, 2003; Sougakoff et al., 2002). The mechanism of cleavage of the β-lactam ring is different for MBL's as compared to β-lactamases, however, both gene products still share a unique αββα fold in the active sites of the enzymes (Ullah et al., 1998). The bla_{IMP} is a foreign gene that is introduced from another species of bacteria and *A. baumannii* only retain the gene in environments where there is selective pressure in the form of the presence of imipenem (Takhashi et al., 2000).

Pandrug-resistant *A. baumannii* (PDRAB) are resistant to nearly all the commercially available antibiotics including amikacin, aztreonam, cefepime, ceftazidime, ciprofloxacin, gentamycin, imipenem, meropenem, ofloxacin, ticarcillin-clavulanate and piperacillin-tazobactam (Hsueh et al., 2002). Carbapenem-resistant *A. baumannii* are usually susceptible to ciprofloxacin, ofloxacin, gentamycin or amikacin (Hsueh et al., 2002). Increasing the use of carbapenems and ciprofloxacin has contributed to the development and spread of PDRAB strains (Hsueh et al., 2002).

Verona integron-encoded MBL (VIM-1) was first identified in Italy in 1997 in a *P. aeruginosa* isolate (Lauretti et al., 1999). *Acinetobacter baumannii* containing the VIM-2 gene has been

reported only in Korea (Yum et al., 2002). *Acinetobacter baumannii* isolates producing metallo-β-lactamases from Korea were reported to be incredibly diverse, containing Seoul imipenemase (SIM-1), which is a novel metallo-β-lactamase (Lee et al., 2005).

7.3 Non-enzymatic mechanisms of antibiotic resistance

In *A. baumannii* isolates from Madrid the loss of the 22-kDa and 33-kDa outer membrane proteins combined with the production of OXA-24, resulted in resistance to carbapenems (Bou et al., 2000). A homologue of OprD, a 43-kDa protein was identified in *A. baumannii* (Dupont et al., 2005). The 43-kDa protein is a well studied porin, which is frequently associated with imipenem resistance in *P. aeruginosa* (Dupont et al., 2005). Confirming resistance to imipenem and meropenem in *A. baumannii* is the channel formation of CarO, a 29-kDa outer membrane protein (Limansky et al., 2002; Mussi et al., 2005; Siroy et al., 2005). Reduced expression of PBP-2 within isolates from Seville, Spain explained the resistance of *A. baumannii* to carbapenems (Fernandez-Cuenca et al., 2003).

7.3.1 Efflux pumps as mechanisms of resistance in *Acinetobacter baumannii*

Efflux pumps cause resistance against several different classes of antibiotics and mediate the efflux of compounds that are toxic to the bacterial cell, including antibiotics, in a coupled exchange with protons (Poole, 2005). The distinct families of efflux pumps the major facilitator superfamily, the small multidrug resistance superfamily, the multidrug and toxic compound extrusion superfamily and the resistance-nodulation-cell division family are found in various species of bacteria (Poole, 2005). Over expression of the AdeABC efflux pump, which forms part of the resistance-nodulation-cell division family, confers high-level resistance to carbapenems, together with carbapenem-hydrolyzing oxacillinase (Marque et al., 2005). The mechanism, which controls the expression of the efflux pump, functions as a two-step regulator (adeR) and sensor (adeS) system (Marchand et al., 2004). A single point mutation within the *ade*R and *ade*S genes results in increased expression and increased efflux (Marchand et al., 2004).

8. Resistance of *Acinetobacter baumannii* to various antibiotics

Resistance to aminoglycosides is mediated by aminoglycoside-modifying enzymes (AME's) (Perez et al., 2007). Examples of such enzymes include aminoglycoside phosphotransferases (aph), aminoglycoside acetyltransferases (acc) and aminoglycoside adenyltransferase (aad) (Perez et al., 2007). *Acinetobacter baumannii* have transposon mediated efflux pumps, which involves tetracycline A (Tet) and TetB (Guardabassi et al., 2000). Tetracycline A allows for the efflux of tetracycline, while TetB allows for the efflux of both tetracycline and minocycline (Huys et al., 2005). The other mechanism of resistance to the tetracyclines is due to the ribosomal protection protein (Perez et al., 2007). The ribosomal protection protein is encoded by the tetracycline M gene and protects the ribosome from the action of tetracycline, minocycline and doxycline (Ribera et al., 2003).

Modification in the structure of the DNA gyrase decreases the affinity of the enzyme to quinolones (Seward & Towner, 1998); therefore *A. baumannii* becomes resistant to quinolones (Perez et al., 2007). Modifications of the lipopolysaccharides (LPS's) in A. *baumannii* cause the bacterium to become resistant to polymyxins (Perez et al., 2007).

Modifications to the LPS in *A. baumannii* include acylation, presence of antigens and acidification, which all interfere with the binding of the polymyxins to the cell membrane (Peterson et al., 1987).

9. Spread and control of *Acinetobacter baumannii*

Infection control is critical concerning *A. baumannii* given its ability to cause outbreaks (Boyce & Pittet, 2002; Pittet, 2004). Contact precautions, hand washing and alcohol hand decontamination are rarely applied however are universally encouraged and important (Boyce & Pittet, 2002; Pittet, 2004). However, the applications of meticulous environmental decontamination and aggressive chlorhexidine baths as temporary measures to control outbreaks are the favourable approach (Maragakis et al., 2004; Wilks et al., 2006). However these methods are expensive, labour-intensive and must be clinically proven through trials (Maragakis et al., 2004; Wilks et al., 2006). The key to infection control measures lies within preventing dissemination of MDR clones (Maragakis et al., 2004; Wilks et al., 2006). The use of molecular tools for investigation of outbreaks to establish clonality among isolates allows for a more effective implementation of infection control measures and aids in the identification of environmental sources (Maragakis et al., 2004; Wilks et al., 2006). Polymerase Chain Reaction followed by electronspray ionization mass spectrometry and base composition analysis are used to determine clonality (Ecker et al., 2006; Hujer et al., 2006). Restriction of the use of especially broad-spectrum activity antibiotics is necessary for infection control strategies (Chakravarti et al., 2000; Hughes, 2003). The refinement of genomic and proteomic techniques represents hope for the discovery of new antimicrobials active against MDR organisms and for the development of vaccines (Chakravarti et al., 2000; Hughes, 2003). The success of these and other approaches for the containment of MDR *A. baumannii* depends on the commitment of clinical practitioners, scientists, hospitals and public health administrators and on the support of the informed public (Chakravarti et al., 2000; Hughes, 2003).

10. Diagnosis and detection of *Acinetobacter baumannii*

Monitoring the geographical spread of virulent or epidemic pathogens is achieved through the identification and typing of bacteria (Grundmann et al., 1997). Traditional methods for the identification of *A. baumannii* are unsatisfactory (Gerner-Smidt et al., 1991), due to the difficulty in distinguishing *A. baumannii* from *A. calcoaceticus* phenotypically (Giamarellou et al., 2008). *Acinetobacter baumanni* is predominantly diagnosed from sputum, blood, central venous catheter tips, pleural fluid, wound pus, bronchial washing and urine (Hsueh et al., 2002).

10.1 Direct phenotypic detection of *Acinetobacter baumannii*

Phenotypic methods of detecting *A. baumannii* include growing the isolates on fluorescence-lactose-denitrification media (FLN) in order to determine the amount of acid produced by the metabolism of glucose (http://microbewiki.kenyon.edu/index.php/Acinetobacter baumannii). This method is used to differentiate the respective species within the *Acinetobacter* genus (http://microbewiki.kenyon.edu/index.php/Acinetobacter_baumannii). Crude enzyme extracts and β-lactamase activity assays are other phenotypic methods used to

detect antibiotic resistant strains of *A. baumannii* (Takahashi et al., 2000). Biochemical tests used to differentiate *A. baumannii* from other species of the *Acinetobacter* genus include the following: haemolysis test (-), histamine assimilation test (-), glucose oxidation test, citrate assimilation test (+), gelatin liquefaction test (-) (Prashanth & Badrinath, 2000).

10.1.1 Automated detection of *Acinetobacter baumannii*

A Vitek GNI card (bio Mérieux, France) is used for the detection of carbapenemase activity in clinical isolates. The results of the Vitek test are confirmed using the API 20NE system (bio Mérieux, France) (Clinical and Laboratory Standards Institute, 2009).

10.1.2 Manual methods of detection of *Acinetobacter baumannii*

The E-test (AB Biodisk, Sweden) is used to identify metallo-β-lactamase production by determining the minimum inhibitory concentration (MIC), which allows for the detection of the production of VIM or IMP enzymes (Walsh, 2005). Susceptibility testing can be performed using broth microdilution according to Clinical and Laboratory Standards Institute standards (2009) and the Kirby-Bauer double disk synergy test (Peleg et al., 2005). The disk approximation test with 2-mercaptopropionic acid or EDTA is used to screen for metallo-β-lactamase producers (Arakawa, 2000; Yong et al., 2006). Ethylenediaminetetraacetic acid (EDTA) is a chelator of Zn^{2+} and other divalent cations and therefore inhibits the metallo-β-lactamases that have zinc ions in their active sites (Lim et al., 1988).

The imipenem (IMP)-EDTA double-disk synergy test (DDST) can distinguish metallo-β–lactamase producing from metallo-β–lactamase non-producing Gram-negative bacilli (Lee et al., 2001). However, occasional isolates show false negative results due to a deficiency of zinc within the isolate's active site (Yigit et al., 2001). The test can be improved by using an IMP disk to which 10 µl of 50 mM zinc sulfate (140 µg/disk) has been added, to compensate for the lack of zinc or by using Mueller-Hinton agar to which zinc sulfate has been added to a final concentration of 70 $\mu g.ml^{-1}$ (Yigit et al., 2001).

The Hodge test is a simple method for screening metallo-β-lactamase producing isolates of Gram-negative bacilli (Lee et al., 2001). The Hodge test/cloverleaf test is a microbiological assay of carbapenemase activity, where an extract of the whole cell or the suspected isolates are tested against imipenem on an agar plate (Hornstein et al., 1997). It is unnecessary to test an isolate for a carbapenemase using the modified Hodge test when all of the carbapenems that are reported by a laboratory test are either intermediate or resistant (Clinical and Laboratory Standards Institute, 2009). However, the modified Hodge test is used for infection control and epidemiological purposes (Clinical and Laboratory Standards Institute, 2009). The imipenem disk test is a poor screening method for carbapenemases (Clinical and Laboratory Standards Institute, 2009).

10.2 Molecular detection of *Acinetobacter baumannii*

Molecular methods based on PCR for the detection of carbapenemase producing genes are used due to the problems with the direct phenotypic detection methods e.g. difficulty in distinguishing between species of the *Acinetobacter* genus (Vaneechoutte, 1996). The molecular methods include a PCR with primers for detecting OXA-23, OXA-24, OXA-51,

OXA-58, IMP-1, IMP-2, IMP-4, VIM-1, VIM-2, SPM-1, GIM-1, and SIM-1 genes can be used to detect all families and subgroups of the presumed carbapenemases (Petropoulou et al., 2006). The genotypic tests used to determine the clonal relatedness of the isolates include, Random amplified polymorphism DNA (RAPD PCR)-fingerprinting with the primers M13, Enterobacterial Repetitive Intergenic Consensus (ERIC2) and Pulsed Field Gel Electrophoresis (PFGE) using the *ApaI* enzyme can be performed (Seifert et al., 2005). The main advantages of the molecular techniques in comparison with the traditional phenotypic methods are high reproducibility and applicability to a wide variety of bacteria and time saving (Grundmann et al., 1997).

10.3 Indirect diagnosis of *Acinetobacter baumannii*

Gram-negative bacteria contain lipopolysaccharides (LPS's) on their outer membranes, which consist of covalently linked lipid A (anchors the LPS into the outer membrane) and the core polysaccharide (O-polysaccharide or O-antigen), which is linked to the lipid A (Pantophlet et al., 1999). The type of LPS found within *A. baumannii* has the smooth or S-form phenotype and can be used in clinical microbiology laboratories for clinical research purposes (Pantophlet et al., 1999).

11. Materials and methods

Ninety-seven imipenem/meropenem resistant *A. baumannii* isolates were collected between March and April 2009 from a Tertiary Academic hospital. These isolates were all given a unique number. The *A. baumannii* isolates were analysed by the Diagnostic Division of the department of Medical Microbiology, National Health Laboratory service (NHLS) at the University of Pretoria. The isolates were identified as *A. baumannii* and underwent susceptibility testing. The Vitek 2 (bioMérieux, France) automated system was used to phenotypically test for the presence of carbapenemases within the *A. baumannii* isolates. Ninety-seven imipenem/meropenem resistant isolates were streaked out onto 5% sheep blood agar plates (Diagnostics Media Products, NHLS, South Africa). The plates were incubated (Horo incubator) overnight at 37ºC. Gram-staining was performed for each isolate. (2002). Brain-Heart infusion broth (Biolab, Wadeville, South Africa) was prepared and aliquoted into Bijou culture bottles before sterilization. The broth bottles were inoculated from overnight plate cultures of *A. baumannii* grown on 5% sheep blood agar (Diagnostic Media Products, NHLS, South Africa) by adding 3 to 4 colonies of an isolate into the broth. The inoculated broths were incubated in a Labcon shake incubator at 37°C overnight. A volume of 900 µl of the inoculated turbid broth and 900 µl of sterile glycerol was added to a sterilized cryotube and were stored at -70°C. The CLSI (2009) guidelines for the performance of the modified Hodge test and for the double disk synergy test were followed for the detection of carbapenemase production.

A MagNA Pure Compact Nucleic Acid Isolation Kit 1 (Roche, Germany) was used to perform automated whole cell DNA extraction according to the manufacturer's guidelines. A volume of 400 µl of each of the *A. baumannii* broth culture samples was added to a Magna Pure sample tube for automated DNA extraction. Sealed cartridges with the necessary reagents were added to each lane. The purified nucleic acids (100 µl of pure *A. baumannii*

DNA) were eluted and stored at -20°C for further analysis. The Nanodrop Spectrophotometer ND-1000 instrument was used to measure the DNA concentration for each of the samples.

Two multiplex PCR assays: 1) Multiplex PCR I reaction of OXA-23, OXA-24, OXA-51 and OXA-58 genes (Woodford et al., 2006) and 2) Multiplex PCR II reaction of IMP, VIM, GIM-1, SPM-1 and SIM-1 genes (Ellington et al., 2007) were performed using the QIAGEN Multiplex PCR 1000 kit (Promega, Madison, USA), which was set up according to the manufacturer's guidelines. The QIAGEN Multiplex PCR 1000 kit contains Multiplex PCR Master mix, RNAse free water and Q solution. The thermocycling was performed using the Eppendorf, Mastercycler epgradient S (Hamburg, Germany). The DNA gel electrophoresis (Elite 300 power pack, Wealtec, South Africa) was performed on a 2% agarose gel (Whitehead Scientific, Brackenfell, Cape Town), which contained 0.5 $\mu g.ml^{-1}$ ethidium bromide (Promega, Madison, USA). The loading dye used was Fermentas 6X orange loading dye solution (Fermentas UAB, Lithuania). The ready to use 100 bp ladder (Promega, Madison, USA) was used as a molecular size marker. A 10% solution of TBE buffer 10X (Promega, Madison. USA) was used for the preparation and running of the gels.

12. Prevalence of antibiotic resistance genes in *Acinetobacter baumannii* isolates in a clinical setting in the Pretoria area, South Africa

The origins of the *Acinetobacter baumannii* isolates collected in this study were 58% (56/97) from sputum specimens, 7% (7/97) from urine specimens, 11% (11/97) from blood cultures and 24% (23/97) from diverse specimens. The *A. baumannii* isolates collected for this study were both imipenem and meropenem resistant with a minimum inhibitory concentration (MIC) of >=16. The 97 *A. baumannii* isolates were subjected to susceptibility testing using the Vitek 2 instrument. The panel consisted of 18 antibiotics to determine the overall pattern of resistance. The selection of *A. baumannii* isolates used in this study was based on the Vitek 2 instrument. Both imipenem and meropenem resistant *A. baumannii* isolates were included in this study. All of the *A. baumannii* isolates showed 100% resistance to the following antibiotics in the panel: ampicillin; amoxicillin/clavulanic acid; cefuroxime; cefuroxime axetil; cefepime; imipenem; meropenem; nitrofurantoin and trimethoprim/sulfamethoxazole. The *A. baumannii* clinical isolates were all susceptible (0% resistance) to colistin (Table 2).

The Hodge test showed that 74% (72/97) of the *A. baumannii* isolates were positive for carbapenemase production and 26% (25/97) of the *A. baumannii* isolates were negative for carbapenemase production (Figure 2). These results are similar to the findings of the study conducted in Korea by Lee et al. (2003), which reported a prevalence of 66% positive for carbapenemase production, 26% negative for carbapenemase production and 8% data unknown.

The Cloverleaf or Hodge test is cumbersome and imperfect. False positives occur due to AmpC and impermeability, not due to β-lactamase production. Weak false positives occur due to AmpC hyperproducers. AmpC hydrolysing β-lactams are produced by Gram-negative bacteria [Presentation by David Livermore on "Detecting carbapenemases" at the 49th Interscience conference on antimicrobial agents and chemotherapy (ICAAC)]. Some *A. baumannii* isolates are resistant to ertapenem, but are rarely resistant to any of the other

Antibiotic Tested	Percentage resistance
Ampicillin	100% (97/97)
Amoxicillin/Clavulanic acid	100% (97/97)
Piperacillin/Tazobactam	99% (96/97)
Cefuroxime	100% (97/97)
Cefuroxime Axetil	100% (97/97)
Cefotaxime	99% (96/97)
Ceftazidime	49% (48/97)
Cefepime	100% (97/97)
Imipenem	100% (97/97)
Meropenem	100% (97/97)
Amikacin	25% (24/97)
Gentamicin	89% (86/97)
Tobramycin	5% (5/97)
Nalidixic acid	95% (92/97)
Ciprofloxacin	91% (88/97)
Nitrofurantoin	100% (97/97)
Colistin	0% (0/97)
Trimethoprim/sulfamethoxazole	100% (97/97)

Table 2. Antibiotic resistance patterns in *Acinetobacter baumannii* isolates from a Tertiary Academic Hospital

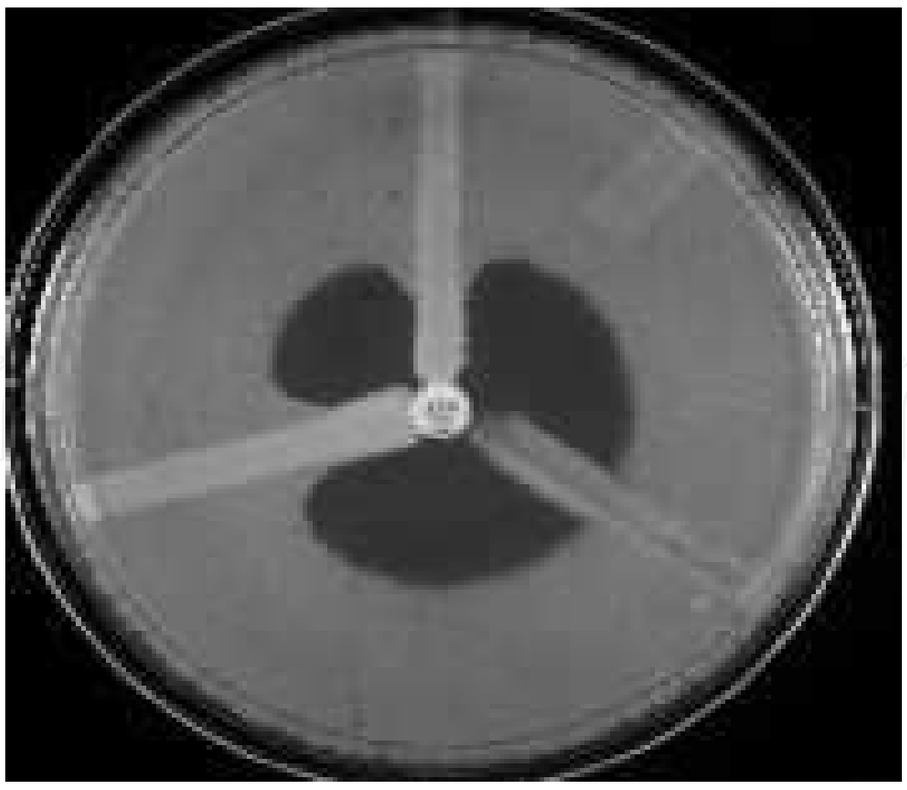

Fig. 2. Hodge or cloverleaf test of three *Acinetobacter baumannii* isolates

carbapenems. The Hodge test is very time consuming to set up and the reading of the results is subjective. Some strains produce bacteriocins, which kill the indicator organism [Presentation by David Livermore on "Detecting carbapenemases" at the 49th Interscience conference on antimicrobial agents and chemotherapy (ICAAC)]. Beta-lactamase production affects the porins of the outer membrane, thus making *A. baumannii* impermeable to antibiotics and therefore resistant to antibiotics e.g. carbapenems [Presentation by David Livermore on "Detecting carbapenemases" at the 49th Interscience conference on antimicrobial agents and chemotherapy (ICAAC)]. The class of carbapenemase cannot be determined by the results of the Modified Hodge test. Some isolates show a slight indentation, but do not produce carbapenemase (Standard operating procedure of the Department of Health and Human Services, Centres for Disease Control and Prevention: "Modified Hodge Test for Carbapenemase Detection in Enterobacteriaceae").

The double disk synergy test showed that 33% (32/97) of the *A. baumannii* isolates were susceptible to both ertapenem and EDTA and 19% (18/97) of the isolates did not grow (Figure 3). A prevalence of 45% (44/97) was recorded for *A. baumannii* isolates that were ertapenem resistant and EDTA susceptible and a prevalence of 3% (3/97) was recorded for both ertapenem and EDTA resistance. These findings are lower than the results of the study conducted in Korea by Lee et al. (2003), which reported a prevalence of 94% (75/80) of *A. baumannii* isolates susceptible for both imipenem and EDTA; all the isolates grew in that study; 5% (5/97) of the *A. baumannii* isolates were resistant to imipenem and susceptible to EDTA and 0% (0/97) isolates were resistant to both imipenem and EDTA.

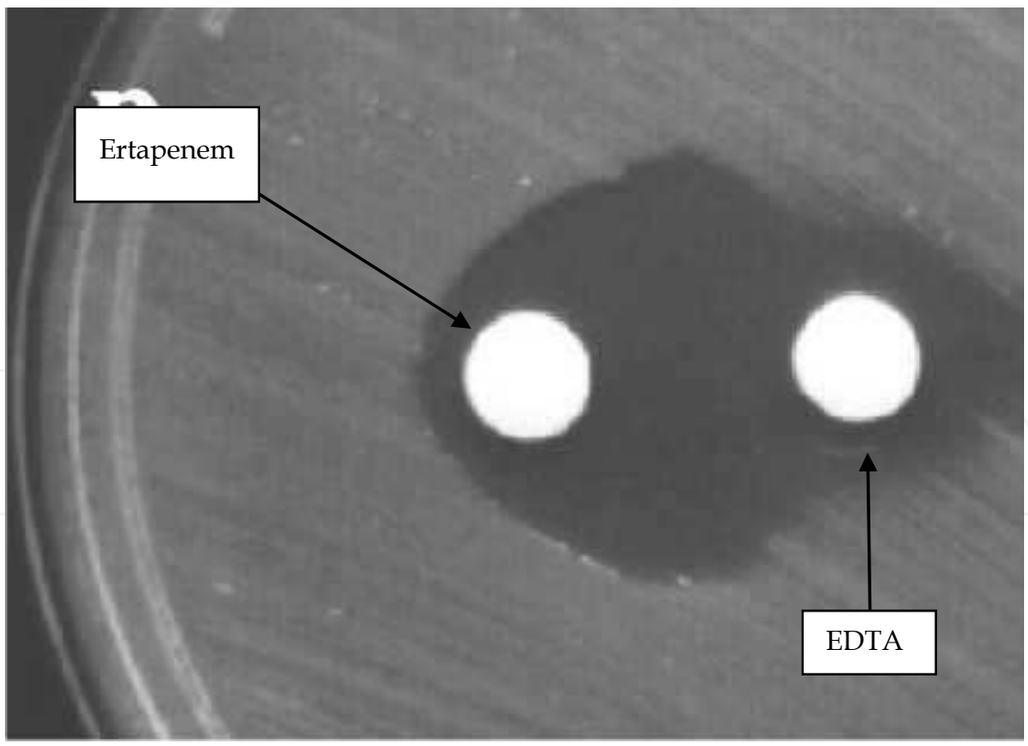

Fig. 3. Double disk synergy test of one *Acinetobacter baumannii* isolate

EDTA permeabilizes the bacterial cell, is a chelator of zinc and disrupts OXA dimers, which may be stabilized by zinc [Presentation by David Livermore on "Detecting carbapenemases"

at the 49th Interscience conference on antimicrobial agents and chemotherapy (ICAAC)]. Therefore EDTA disrupts the function of the carbapenemase producing genes and hence the *A. baumannii* isolates are more susceptible to ertapenem in the presence of EDTA than without EDTA. The discrepancies in the results of this study compared to the results of the study conducted by Lee et al. (2003) were due to the use of different carbapenems. Ertapenem was used in this study, while Lee et al. used imipenem in their study conducted in 2003. Imipenem disks perform poorly as a screen for carbapenemases (Clinical and Laboratory Standards Institute, 2009) and thus ertapenem was used in this study.

The *A. baumannii* clinical specimens were cultured and two different multiplex PCR assays were performed on the extracted DNA sample of each isolate. The first multiplex PCR assay (Multiplex PCR I) was performed to screen for the presence of the OXA-group genes (OXA-23, OXA-24, OXA-51 and OXA-58). The second Multiplex PCR assay (Multiplex PCR II) screened for the presence of the Metallo-β-lactamase genes (IMP, VIM, SIM, SPM and GIM). Multiplex PCR I showed that 80% (78/97) of the *A. baumannii* isolates were positive for OXA-51, 52% (50/97) were positive for OXA-23, 1% (1/97) were positive for OXA-58 and 2% (2/97) were positive for OXA-24 (Figure 4). Figure 5 showed the gel electrophoresis pattern of the OXA-51, OXA-23, OXA-58 and OXA-24 genes.

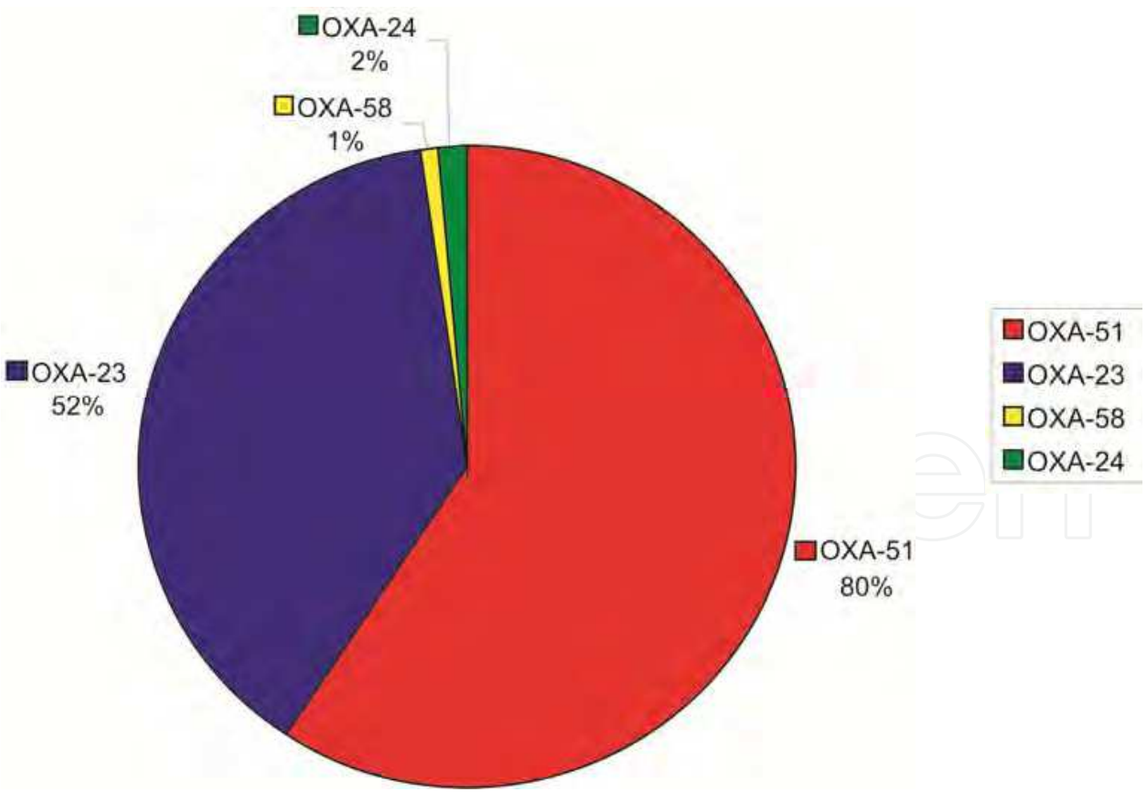

Fig. 4. Pie chart showing the results of the Multiplex PCR I for the prevalence of the OXA genes in the 97 *Acinetobacter baumannii* isolates obtained from a Tertiary Academic Hospital

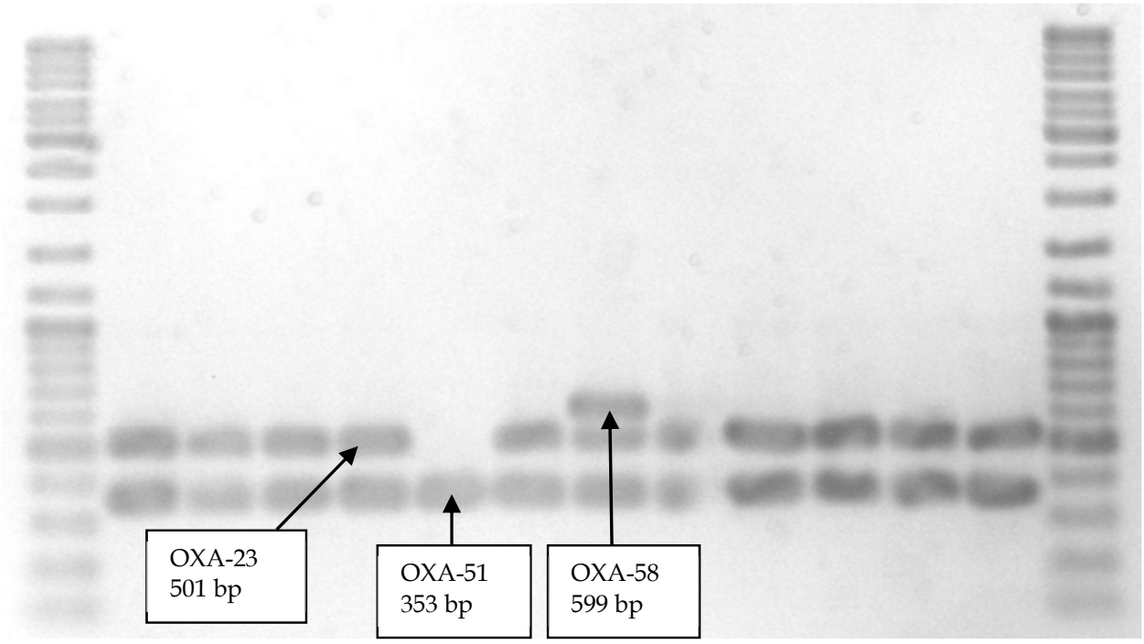

Fig. 5. Results obtained after Multiplex PCR I was performed, using a 2% agarose gel for the detection of OXA genes in the *Acinetobacter baumannii* isolates. Lanes 2, 3, 4, 5, 7, 9, 10, 11, 12 and 13 were positive for both OXA 51 and OXA-23. Lane 6 was positive only for OXA-51. Lane 8 was positive for OXA-23, OXA-51 and OXA-58. Lanes 1 and 14 contain the molecular weight markers (100 bp DNA ladder).

The multiplex PCR I results showed there were four distinctive strains of *A. baumannii* circulating in a Tertiary Academic hospital in Gauteng, South Africa. The first group of strains were positive for both OXA-51 and OXA-23 (52%). The second group of strains was positive for OXA-51 (26%) alone. The third group of strains were positive for both OXA-51 and OXA-24 (2%) and the fourth group of strains were positive for both OXA-51 and OXA-58 (1%) (Figure 6).

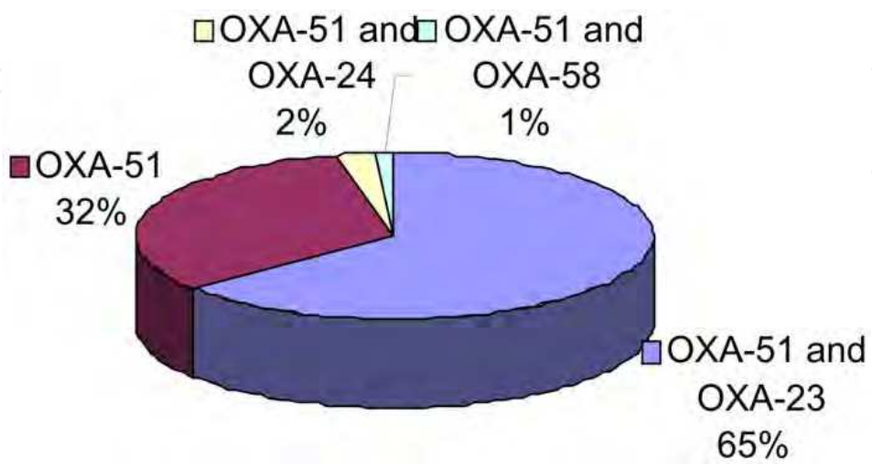

Fig. 6. Pie chart showing the results of the Multiplex PCR I for the four strains of *Acinetobacter baumannii* circulating in a Tertiary Academic Hospital

OXA-51 is an abiquitous or naturally occurring gene within *A. baumannii* (Merkier & Centron, 2006). OXA-51 is a chromosomally located gene, which needs to be regulated upstream by IS_{Aba1} to provide resistance [Presentation by David Livermore on "Detecting carbapenemases" at the 49th Interscience conference on antimicrobial agents and chemotherapy (ICAAC)]. The prevalence of OXA-51 in clinical isolates of *A. baumannii* in this study was 80% (78/97). This figure is lower than the findings of a study conducted in Iran by Feizabadi and colleagues (2008), which reported a 100% prevalence of the OXA-51 gene in clinical isolates of *A. baumannii*.

The prevalence of OXA-23 in clinical isolates of *A. baumannii* in this study was 52% (50/97). This finding was similar to the OXA-23 prevalence of 66.5% in the Asia Pacific nations (India, China, Thailand, Singapore, Hong kong and Korea) (Mendes et al., 2009). However, based on other studies conducted in Iran by Feizabadi and colleagues (2008) who reported a prevalence of 36.5% in clinical isolates of *A. baumannii* the prevalence of OXA-23 varies worldwide. OXA-23 forms part of the class D metallo-β-lactamases and is an acquired carbapenemase gene, thus a varied prevalence is observed as not all *A. baumannii* isolates will obtain the gene compared to OXA-51 which is a chromosomal carbapenemase gene occuring naturally in *A. baumannii* (Merkier & Centron, 2006). The carbapenemase gene occurs in widespread clones and contributes to the multidrug resistant nature of *A. baumannii* [Presentation by David Livermore on "Detecting carbapenemases" at the 49th Interscience conference on antimicrobial agents and chemotherapy (ICAAC)].

The OXA-24 genes also form part of the class D metallo-β-lactamases and are acquired carbapenemase genes, which occur within widespread clones [Presentation by David Livermore on "Detecting carbapenemases" at the 49th Interscience conference on antimicrobial agents and chemotherapy (ICAAC)]. The prevalence of OXA-24 in A. *baumannii* in this study was 2% (2/97). This finding was similar to the OXA-24 prevalence of 5.6% in *A. baumannii* clinical isolates from a study conducted in Thailand, Taiwan and Indonesia in 2008 (Mendes et al., 2008). However, a prevalence of 26% of OXA-24 was reported in the study conducted by Feizabadi and colleagues in Iran in 2008. The varied prevalence results are due to OXA-24 being an acquired gene within *A. baumannii* and thus not all isolates will contain the gene (Merkier & Centron, 2006).

The prevalence of OXA-58 in clinical isolates of *A. baumannii* in this study was 1% (1/97). This finding was lower than the OXA-58 prevalence of 15% in *A. baumannii* isolates in the study conducted by Feizabadi and colleagues in Iran in 2008. The differences in the results of this study and other studies are due to OXA-58 being an acquired carbapenemase gene and the presence of this gene within widespread clones Presentation by David Livermore on "Detecting carbapenemases" at the 49th Interscience conference on antimicrobial agents and chemotherapy (ICAAC)].

No metallo-β-lactamase genes were detected in any of the *A. baumannii* isolates. IMP, VIM, SIM, SPM and GIM genes belong to the class B metallo-β-lactamases and are acquired carbapenemase genes. IMP (primarily detected in South Korea) and VIM (previously detected in China) are the two metallo-β-lactamase genes, which are the most frequently detected genes in *A. baumannii* isolates (Coelho et al., 2006). The prevalence of the metallo-β-lactamase genes is generally low within *A. baumannii* isolates as illustrated in a study by Mendes and colleagues (2009) where the prevalence was 0.8% in Taiwan. VIM, SIM, SPM, IMP and GIM have not been

detected in South Africa yet. Therefore the results of this study compared with the literature in that the selected *A. baumannii* isolates were negative for all metallo-β-lactamase genes.

13. Conclusions

Acinetobacter baumannii is an important opportunistic pathogen and causes a variety of nosocomial infections especially within the ICU of the Tertiary Academic Hospital in Gauteng, South Africa. The results of the phenotypic analysis in the form of the Hodge test and Double disk synergy test were similar to the results obtained from the study conducted by Lee et al. in Korea in 2003. The discrepancies with the results of the two studies can be largely due to the use of different carbapenem antibiotic disks. The Hodge test is imperfect as false positives occur due to AmpC production and impermeability of the bacterium to antibiotics due to β-lactamase ability to affect the porins of the outer membrane.

After completion of this study it is evident that the OXA group of genes (class D carbapenemases) are a problem in clinical isolates of *A. baumannii* from the Tertiary Academic Hospital. It was found that OXA-51 genes (80%) and OXA-23 genes (52%) were highly prevalent in this study and these prevalence rates were similar to the worldwide prevalence of OXA genes, which are widespread in *A. baumannii* throughout the world (Feizabadi et al., 2008). Metallo-β-lactamase (MBL) genes were not prevalent in the selected clinical isolates of *A. baumannii* due to the contained spread of the genes and thus no metallo-β-lactamase has been detected in South Africa thus far. According to Livermore molecular tests are definitive, but a few isolates with strong carbapenemase activity were negative in all molecular tests [Presentation by David Livermore on "Detecting carbapenemases" at the 49th Interscience conference on antimicrobial agents and chemotherapy (ICAAC)].

The multiplex PCR assays proved to be a rapid technique for antimicrobial susceptibility testing, however, there is much work to be done in order to investigate the possibilities of multiplex PCR assays as an alternative to current antimicrobial susceptibility testing. Continuous research and surveillance is necessary to monitor the prevalence of antibiotic resistance genes associated with *A. baumannii* in clinical settings. The ability of *A. baumannii* to grow in biofilms poses a threat concerning the possibilities of the spread of both the bacteria and the antibiotic resistance genes, which should be investigated in future research.

14. References

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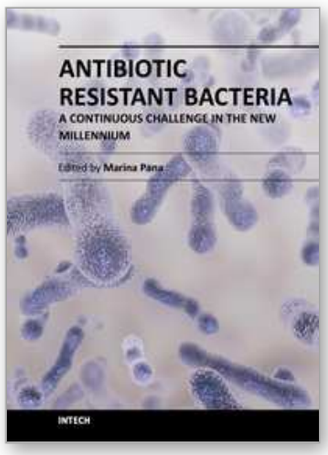

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Antibiotic-resistant bacterial strains remain a major global threat, despite the prevention, diagnosis and antibiotherapy, which have improved considerably. In this thematic issue, the scientists present their results of accomplished studies, in order to provide an updated overview of scientific information and also, to exchange views on new strategies for interventions in antibiotic-resistant bacterial strains cases and outbreaks. As a consequence, the recently developed techniques in this field will contribute to a considerable progress in medical research.

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