THE DETERMINATION AND CHARACTERISATION OF BETA LACTAM RESISTANCE IN CLINICAL ISOLATES OF *ACINETOBACTER BAUMANNII*

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Thesis Presented for the Degree of

MASTERS OF SCIENCE IN MEDICINE

Division of Medical Microbiology

Faculty of Health Sciences

University of Cape Town

February 2002

Dedicated with love to my parents,

Mirza and Nazeema Zavahir

DECLARATION

I, **Minha Shabnam Zavahir**, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise), and that neither the whole nor any part of it has been, is being, or is to be submitted for any other degree at this or any other university.

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ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Gay Elisha for the time and that you have spent with me and for the effort that you have put in during the write up of my thesis.

I would secondly like to thank Dr. Jenny Ramesar for her invaluable input and support; the countless hours she put into helping me get through my Masters programme.

I would like to thank the members of the Medical Microbiology team for the unfailing support and assistance during the duration of my course; Zeino, Karen, Ros, Zuleika, Elton, Rhett, Gavin and Robin for their assistance and advice.

I would like to thank the board of FIP (The Hague) for the Fellowship that was bestowed upon me for the second year of my Masters.

I would like to thank all those who have played a vital role in getting me through this degree, but especially my mother, father and brother for their unconditional and unwavering support.

BRIEF CONTENTS

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ABBREVIATIONS

A.	*Acinetobacter*
amps	amperes
Amp	ampicillin
Asp	aspartate
β	beta
bp	base pair(s)
°C	degrees Celsius
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
E.	*Escherichia*
EDTA	ethylene diamine tetra-acetic acid
EtBr	ethidium bromide
EtOH	ethanol
g	gram(s)
Gln	glutamine
Glu	glycine
His	histidine
H_20	water
hr	hour(s)
Ile	Isoleucine
kb	kilobase(s)
M	molar
MCS	multiple cloning site
mg	milligram(s)
$MgCl_2$	magnesium chloride

MIC(s)	minimum inhibitory concentration(s)
min	minute(s)
µl	microlitre(s)
ml	millilitre(s)
mM	millimolar
Na	sodium
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram(s)
nt	nucleotide(s)
O_2	oxygen
OD	optical density
O/N	overnight
%	percent
PCR	polymerase chain reaction
pH	percent hydrogen
pmol	picomole(s)
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second(s)
Ser	serine
spp.	species
ssp.	subspecies
TAE	Tris-acetate EDTA buffer
TE	Tris-EDTA buffer

U	enzyme unit
UV	ultraviolet
V	voltage
v/v	volume per volume
W	watts
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-Galactosidase

ABSTRACT

Strains of *Acinetobacter baumannii* (*A. baumannii)* were isolated from patients in disparate wards at Groote Schuur Hospital. *A. baumannii* are opportunistic nosocomial pathogens that are gaining widespread interest from scientists globally. They are responsible for infections in immunocompromised patients and those receiving intensive care. *A. baumannii* infections can result in pneumonia, bacteraemia and in rare cases meningitis. For the past four years, beta lactams have been the choice of treatment for these infections at Groote Schuur Hospital. The emergence of resistance is a cause for concern, as the choice for treatment becomes more limited. Ten resistant isolates, one intermediate resistant isolate and one susceptible isolate were included in this study. The resistance to piperacillin-tazobactem was taken as an indication of beta lactam resistance, following which the strains were subjected to further antibiotic susceptibility testing. The resistant strains were resistant to co-amoxyclav, amoxycillin, cefuroxime, cefotaxime, ceftazidime and piperacillin. Poly-GTG typing was carried out to determine the epidemiology of the isolates. Poly-GTG typing determined that all the clinical strains (12/12) had a common origin, suggesting organism spread. Iso-electric focussing showed the presence of a TEM-type beta lactamase only (pI 5.3) in both the resistant test isolate (Strain 2) and sensitive test isolate (Strain S). SHV related genes were not detected following PCR assays (0/12).TEM related genes were detected via PCR in 10/12 strains tested. No TEM genes were detected in test strains 3 and 9. Amino acid sequence analysis of the structural region of the TEM β lactamase showed two previously undesribed mutations: At codon 205, H→Q and at codon 213, E→D . These correspond to His→Gln and Glu →Asp respectively. An AmpC β lactamase was detected in all the strains (12/12) suggesting that the AmpC β lactamase was intrinsic to *A. baumannii. E.coli* (p101) carrying the *ampC* gene was found to confer resistance to amoxycillin, co-amoxyclav, piperacillin, pipercillin-tazobactem and cefuroxime but not ceftazidime. Outer membrane protein (OMP) analysis showed the presence of two porins: a 40kDa porin in resistant strain 2 and a 35 kDa porin in the sensitive strain. In addition, OMPs of size 55kDa and 67kDa respectively were detected in the intermediate resistant strain and resistant strain 2, which formed part of a transport system.

CHAPTER 1

INTRODUCTION

1.1 *Acinetobacter* spp.: A brief overview

Over the past 30 years, a number of nosocomial pathogens have been drawing attention from clinicians. Amongst these, the multiple antibiotic-resistance displaying Gram-negative bacteria have proven to be the most problematic (Bergogne-Bérézin *et al.*, 1996). The *Acinetobacter* genus as described by Brisou and Prevout (1954) is one such example of these Gram-negative cocco-bacilli (Henriksen, 1973). These saprophytic bacteria are ubiquitous, non-fastidious and have been shown to utilise a wide range of substrates for growth (Juni, 1978). Thus, they are able to survive in relatively harsh conditions, such as inanimate sources in hospitals (Bergogne-Bérézin *et al.*, 1996).

The past few decades have seen hospital-acquired infection gaining widespread importance on a global scale. In the last twenty years, the genus *Acinetobacter*, specifically the species, *Acinetobacter baumannii (A. baumannii)* has emerged as an opportunistic nosocomial pathogen and has been implicated in a number of serious infections in patients, especially those receiving intensive care (Bergogne-Bérézin and Towner, 1996). Over a decade ago, The Centers for Disease Control (1987) reported that infections due to *Acinetobacter* spp. were quite uncommon, accounting for <1% of the total infections in most hospitals. In 1999, 10% of nosocomial infections were attributed to *Acinetobacter* spp (Bergogne-Bérézin, 2000). Recently, investigations have shown that *Acinetobacter* spp. have contributed to almost 30% of infections, second only to *Pseudomonas* spp. infections in some hospitals (Eltahawy and Khalaf, 2001).

The rise in frequency of *Acinetobacter* infections is a growing concern amongst clinicians (Chang *et al.*, 2000). Previously, *Acinetobacter* spp. were clinically insignificant bacteria, which came to fill the void created by the elimination of other bacteria following successful drug therapy and thus, the emergence of *Acinetobacter* spp. infections has

resulted from the selective pressure exerted by the widespread use of antibiotics. The concomitant increase of *Acinetobacter spp.* infections with its widespread antibiotic resistance has lead to a clinical awareness of this genus (Towner, 1996). It has been noted that treatment of this opportunistic pathogen is difficult and is complicated by its multi-drug resistance to the major antibiotics and hence, various combinations of therapy have been sought for effective treatment (Bergogne- Bérézin and Towner, 1996).

1.2 Taxonomy

It is important to note that a bacterial species needs to be identified and classified correctly before it can be implicated in disease, identified as a pathogen or particularly pathogenic (Bergogne- Bérézin, 1996). In the case of *Acinetobacter,* this process of classification has been a long one. *Acinetobacter* has been through significant taxonomic changes over the years. They have been classified under at least 15 different genera including *Bacterium* (Shaub and Hauber, 1948) *Achromobacter*, *Alcaligenes*, *Neisseria*, *Diplococcus*, *Micrococcus*, *Cytophyga* (Juni, 1972) , *Mima*, *Herellea* (De Bord, 1939) and *Moraxella* (Piechaud *et al.,* 1956).

The classification of *Acinetobacter* comprising its own genus was initially conceived by French microbiologists, Brisou and Prevout in 1954. This classification included Gram-negative, non-motile oxidase-positive (*Moraxella*) and oxidase-negative saprophytes, which were distinguished from the other bacteria by the absence of pigmentation (Ingram and Shewan, 1960). Baumann *et al.* (1968) carried out nutritional studies that clearly showed that the oxidase-positive strains differed significantly from the oxidase negative strains. With this new data in hand, following the Subcommittee on Taxonomy of Moraxella and Allied Bacteria in 1971, it was ' d to confine the *Acinetobacter* genus to oxidase negative strains only (Lessel, 1971). Transformation tests carried out by Juni (1972) served only to confirm the recent re-classification.

Initially, according to the Bergey's Manual of Systematic Bacteriology, the genus *Acinetobacter* was classified in the family *of Neisseriaceae* (Juni, 1984) and comprised

only one species, *A. calcoaceticus* and two varieties, var. *anitratus* (formerly *Herellea vaginicola*) and var. *lwoffii* (formerly *Mima polymorpha*). The *Acinetobacter* genomic species was further defined and differentiated into the current classification by Bouvet and Grimont in 1986. This was reinforced by Rossau *et al.* (1991), who proposed changes based on recent taxonomic developments, suggesting that the members of the genus should be classified into a new family *Moraxellaceae,* which includes *Moraxella*, *Acinetobacter* and *Psychrobacter* and other related organisms. This move was based on 16S rRNA studies and RNA: DNA hybridisation studies. It is in this new family that *Acinetobacter* still finds itself.

Wayne *et al.* (1987) proposed a formal definition of species which stated, that a species should include strains with approximately 70% or greater DNA: DNA relatedness. Modern methods of taxonomy and identification such as DNA hybridisation, genetic transformation, rRNA sequence data and biochemical studies have also contributed to the classification studies (Bouvet and Jeanjean, 1989; Nishimura *et al.,* 1988; Bouvet and Grimont, 1986). Based on Wayne's definition as well as the additional criteria mentioned above, the genus *Acinetobacter* has been classified into 19 different DNA-DNA homology groups or genomic species. Of the 19 different genomic groups, 10 have been designated formal names: *A. calcoaceticus*, *A. baumannii*, *A. junnii, A. haemolyticus, A. lwoffii, A. radioresistams, A. johnsonii, A.venetianus, A. ursingii and A. schindleri* (Nemec *et al.*, 2001) The remaining genomic species have not yet been assigned a name and are still designated numerically.

Different laboratories have carried out extensive studies in order to identify and classify the different *Acinetobacter* strains. A definitive numbering scheme was used initially and Table 1.1 shows a comparative delineation of the *Acinetobacter* genomic groups by these different laboratories (Towner, 1996). Most of the species have been identified and classified into the same group, however, as can be seen from the table, there are minor discrepancies. For the most part, the species definitions from the different laboratories correlate well. Interestingly, because of its frequent isolation as a clinical cluster, Acinetobacter groups 1, 2 and 3 (Bouvet and Grimont, 1986) and group 13 (Tjernberg

and Ursing, 1989) are sometimes referred to by some research groups as "*A.calcoaceticus-A.baumannii* complex" (Gerner-Smidt *et al.,* 1991). Delineation of the species is still an ongoing process.

The table below shows the formal designations as well as the numerical designations of some of the various species of *Acinetobacter* (adapted from Towner, 1996).

Table 1.1 Comparison of the delineation of *Acinetobacter* genomic species by different laboratories

Species name	Genomic species numbering according to References 1 and 2	Reference 3	References 4 and 5	Type Strain
A. calcoaceticus	1	1	1	ATCC23055
A. baumannii	2	2	1	CIP 70.34
	3	3	NT	ATCC 19004
	UG	13	NT	ATCC 17903
A. haemolyticus	4	4	4	ATCC 17906
A. junii	5	5	NT	ATCC 17908
	6	6	4	ATCC 17979
A. johnsonii	7	7	3	ATCC 17909
A. lwoffii	8	8	2	ATCC 15309
	9	8	NT	ATCC 9957
	10	10	UG	ATCC 17924
	11	11	UG	ATCC 11171
A. radioresistens	(12)*	12	UG	IAM 13186
	13	14	5	ATCC 17905
	14	NT	NT	Bouvet 382
	15	NT	NT	Bouvet 240
	16	UG	NT	ATCC 17988
	17	NT	NT	Bouvet 942
	NT	15	NT	Tjernberg 151a

Note: UG= ungrouped; NT=not tested; * unpublished result

Reference: (1) Bouvet and Grimont (1986); (2) Bouvet and Jeanjean (1989); (3) Tjernberg and Ursing (1989); (4) Nishimura *et al.,* (1987) and (5) Nishimura *et al.,* (1988)

1.3. Biology of *Acinetobacter*

The essential features of the genus have been defined clearly (Bergogne- Bérézin and Towner, 1996). The *Acinetobacter* genus (Brisou and Prevout, 1954) comprises a ubiquitous group of Gram-negative bacteria. They appear under the microscope as short plump coccobacilli. They have a DNA G: C content of between 39-47% and are non-fermentative, non-fastidious saprophytes. These bacteria once thought to be predominantly soil organisms, have recently, more importantly, been found in clinical environments. Most of the clinical isolates will grow at 37°C (Bergogne- Bérézin and Towner, 1996), whereas the environmental strains prefer 20-30 °C. All *Acinetobacter* strains are oxidase-negative and are catalase-positive and it is this oxidase-negative reaction that serves to distinguish these bacteria from other genera in the family of *Moraxellacaea* (Rossau *et al.,* 1991). Most strains of *Acinetobacter* can grow in minimal media containing only a single carbon source or ammonium and nitrate salts. Its ability to utilise a variety of carbon sources as substrates as well as its ability to survive in dry and inanimate surfaces in the presence of limited nutrients have contributed to its success as a nosocomial pathogen.

1.4 Habitats

Acinetobacter spp. can be categorised on the basis of their habitat: environmental or clinical (Bouvet and Grimont, 1987) and the *Acinetobacter* spp. have been isolated from both soil and water samples under the correct growth conditions (Baumann, 1968; Joly-Guillou and Brun-Buisson,1996). *Acinetobacter* strains have been isolated from the natural environment, as mentioned previously, as well as from inanimate sources such as ventilator shafts in which the conditions are not conducive to survival (Bergogne-Bérézin, *et al.,* 1996).

Isolates of *A. baumannii* are the predominant clinical species and account for up to 90% of all *Acinetobacter* strains isolated from hospitalised patients (Joly-Guillou, *et al.,* 1991). The primary reservoir is humans and other reservoirs have not yet been conclusively identified. (Joly-Guillou and Brun-Buisson, 1996). *A. baumannii* has been isolated from

respiratory tract specimens, blood cultures and wound swabs. It is thought that the presence of this species in a clinical setting has more often resulted from contamination from an infected or colonised patient (Bouvet and Grimont, 1987).

While *A. baumannii* has been mainly isolated from clinical specimens, *A. calcoaceticus* has been shown to predominately inhabit soil (Bergogne-Berezin and Joly-Guillou, 1985). Although *A. calcoaceticus* has very rarely been implicated in human infections, there have been reports on the isolation of this organism from clinical specimens in nosocomial respiratory tract infections (Buxton *et al.,* 1978; Baumann, 1968).

Both environmental and clinical strains of *A. junii* have been isolated (Joly-Guillou, 1996). *A. johnsonnii* and *A. lwoffii* have also been isolated from a variety of sources including soil, animals and animal by-products, skin of hospitalized patients as well as hands of uninfected staff (Joly-Guillou and Brun-Buisson, 1996).

A. radioresistens has also been recovered from soil and more recently hospital patients (Nishimura *et al.,* 1989; Christensen *et al.,* 1991). *A. haemolyticus* and species 6 are mainly environmental organisms but they have been isolated from the hospital on rare occasions. However, their isolation from clinical specimens is thought to be more as a result of contamination rather than true infection. These rather uncommon *Acinetobacter* species have been isolated from cases of bacteremia (Joly-Guillou *et al.,* 1991). The remaining *Acinetobacter* species' habitats have not yet been implicated in clinical infections nor have they been well-defined (Joly-Guillou and Brun-Buisson, 1996).

1.5 Sources of Clinical infections

Acinetobacters form part of the natural bacterial flora of the skin, specifically the moist regions, such as the axillae, groin and toe-webs (Taplin *et al.,* 1963). They have been isolated in hospitals, from human sources, such as oral cavities, respiratory tracts and even rectums of healthy individuals (Rosenthal and Tager, 1975; Glew *et al.,* 1977). It has been shown that the skin is more often than not, the source of contamination in

outbreaks and infections (Al-Khoja and Darrell, 1979; French *et al.*, 1980). Joly-Guillou and Brun-Buisson (1996) noted that *Acinetobacter* strains have played a significant role in colonization and infection of patients admitted to hospitals in intensive care units (ICUs). In an ICU setting, antibiotic resistance can be highly problematic. This is due to the range of antibiotics used in therapy and the high selection pressure exerted by the use of these broad-spectrum antibiotics, thereby selecting out resistant strains of *Acinetobacter*. These resistant strains are known to be rapidly disseminated in a number of ways; in addition to dissemination by human carriage, inanimate reservoirs have been reported to be responsible for the transmission of infections. These include soaps, taps, basins, wet mattresses and plastic apparatus such as urinals, blood collection tubes, respirometers, humidifiers and ventilator equipment (Siegman-Igra *et al.*, 1993; Abrutyn *et al.*, 1978; Smith and Massanari, 1977).

1.6 Clinical infections (*Acinetobacter* in human disease)

There are two types of clinical infections caused by *Acinetobacters*; community-acquired infections and hospital-acquired infections. One of the main reasons for the increased interest in this genus is the fact that these bacteria are often responsible for difficult-to-treat infections because of their frequent multiple antibiotic resistance to major antibiotics (Towner, 1996). These opportunistic pathogens are responsible for a wide variety of nosocomial infections (Bergogne- Bérézin *et al.*, 1996). Of particular concern are infections in ICU patients, mentioned previously, where *A. baumannii* can cause clinically significant infections. Here it manifests itself as conditions of the respiratory tract, infections in post-surgery wounds and burns wounds. *A. baumannii* has also been known to result in infections in neonates.

1.6.1 Community acquired infections

Community acquired infections have been reported sporadically. *Acinetobacter spp.* has been known to be responsible for community acquired-bacteremia, -pneumonia, -urinary tract infections and even dental infections (Barnes *et al.*, 1988). Community-acquired

pneumonia caused by *Acinetobacter* is relatively rare and sporadic cases have been reported from the US (Cordes *et al.,* 1981). Despite their rarity, these *Acinetobacter*-pneumonia infections were shown to carry a high mortality rate, making rapid diagnosis and treatment a priority. Chronic conditions such as diabetes and tobacco and alcohol consumption appear to be the main predisposing factors contributing to the incidence of community-acquired infections. The most commonly administered treatment in these cases of *Acinetobacter* community-acquired pneumonia was an aminoglycoside, an extended spectrum penicillin or third generation cephalosporin (Hoffmann *et al.,* 1982).

1.6.2 Hospital acquired infections

Due to the fact that *Acinetobacter spp.* form part of the natural flora of the skin it is not always very easy to distinguish between true infection and colonization. (Streulens *et al.,* 1993).

The most common illnesses associated with *A. baumannii* are the respiratory tract infections, and one such example is pneumonia (Bergogne-Bérézin and Joly-Guillou, 1991). Large outbreaks of *Acinetobacter* pneumonia have been reported and it was found to be most common in elderly patients with a chronic condition, such as those who had been on prolonged ventilator assistance and those who had been receiving prolonged antibiotic therapy as well (Hartstein *et al.,* 1988). These infections were usually acquired via aspiration. Factors associated with higher colonization include use of inhalation equipment, intubation and selective use of antibiotics. The prevalence of *Acinetobacter*-associated pneumonia is quite serious considering that the *Acinetobacter* pneumonia mortality rate is 72% as compared to a mortality rate of 54% in pneumonia caused by other organisms (Fagon *et al.,* 1993).

A. baumannii has also been isolated from infections resulting in bacteremia. As is the case with most *Acinetobacter* infections, it was very difficult to distinguish between bacteremia and contamination. However, Tilley and Roberts (1994) proposed a list of

criteria in order to distinguish between bacteremia and contamination, in the case of *Acinetobacter* infections. Based on these criteria, bacteremia is not a very common occurrence. However, in some of these rare cases where bacteremia has been observed, it is most commonly found in immunocompromised patients, those with malignant disease, trauma, burns, and in some patients, bacteremia is the result of a respiratory tract infection.

Acinetobacter has rarely been implicated in nosocomial urinary tract infections (UTIs) but in the isolated cases that they have been reported in, the infections have been quite severe (Levi and Rubinstein, 1996). As mentioned previously, *A. baumannii* infections are predominantly found in immunocompromised patients and in the case of UTIs, it occurs most commonly in elderly and debilitated patients. In one study, Pedraza *et al.* (1993) identified most of the patients as being male and ascertained that the infection was most probably as a result of catherisation.

Acinetobacter infections in meningitis have only been reported in the last decade. Most of these sporadic cases of meningitis caused by *Acinetobacter*, have been described following head trauma and surgical procedures to the head. In addition to infections in adults, only a few cases have been reported about meningitic infection in neonates (Kellar *et al.*, 1995).

1.7 Chemotherapy for *Acinetobacter* infections

The widespread use of antibiotics has proven successful in treating a number of bacterial infections and eliminating the source of infection (Barriere and Conte, 1980). In the absence of competition from other bacteria, *Acinetobacter* has come to occupy this previously unavailable niche. The selection pressure of antibiotic use has favored the *Acinetobacter* lifestyle and hence contributed to its spread. As previously mentioned, chemotherapy for *Acinetobacter* infections often proves quite difficult due to the fact that this species often exhibit multiple resistance to antibiotics and combination therapy is usually the route taken (Bergogne-Bérézin 1996; Gould *et al.*, 1994). The normal course

is to treat the patient with combination therapy such as an aminoglycoside with a carbapenem or a broad-spectrum cephalosporin with an inhibitor (Barriere and Conte 1980; Levi and Rubinstein, 1996). A number of factors have been described which are thought to contribute to the overall resistance of this successful pathogen.

The ability of *Acinetobacter*, specifically *A. baumannii* to acquire and develop resistance to antibiotic therapy has contributed to its emergence as a successful nosocomial pathogen (Bergogne- Bérézin *et al.*, 1996). Over two decades ago, *Acinetobacter* infections were treated successfully with a variety of different antibiotics including nalidixic acid, gentamicin, ampicillin and carbenicillin, which were administered alone or as part of a combination therapy (Bergogne-Bérézin and Towner, 1996). Other combinations included an aminoglycoside with a carbapenem or other broad-spectrum cephalosporin (Levi and Rubinstein, 1996).

The resistance to the beta-lactams (β lactams) is known to be sometimes mediated by enzymes, beta-lactamases (β lactamases). There are many strategies adopted to help deal with the problem of β-lactamase producing bacteria. The specific use of β-lactamase inhibitors has been the most popular method of treating Gram positive and Gram negative infections globally. Among these, clavulanic acid, sulbactam and tazobactam which act as suicide inhibitors, produce irreversible inhibition of β-lactamases. In combination with β lactam antibiotics such as ampicillin and piperacillin, these inhibitors provide broad-spectrum activity (Kadima and Weiner, 1997). However, in 1987, amoxicillin-clavulanate (co-amoxyclav) resistant strains of *E. coli* (Pitout *et al.,* 1998) have been isolated and since then organisms resistant to various inhibitor combinations have been isolated more and more frequently.

At Groote Schuur Hospital (GSH), Cape Town, a combination therapy of piperacillin (βlactam) and tazobactam (inhibitor) was introduced to treat *Acinetobacter* infections in 1996 (GSH statistics). This combination proved successful for a limited period only, with an emergence of resistant strains being detected in the latter part of 1999 (GSH, January

2000). Since then the drug regimen has changed and the current choice of treatment for *Acinetobacter* infections is a carbapenem, specifically meropenem. (GSH statistics).

1.8 Antibiotic resistance and mechanisms of resistance

Of all its properties, *Acinetobacter* is probably most well known for it multiple antibiotic resistance, since it was initially described as a nosocomial pathogen (Bergogne-Bérézin 1996). It has been hypothesized that the ability of *Acinetobacter* spp. to display such recalcitrance to antibiotics is due to its long-term evolutionary exposure to antibiotic producing soil organisms and hence as a result has developed the propensity to develop such rapid resistance (Levy, 1997). Other nosocomial pathogens such as *Klebsiella* spp. and *Pseudomonas* spp., exhibit resistance-acquisition at a much slower rate than *Acinetobacter* (Levy, 1997). As a result of this evolutionary advantage, it appears that *Acinetobacter* poses a fairly serous threat as a nosocomial pathogen (Levy, 1997).

It has been noted in the past, that these multiple-resistant organisms are quite rare in a relatively antibiotic free environment. Therefore, it can be seen how the success of *Acinetobacter* as a successful nosocomial pathogen is dependent on these organisms being selected out due to their ability to respond rapidly to changing antibiotics as well as the unchecked use of antibiotics in hospitals (Bush, 1999). Interestingly, proof of this selection theory was well demonstrated, where in an isolated case, a clinical outbreak of *A. baumannii* spontaneously subsided when the selective pressure of antibiotics was removed (Siegman-Igra *et al.,* 1993).

Acinetobacter has exhibited resistance towards most of the current antibiotics in use including the beta lactams, the aminoglycosides as well as the quinolones. Resistance to these drugs and other combinations was detected as early as 1971 (Towner, 1996) and since then *Acinetobacter* has become resistant to virtually all the commercially available antibiotics. There are documented cases of resistance to the aminopenicillins, ureidopenicillins, cephalosporins, cephamycins, most aminoglycoside, chloramphenicol, trimethoprim and tetracycline (Dowding, 1979; Devaud *et al.,* 1982; Garcia *et al.,* 1983;

Goldstein *et al.,* 1983). The minimum inhibitory concentrations (MICs) of most of the antibiotics for *A. baumannii* specifically, are increasing and there appears to be growing resistance towards some of the broad-spectrum cephalosporins such as cefotaxime and ceftaxidime (Bou *et al.,* 2000).

Initially, imipenem had complete activity against *A.baumannii* infections, but in 1994, Go *et al.,* reported an *Acinetobacter* infection which displayed resistance to nearly all the antibiotics including imipenem. Scaife *et al.,* (1995) reported a case of transferable imipenem resistance in a clinical isolate of *Acinetobacter*. The nosocomial spread of imipenem resistance amongst *Acinetobacter* strains poses a serious threat for the successful treatment of *Acinetobacter* infections in the future (Chu *et al.,* 2001), as this is one of the drugs of choice. This is the case for chemotherapy both locally and globally.

Resistance to different families of antibiotics has been described in *Acinetobacter*. A number of different mechanisms contributing to the overall resistance have also been described and will be dealt with in the following sections.

1.8.1 Resistance to aminoglycosides

One of the most frequently described modes of resistance to the aminoglycosides is enzymatic inactivation (Shaw *et al.,* 1993). This inactivation has been extensively described and is mediated by three types of enzymes, acetyltransferases (AAC) (Rudant *et al.,* 1998), adenylyl or nucleotidyl transferases (AAD or ANT respectively) and phosphotransferases. These enzymes can be further categorised on the basis of their substrate profile. The three types of aminoglycoside modifying enzymes (AMEs) mentioned above have been described in *Acinetobacter* (Shaw *et al.,* 1993; Lambert *et al.,* 1993) and specifically, the aac' genes have shown variation based on their geography (Shaw *et al.,* 1993)

1.8.2 Resistance to quinolones

Resistance to the quinolones has been described in *Acinetobacter* (Amyes and Young, 1996). Even though the reduced outer membrane permeability of *Acinetobacter* OM has thought to play a role in quinolones resistance, mutations in the DNA gyrase have been described and it is thought that these mutations may also contribute significantly to resistance in *Acinetobacter* (Vila *et al.,* 1995). The DNA gyrase and topoisomerase IV are the principle targets of the fluorinated- 4- quinolones. Resistance to this group of antibiotics in other bacteria, such as *E. coli*, has been attributed to the changes in structure of the DNA gyrase sub-unit (Vila *et al.*, 1995). The mutations in *E.coli* gyr A leading to resistance, was traced back to the Gly 81, Ser83 and Ala 8 residues (Vila *et al.*, 1995). However, it was shown that only one mutation, at the Ser83 codon of the gyrA gene, was contributing to the flouroquinolone resistance in *A. baumannii* from some Spanish hospitals (Vila *et al.,* 1997)

1.8.3 Resistance to β-lactams

One of the most commonly described mechanisms of resistance to the β lactam family of antibiotics is that, mediated by the enzymes β-lactamases as previously mentioned. As this thesis focuses on β-lactam resistance in *Acinetobacter*, this mechanism of resistance will be described in detail in the subsequent sections.

1.8.3.1 βLactamases (Chromosomal- and plasmid- mediated resistance genes)

There are two types of βlactamases in *Acinetobacter*; those, which are chromosomally encoded, and those, which are plasmid, encoded. Even though the contribution of chromosomal cephalosporinases appears to be significant, this is only part of the whole contribution and other mechanisms such plasmid mediated βlactamases and altered permeability due to outer membrane proteins, may also confer some inherent resistance (Bush, 1996).

1.8.2.1 Chromosomal beta lactamases

It is thought that chromosomal AmpC, a β-lactamase, which is found in many bacteria, including *E.coli* was present pre-dating the antibiotic era. The AmpC may have had some physiological roles in peptidoglycan assembly or may have evolved to defend bacteria from the beta lactams produced by fungi or other environmental bacteria (Livermore, 1994; Levy, 1997). It appears, however, that the current misuse of beta lactams, is a major selection factor influencing β lactamase production

As early as 1976, chromosomal cephalosporinases were demonstrated in a strain of Acinetobacter by Mathew and Harris and this data was confirmed by work done by Sykes and Matthew (1976). Subsequent analysis of the strain identified it as *A. lwoffii* (Hood and Amyes, 1991) and this was the first report of a chromosomal beta-lactamase.

In the subsequent decade, based on IEF, molecular studies and biochemical properties at least four different chromosomal β-lactamases were identified and designated ACE 1- 4 (*Acinetobacter* chromosomal enzymes). All four of these enzymes are cephalosporinases with limited activity against penicillin (Hood and Amyes, 1991). However, these enzymes did not exhibit any hydrolyzing activity against ceftazidime or cefotaxime. ACE 1 also showed some activity against cefuroxime (Hood and Amyes 1991). This is unusual, as the extended spectrum of activity is usually not chromosomally mediated, rather plasmid mediated. In addition to this anomaly, there have been conflicting reports regarding the regulation of these enzymes; whether they are inducible or constitutively expressed (Amyes and Young, 1996). Despite the ambiguity of the regulation of the chromosomal β-lactamases, it has been suggested that although the contribution of these enzymes is important to the resistance profile, they work in tandem with other mechanisms such as the inherent resistance conferred by the reduced outer membrane permeability and altered penicillin binding proteins (Sato and Nakae 1991, Obara and Nakae 1993, Amyes and Young, 1996). Recently, a chromosomal cephalosporinase was identified from a clinical strain of *A. baumannii*, characterised and designated AmpC (Bou and Martinez-Beltran, 2000). It is likely, that this AmpC is one of the four described

ACE genes. The clinical strain was resistant to a number of β lactam antibiotics including ampicillin, ticarcillin, cefuroxime, cefotaxime and ceftazidime. The AmpC itself, however, displayed only weak activity against these β-lactams.

Since 1976, more chromosomal cephalosporinases have been identified. In one instance, two *Acinetobacter* species; *A.lwoffii* and *A.baumannii* Perilli *et al.,* (1996) were found to have them. Both species displayed high MICs against piperacillin and cephaloridine (32 and 256 μg/ml; 256 and 128 μg/ml respectively). These beta lactamases were not inhibited by clavulanic acid (a commonly administered beta lactamase inhibitor). AS was the case with the AmpC, a comparison of the susceptibility data with the kinetic studies suggested that these enzymes were only partially contributing to the overall resistance profile.

1.8.3.2 Plasmid mediated β-lactamases

Resistance to all the beta lactams is due, in part, to acquisition of extra-chromosomal beta lactamases, in addition to the chromosomal cephalosporinases. The high incidence of beta lactam resistance in the early days of antibiotics suggested the presence of plasmids, carrying beta lactamase genes (Amyes and Young, 1996). Despite the fact that there have been many incidences of plasmid mediated beta lactamases, it is more than likely that all these enzymes had a chromosomal origin, although the source organisms are not yet known (Levy, 1997).

One of the earliest reports of *Acinetobacter* beta lactam resistance was due to a plasmid-encoded Class A TEM-1 beta lactamase in an epidemic strain as early as 1980 (Phillipon *et al.*). Subsequent isolation of resistant strains, and protein analysis showed the presence of a number of other beta lactamases. These included TEM-2 beta lactamase and CARB-5. CARB-5 was shown to hydrolyse both ampicillin and carbenicillin but not the cephalosporins (Devaud *et al.,* 1982). Despite the presence of these enzymes, which were shown to be associated with a transposable element on a resistance-encoding

plasmid, it has been suggested that chromosomal cephalosporinases are the predominant beta lactamases in *Acinetobacter* (Vila *et al.,* 1993).

Another beta lactamase, ARI-1 has also been described in *Acinetobacter*. This beta lactamase mediates resistance to imipenem (Paton *et al.,* 1993; Donald *et al.,* 2000). ARI-1 is not a metallo beta lactamase (CLASS B) and was thought to be a serine-based enzyme (Class A). Paton *et al.* (1993) showed that ARI 1 gene was carried on a 45kb plasmid, pUK1356 which is self-transmissible within the *Acinetobacter* genus and not to other bacterial species. This was the first report of transferable (plasmid encoded) imipenem resistance. Recently, Donald *et al.*, (2000), re-named this enzyme OXA-23 based on sequence analysis. In 1998 Brown *et al.,* also described another enzyme ARI-2, which was isolated from a clinical strain of *Acinetobacter* from Argentina. But, as was the case for ARI-1, further sequence analysis has resulted in a shift from the Class A to the Class D β-lactamases, and currently ARI-2 is classified as OXA-34 (Bou *et al.*, 2000)

TEM1 and TEM2, which are two progenitors of extended spectrum beta lactamases (ESBL), have been described in *Acinetobacter* strains (Amyes and Young, 1996). In 1993, Nordmann *et al.,* isolated and characterised a novel beta lactamase, from *P.aeruginosa*, which they designated PER-1. This extended-spectrum beta lactamase (ESBL) had activity against the extended spectrum beta lactam antibiotics, which included ceftazidime and cefperazone. Since 1993, this gene has been detected in clinical isolates of both *Pseudomonas* as well as *Acinetobacter* in Turkey (Vahaboglu *et al.,* 1997). The Turkish study showed that almost 46% of the *A.baumannii* strains were found to harbor the PER-1 gene. In the course of routine testing, Poirel *et al.* (1999) identified a strain of *A.baumannii,* in France, which exhibited only slight synergy between the clavulanic acid and ceftazidime. Also, in the presence of clavulanic acid, the MIC for ticarcillin and ceftazidime dropped from 512 to 256 and 512 to 128 μg/ml, respectively, showing only limited inhibition. The authors were expecting complete inhibition. Following PCR assays using primers directed against specific genes for beta lactamases, SHV, TEM and PER, Poirel *et al.*(1999) obtained a product with the PER-1 assay. A plasmid location for the gene was not demonstrated but not ruled out either. Poirel and

his colleagues were able to show, using various typing techniques, that this PER-1 beta lactamase was not clonally related to the PER-1 isolated from Turkey (Vahaboglu *et al.,* 1997). The isolation of a PER-1 type gene, less than 2 years later, from a 90- year old patient in France who had had no contact with anyone in Turkey, makes the possibility of transmission via contact not likely. Interestingly, this suggests some other mechanism by which the PER beta lactamase must have evolved.

The extent of the beta lactamases described in *Acinetobacter* is not limited to those mentioned previously. In 1997 Vila *et al.,* described a gene encoding an oxacillinase from a clinical strain of *A.baumannii* (Ab41). The strain was resistant to all beta lactam antibiotics tested except ceftazadime, ceftriaxone and imipenem. This strain was shown to produce three beta lactamases- a TEM-1 like β-lactamase; a novel OXA derived beta lactamase and chromosomal cephalosporinases. The OXA-derived β-lactamase was designated OXA-21 which fell into the class D classification (Ambler, 1980). Afzal-Shah *et al.,* (2001) have also described and characterized OXA 25, 26 and 27. These integron-associated, chromosomal-based are molecular class D beta lactamases which conferred resistance to the carbepenems and high resistance to the oxacillins (MIC's were >128μg/ml). Despite the high MICs (imipenem 64μg/ml; meropenem >128μg/ml; ampicillin >128μg/ml) to the carbapenems and penicillins, these enzymes demonstrated a weak kinetic activity against these same enzymes (>1% activity) leading to the suggestion that the resistance profile might be as a result of enzymes which work in tandem with secondary resistance mechanisms, such as impermeability and up-regulated efflux (Afzal-Shah *et al.,* 2001)

A tabulated summary of the beta lactamases described so far appears in the table below

Table 1.2 β-lactamases in Acinetobacter (Adapted from Hood and Amyes, 1991)

Enzyme or strain	Location of gene	Predominant substrate	Approximate Iso-electric focus (pI)
TEM-1	Plasmid	Penicillin	5.4
TEM-2	Plasmid	Penicillin	5.6
CARB-5	Plasmid	Penicillin	
ACE-1	Chromosome	Cephalosporins	8.8, 8.6
ACE-2	Chromosome	Cephalosporin s	8.7
ACE-3	Chromosome	Cephalosporins	8.1, 8.2
ACE-4	Chromosome	Cephalosporins	7.7
SHV-like	Unknown	Penicillin	
SHV-like	Unknown	Penicillin	
Amp (Possibly one of the ACEs)	Chromosome	Ampicillin, amoxy-clav, ticarcillin, cefuroxime	9.4
IMP-4	Chromosome	Carbapenem	
PER-1	Plasmid	Piperacillin, Amoxy-clav, Ticarcillin, ceftazidime-clavulanic acid	
OXA-21	Integron-chromosome		7.0
OXA-23 (Previously ARI-1)	Plasmid	Carbapenem	
OXA-34 (Previously ARI-2)	Integron-chromosome	Imipenem, Meropenem ,ampillin, ticarcillin, cefuroxime, cefotaxime, ceftazidime, cefoxitin	>9.0
OXA-25	Integron-chromosome	Imipenem, meropenem, ceftazidime, cefuroxime, cefotaxime, ampicillin, piperacillin	>9.0
OXA-26	Integron-chromosome	Imipenem, meropenem, ceftazidime, cefuroxime, cefotaxime, ampicillin, piperacillin	>9.0
OXA-27	Integron-chromosome	Imipenem, meropenem, ceftazidime, cefuroxime, cefotaxime, ampicillin, piperacillin	>9.0

1.9.1 Outer membrane permeability

The understanding of the contribution of the structural features of the outer membrane, in the overall resistance profile of bacteria is becoming clearer as more extensive studies are carried out (Nikaido, 1989). Many antibiotics have to cross the bacterial outer membrane, to access their targets and such is the case for the beta-lactam antibiotics, which have to traverse this membrane, and thus the outer membrane presents a selective barrier between the bacteria and its environment.

Sato and Nakae (1991) have re-iterated this and have reported the presence of a significant permeability barrier in the *A. calcoaceticus* species. Being unable to attribute the high level of resistance to the beta lactams solely to the beta-lactamases, Sato and Nakae carried out studies on the outer membrane of *A. calcoaceticus* using *E.coli* and *P.aeruginosa* as comparisons. The authors made a number of observations. They observed the extremely slow diffusion of zwitterionic cephalosporins, up to 90% reduced levels, in *A. calcoaceticus* as compared with *E.coli*. They attributed this to three factors: i) the size of the outer membrane pore is small ii) the organisms only produced a limited amount of porins and iii) a combination of the two. In addition, they observed that the diffusion rates of carbapenems were only 1% of *E.coli*'s. Following liposome swelling assays and protein quantification studies, this reduced level of diffusion was attributed to the fact that multidrug resistant *Acinetobacter* contained a very low percentage of porins in its outer membrane (OM). These porins were previously described as serving (mostly), as non-specific channels for the movement of molecules across the bacterial membrane (Parr *et al.,* 1987; Caulcott, 1984). Nikaido (1989) observed that only certain antibiotics, which included the beta lactams, tetracycline, chloramphenicol and aminoglycosides crossed into the bacterial cytoplasm (in *Pseudomonas*) through these porins. This transmission was allowed, while larger molecules are excluded and hence porins act as an effective barrier against the larger more hydrophobic antibiotics, including the macrolides and the hydrophobic beta lactams. In fact, the protein quantification studies also showed that the *Acinetobacter* strain porins content was only 5% of the total OM protein content,

compared to 60% for *E. coli* (Rosenbusch, 1974). It is also interesting to note that this low porin content most probably contributes significantly in preventing entry of antibiotics leading to the broad spectrum of antibiotic resistance.

As part of a routine study, it was noted that an imipenem susceptible isolate of *Acinetobacter* did not produce any detectable levels of beta lactamase (Clark ,1991*)*. Three strains formed the basis of this study; an imipenem resistant isolate, an imipenem susceptible isolate and a control strain. The authors noted that the decreased expression of outer membrane protein 33-36 kDa OMP was associated with the increased resistance to imipenem. Imipenem resistance in one strain was four fold higher when the expression of a 33-35kDa protein was reduced, while the other strain expressed more protein and concomitantly exhibited lower levels of resistance to imipenem. Interestingly, no reference was made to Opr D in *Pseudomonas*, which is a well-studied example of a porin whose expression is involved in the regulation of imipenem influx.

Another outer membrane protein (OMP), not involved in resistance had also been described by Jyothisri *et al.* (1999). They purified and characterised a major 40kDa outer membrane protein in a clinical isolate of *A.baumannii*, which they designated OmpAb. Their studies showed that this major OmpAb was both thermoregulated as well as osmoregulated, and therefore its expression would be dependent on its environment. Following liposome-swelling assays they determined the porin diameter to be 1.3 nm and determined the specific activity of the pore for sugar (arabinose diffusion) to be 760. This additional data indicating diameter and specificity of the pore was comparable to a porin in *E.coli*, OmpF and there was found to be a close correlation between the two. Viz *E.coli*'s OmpF was 1.2 nm and its specific activity for the sugar was 680. OmpF is a general diffusion pore in *E.coli* and so this close comparison led them to propose that this 40kDa protein could play a similar structural role *A. baumannii*.

Another mechanism of resistance to β-lactams that have been described in other Gram-negative bacteria is the pumps. The general efflux pumps and multi-drug efflux pumps

have been well documented in bacteria such as *P.aeruginosa* (Nikaido *et al.*, 1991). However, to date, the only description of an efflux pump is a resistance-nodulation cell division type efflux pump. This pump is associated with aminoglycoside resistance in *Acinetobacter* spp. (Magnet *et al.*, 2001).

1.10 Summary

Over the years, ***Acinetobacter spp.***, specifically *A.baumannii* has emerged as a significant nosocomial pathogen. A number of different factors come into play which contribute to its overall success as a pathogen and these include virulence factors such as beta lactamases and small porins to name a few. Its ability to respond rapidly to the onslaught of antimicrobial therapy to which it is subject means that it appears to always be one step ahead of treatment. It will become important in the future to be able to better understand the underlying mechanisms that contribute to this resistance and therefore be able to treat the infections which it causes.

1.11 The project plan and description

Antibiotic resistant bacteria play an important role in preventing the recovery of patients receiving intensive care (those in ICU) and in those patients post surgery, those who are immunocompromised or on prolonged antibiotic therapy at Groote Schuur Hospital in Cape Town, South Africa. However, it is important to note that this problem is not confined to South Africa alone; it is in fact a global problem as well. Bacterial infections have proved to be the most insidious especially those infections by Gram negative bacteria. Of these *Acinetobacter* spp. is no stranger to the hindrance in the recovery of the above mentioned patients. Its intrinsic nature to be resistant and its ability to acquire resistance to a number of currently used antibiotics has made it a nosocomial pathogen of significant proportions. In particular, the *Acinetobacter baumannii* species has showed its ability to be resistant (to a number of antibiotics), in strains isolated from the hospitals.

It is known that the β lactam resistance is mediated in part by βlactamases, which degrade the β lactam family of antibiotics, including ampicillin, piperacillin, the extended spectrum cephalosporins and other penicillin derivative drugs. The combination of choice at Groote Schuur for treating *Acinetobacter* infections, during the 1999 and the early part of 2000 was piperacillin-tazobactam, which is a penicillin derivative and a β lactamase inhibitor combination. These two work in tandem to combat and halt growth of *A.baumannii* infections. Since the introduction of piperacillin and tazobactam about 4 years ago, this combination therapy is no longer proving effective.

With this in mind the strains isolated from the hospital from patients at Groote Schuur Hospital were sent to our department for analysis. These clinical isolates were to be tested using current molecular techniques.

The **aims** of this project were two fold:

i) to identify the different mechanisms that are contributing towards β lactam resistance. in *Acinetobacter baumannii*

ii) to characterise these mechanisms and hence help better understand the resistance displayed by this organism

CHAPTER 2

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2.5 DISCUSSION

CHAPTER 2

MOLECULAR TYPING OF CLINICAL ISOLATES OF *A. BAUMANNII*

2.1 INTRODUCTION

As a first step in this study, the epidemiology of clinical isolates of *A.baumannii* was investigated by molecular typing. A number of different typing techniques have been described to type *Acinetobacter* spp. and it has not yet been possible to identify the most suitable typing method. For the sake of completeness, the following sections will examine all the techniques described to date.

For epidemiological purposes the most widely used phenotypic procedure has been serotyping but this method usually lacks adequate discriminatory power and therefore there is a well-recognized need for alternative subtyping schemes. Many DNA based (genotypic) subtyping schemes have been developed including pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) analysis and ribotyping. Often combinations of serotyping and genotyping techniques are used, resulting in higher discriminatory power and therefore, more complete strain identification (Gerner-Smidt, 1992).

There are two different categories of typing, general and comparative (also known as fingerprinting) (Dijkshoorn, 1996). The general methods include biotyping (Bouvet and Grimont, 1986), serotyping (Henriksen, 1973) and phage typing (Vieu *et al.*, 1979). The comparative methods include anti-biogram typing (Alexander *et al.*, 1998) but it has been advised that this method be used in correlation with other typing tools as many unrelated strains may exhibit similar antibiogram typing patterns. The most common genotyping methods applied to *Acinetobacter* spp. are RAPD (Mathai *et al.*, 2001), PFGE of chromosomal DNA, plasmid typing, amplified ribosomal DNA restriction analysis (ARDRA), ribotyping and PCR fingerprinting. These various genotyping methods can be compared by a number of criteria, which include sensitivity, reproducibility, and ease of

use, speed, availability and cost. Different laboratories vary in their choice of importance of these criteria. However, discriminatory power is one of the most important factors to consider when such comparisons are carried out between different typing methods.

Only recently have molecular subtyping techniques been developed to address the shortcomings of phenotypic methods (Dijkshoorn *et al.*, 1993). Major advantages of genetically based methods are their enhanced sensitivity and discrimination. Genotyping methods have proved their worth as typing tools and are potentially available worldwide (Struelens *et al.*, 1993).

2.2 COMPARATIVE TYPING METHODS

2.2.1 Pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) is based on the presence of restriction enzyme recognition sites. The enzymes used cut infrequently resulting in large (20-200 kb) fragments. These fragments are separated using defined electrophoretic conditions. Strain variation depends on the presence of the restriction site in the chromosome (Birren and Lai, 1993). PFGE is considered to be one of the most powerful tools available for microbial molecular epidemiology. The technique is highly sensitive in that whole genome restriction site polymorphisms are detected making strain differences easier to distinguish. The various DNA fragments obtained are separated by defined electrophoretic conditions and pulsed electric fields are applied from different positions in the electrophoretic cell.

Even though PFGE has a higher discriminatory power then other typing techniques, there are a number of disadvantages with PFGE: (i) the apparatus required for electrophoresis is specialized and expensive; (ii) considerable variation in restriction enzymes and electrophoretic conditions between laboratories makes comparison of PFGE profiles difficult and (iii) genetic instability can lead to minor or major changes in profiles.

Seltmann *et al.*, (1995) were able to demonstrate the usefulness of this technique to type 49 *A.baumannii* strains from a clinical outbreak. They compared this technique to whole cell protein profiling and plasmid typing and found it to be the most suitable technique due to its high reproducibility and its high discriminatory power. In another instance, Liu and Wu (1997) were also able to successfully investigate the epidemiology of strains of *A.calcoaceticus*, providing further support for the use of this technique.

2.2.2 Randomly amplified polymorphic DNA

Random amplified polymorphic DNA (RAPD) is a polymerase-chain reaction (PCR) based fingerprinting technique. Arbitrarily designed primers are used to amplify random DNA sequences from the genome under conditions of low stringency. Strain variation is the result of the presence, number, distance and mismatch fidelity, of the sites to which the primers anneal (Williams *et al.*, 1990). The method uses the entire genome of the target organism to generate amplified fragments. The number and size of the fragments generated are partially controlled by stringency regulation through manipulations of the annealing temperature. Amplification varies due to the length of the product formed and the efficiency of annealing, resulting in banding patterns consisting of stronger and weaker amplicons of variable length.

The RAPD technique can be used as an effective typing tool. Banding patterns obtained are suitable for comparison of isolates within a bacterial species (Koeleman *et al.*, 1998). These authors demonstrated the usefulness of this technique for discriminating between 31 strains of clinical isolates of *A.baumannii*, and this technique clearly demonstrated the polymorphisms between the different strains, suggesting that they were not part of an outbreak.

A slight variation on the technique is whereby it is used as a specific tool, is in the amplification of genomic DNA fragments. This is achieved by the use of primers specific for enterobacterial repetitive intergenic consensus sequences (ERIC) (Sharples and Lloyd, 1990; Vila *et al.*, 1996). ERIC primers can be used under high stringency to match

the target sequences. These ERIC primers have been used to type *A.calcoaceticus* as well as *A.baumannii* in two separate investigations. RAPD has the advantage of being much quicker and cheaper than PFGE and does not require complex apparatus. However, there have been problems associated with reproducibility in typing *A.baumannii* (Marques *et al.*, 1997)

2.2.3 Ribotyping

Ribotyping makes use of the presence of multiple copies of the ribosomal RNA genes coding for 16S and 23S rRNA at different positions in the chromosome. Ribotyping depends on restriction enzyme recognition sites flanking the ribosomal RNA-genes (16S rRNA) present on the *Acinetobacter* genome. These genes are suitable for subtyping purposes due to the strong conservation of regions within the rRNA genes between bacteria, and the presence of highly variable, non-coding, flanking regions. (Owen, 1989; Grimont and Grimont, 1986). The most commonly used technique is Southern blot hybridisation of restricted DNA, hybridised with a probe specific for rRNA genes. This method has been used quite successfully by Gerner-Smidt (1992) to investigate 29 strains of *Acinetobacter* spp. as part of a clinical outbreak.

Ribotyping is unsuitable as a routine genotyping procedure as it does not have the highest discriminatory power and it is a laborious and time-consuming technique. Development of a completely automated ribotyping procedure for identification and characterisation of bacteria enhances reproducibility and saves time. However, the high costs of equipment and consumables limits the use of this technology (Dijkshoorn *et al.*, 1987)

2.2.4 Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) was originally developed for the genetic analysis of plants (Vos *et al.*, 1995) and has been adapted for genotyping of bacteria (Lin and Kuo, 1995; Janssen *et al.*, 1996). AFLP has proven to be a useful typing tool for *Acinetobacter* (Dijkshoorn *et al.*, 1996).

The method involves chromosomal DNA digestion with two restriction enzymes. The enzymes of choice are dependent on the G+C content and the size of the genomic DNA. Amplification by PCR of the digested products is based on the restriction sites and designed such that only those fragments flanked by both restriction sites are amplified. The primers are either radioactively labelled (Vos *et al.*, 1995) or fluorescently labelled (Kokotovic and On, 1999). The labelled PCR products are analysed on denaturing polyacrylamide gels. Optimally 80-100 bands are generated which can be reduced by incorporation in the PCR primers, of one or more specific nucleotides adjacent to the restriction site. Therefore, only fragments with specific nucleotides adjacent to restriction site will be detected and analysed (Koeleman *et al.*, 1998) on an automated DNA sequencer.

AFLP has also been successfully used for the differentiation between 31 isolates of *A. baumannii.* (Koeleman *et al.*, 1998), as part of a comparative study into the suitability of typing tools. An advantage of this technique is that a random proportion of the complete genome can be analyzed. AFLP is rapid and easily standardised; however, the equipment is expensive. With the digitization of AFLP results, accurate interpretation, ease of data storage and data exchange between research institutes is possible.

2.2.5 Poly- GTG typing

Poly-GTG typing is the use of a $(GTG)_5$ oligonucleotide, which is a repetitive marker in the chromosome. The poly-GTG oligonucleotide was initially used as a probe in DNA: DNA hybridisation studies investigating *Salmonella* and *Shigella* isolates, respectively. (Doll *et al.*, 1993). Since then, this method was shown to be successful in the identification of *Mycobacterium tuberculosis* (Sechi *et al.*, 1996) where it was used as a probe in fingerprinting studies of clinical strains from Italy. This oligonucleotide has also been used successfully as a probe in DNA: DNA hybridization experiments with respect to other *Mycobacterium* strains (Warren *et al.*, 1996; Cilliers *et al.*, 1997).

The use of poly-GTG primers as a PCR based fingerprinting technique was initially used in our department for the typing of *Campylobacter* strains (Matsheka, 2000). The method used to type the *Campylobacter* strains was a modification of the amplification protocol for arbitrarily primed PCR (AP-PCR) described by Williams et al., (1990). This PCR-based fingerprinting method was shown to have good discriminatory power. The PCR amplicons were resolved using agarose gel electrophoresis and were separated on the basis of their relative molecular masses and gave good electrophoretic profiles. The availability of the poly-GTG primers recommended the use of this for typing.

2.3 EXPERIMENTAL PROTOCOL

2.3.1 Bacterial Isolates and Susceptibility Testing

During the early part of 2000, a cluster of clinical strains of *A.baumannii* was isolated from patients being treated with the piperacillin-tazobactem combination drug-therapy at Groote Schuur Hospital in Cape Town. The strains were isolated from disparate wards and were identified as *A. baumannii* by the Clinical Microbiology diagnostic laboratory of the hospital using standard morphological and biochemical tests, demonstrating the characteristics consistent with the genus *Acinetobacter*. Ten strains resistant to the combination of piperacillin-tazobactem and one intermediate resistant strain were included in the study. These 11 isolates were also resistant to the other penicillin derivative drugs. A sensitive strain was used as a reference and control in the study. This sensitive strain was isolated from a patient in the same time period. In addition to these strains, an outgroup strain of *A.baumannii*, isolated from a later period (approximately a year later) was used as part of this epidemiological study.

Antibiotic sensitivity testing was determined by the disc diffusion test. Disc susceptibility testing was carried out according to the National Committee for Clinical Laboratory Standards (NCCLS) criteria (National Committee for Clinical Laboratory Standards criteria, 1999b).

The *A. baumannii* strains used in this study are listed in the table below.

Table 2.1 Description of the *A.baumannii* strains used in the study

Strain Designation	Date of isolation	Source of strain (Patient)	Source of strain (Ward)
S	25/02/2000	Abdominal wound	C 15
I	4/02/2000	Abdomen	C 21
1	02/02/2000	Unknown	F 26
2	17/02/2000	Leg	F 13
3	28/01/2000	Right hip	F 22
4	03/02/2000	Abdomen	D 12
5	04/02/2000	Small hole in back	C 27
6	25/01/2000	Liver abscess	F 25
7	22/01/2000	Arterial site	C 27
8	17/02/2000	Right leg	F 13
9	15/02/2000	Leg	C 5
10	08/02/2000	Arm	F 23
11	02/2001	Abdomen	C13

2.3.2 Genomic DNA extraction

Genomic DNA was extracted by the method described by Ausubel *et al.* (1987). A single colony of each of the acinetobacters was inoculated into 10ml of 2XTY broth and incubated aerobically for 18 hours at 37°C. The bacterial cells were harvested in a Beckman Model J2-21 centrifuge at 6000 rpm for 10 minutes. The bacterial pellets were resuspended in 567ml of Tris EDTA by repeated pipetting and the cell walls were lysed by the addition of 30ml 10% SDS. Three microlitres of proteinase K was added to digest cellular proteins as a result of cell wall lysis. This suspension was mixed thoroughly; lysis and protein digestion were allowed to continue at 37 ° C for an hour.

After lysis, the solution became viscous and 100 ml of 5M NaCl (Appendix A) and 80 ml of CTAB/NaCl (Appendix A) were added, the solution mixed thoroughly and incubated at 65 ° C for 10 minutes. This removes the cell wall debris, denatured/digested protein and polysaccharides, which become complexed to CTAB by selective precipitation. The presence of the 5M NaCl prevents the formation of a CTAB-nucleic acid precipitate and the nucleic acids remain in solution. The CTAB-protein/ polysaccharide complexes were then extracted with an equal volume of chloroform-isoamylalcohol (Appendix A). After centrifuging the mixture for 10 minutes in a microfuge (Eppendorf Centrifuge 5415C) at 140000 rpm a white interface was visible.

The aqueous phase was removed to a clean microfuge tube and the DNA was further purified by extraction with an equal volume of phenol chloroform isoamylalcohol (Appendix A) and centrifuged for 10 minutes at 14000 rpm in microfuge ((Eppendorf Centrifuge 5415C). This step was repeated until the aqueous phase was completely clear of the CTAB-protein polysaccharide complex. Once the aqueous phase was clear, the DNA was precipitated with 0.6 volumes of isopropanol. Shaking the tube back and forth allowed visualization of stringy white DNA precipitate. The DNA was collected by centrifugation for 10 minutes in a microfuge at 14000 rpm. With certain preparations, no stringy precipitate was visible since the DNA was sheared during the extraction process. However, the DNA could still be pelleted by centrifugation. DNA pellets were washed

with 70% ethanol to remove the residual CTAB and recentrifuged. The supernatant was carefully removed and DNA was allowed to dry for 10-15 minutes at 37 degrees. The DNA was then resuspended in 70-100µl of TE at 4°C overnight. The concentration of DNA in each genomic DNA preparation was determined using fluorescent quantitation. Ethidium bromide intercalates between the DNA bases and there is a relationship between the amount of fluorescence and the quantity of DNA in the sample that is, the more DNA in a sample the more it will fluoresce. Therefore to estimate the concentration of DNA in a sample, ethidium bromide was added and it was subjected to agarose gel electrophoresis alongside a sample of DNA of known concentration. The fluorescence emitted by the sample of unknown DNA Concentration was compared to that of the known DNA concentration.

2.3.3 DNA Concentration Determination

DNA concentration was determined using two different techniques: genomic DNA concentration was determined by either agarose gel electrophoresis and/or confirmed by using a UV spectrophotometer. Aliquots of sample DNA were electrophoresed next to increasing titrated concentrations of lambda-DNA (λ-DNA), commencing with 50 ng-500 ng. DNA concentration was estimated by visual comparison of band intensity with λ-DNA of known concentrations.

A UV spectrophotometer was also used to determine DNA concentration of genomic DNA. The DNA was diluted 100-fold and the DNA concentration was determined by measuring the absorbency at 260 nm in a Hewlett Packard Diode Array Spectrophotometer using the HP 8452 Win System and the formula 1 OD unit (OD_{260}) = 50mg/ml DNA. DNA was diluted to approximately 200 ng/µl for use in later applications.

2.3.4 Agarose gel electrophoresis

Agarose gel electrophoresis (AGE) resolves DNA fragments on the basis of their differing rates of migration in an agarose gel (Ausubel et al., 1987). Smaller fragments will migrate faster than the larger ones when an electric field is applied across the gel. Since DNA is negatively charged, it migrates from the cathode to the anode.

DNA fragments were separated in horizontal gels of 1-2.0% (w/v) agarose dissolved in 1x TAE (0.4M Tris Acetate, 0.01M EDTA) (Appendix A). Prior to the addition of DNA, the agarose was dissolved by heating the solution to boiling point in a microwave oven. Ethidium bromide was added at a concentration of 10µg/ml, such that the DNA could be visualized by UV transillumination at 302 nm (The wavelength at which the bound dye absorbs the ultraviolet radiation) (Sambrook *et al.*, 1989).

The gel was poured into a mould and a comb inserted. Once the gel had polymerized, the comb was removed and the gel was submerged in 1xTAE (Appendix A) in an electrophoresis tank.

Before the DNA samples were loaded into the wells, they were mixed with a gel tracking dye (Appendix A) which served three purposes: (I) sucrose increased the density of the sample ensuring that the DNA remained in the well, (II) the bromophenol blue in the dye allowed one to monitor the rate of migration of the DNA, and (III) the EDTA chelated the Mg2+ ions thereby inhibiting enzymatic activity in those reactions which involved DNA digestion.

Molecular weight markers were included so that the DNA fragment sizes could be determined. Molecular weight marker VI (Beohringer Mannheim, Germany) and the 1kb ladder (Promega, Madison) were used on separate occasions during this study (Appendix B). Electrophoresis was performed at 2-10 volts/cm for 1-10 hours. The fluorescence was detected by means of a Kodak digital camera DC 120.

2.3.5 Polymerase chain reaction

The Polymerase chain reaction (PCR) is a primer-directed enzymatic amplification of DNA using a thermostable DNA Polymerase isolated from *Thermus aquaticus*, called Taq DNA Polymerase (Saiki *et al.*, 1988). The reaction involves alternate cycles of template denaturation, primer annealing and DNA strand extension by the Polymerase (Saiki *et al.*, 1988). The thermostable DNA Polymerase is used since it is not denatured or inactivated at high temperatures required to denature the double stranded template (Saiki *et al.*, 1988)

2.3.6.1 16S rDNA Gene Amplification

To determine the suitability of template DNA for PCR, the 16S rDNA was amplified using primers designed to amplify an approximately 1,000-bp fragment. The forward and reverse primers used were pA (5'-AGAGTTTGATCCTCGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGA-3'), respectively (Edwards et al., 1989). Primers were synthesized by GIBCO-BRL (Life Technologies). The 16S PCR amplification method was performed in a 50 µl reaction volume containing approximately 300 ng DNA, 0.5 µM each primer, 10× PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin (TaKaRa), 2.5 mM of each dNTP (TaKaRa), 0.5 units *Taq* Polymerase (TaKaRa). PCR amplification was performed in a Perkin Elmer GeneAmp PCR System 2400. Initial denaturation was carried out at 95°C for 120 seconds, followed by 30 amplification cycles, each consisting of 94°C for 60 seconds, 52°C for 45 seconds and 72°C for 120 seconds. A final primer extension at 72°C for 10 minutes was included. Aliquots (10 µl) of PCR products were electrophoresed on a 1.5% agarose gel 1-½ 100V for 1 ½ hours. The resolution was visualized using a Kodak digital camera DC 120. The PCR conditions are summarised in the Table 2.1 below.

Table 2.2 PCR conditions for 16S rDNA amplification

Step	Temperature (°C)	Time (seconds)
Initial denaturation	95	120
Secondary denaturation	94	60
Annealing	52	45
Extension	72	120
Final Extension	72	600

2.3.7 PCR fingerprinting using Poly-GTG typing

The reaction components and thermocycle profile were described previously (Matsheka, 2000) and no alteration was made to this protocol. The reactions were performed in 25µl reaction volumes containing 50pmol of $(GTG)_5$ primer, 200µl dNTP, 1.25 Units of TaKaRa *Taq* and 1x TaKaRa reaction buffer which contained 1.5 mM $MgCl_2$. Takara Biotechnology supplied all the components. An amount of 400ng of genomic DNA was used as template per reaction volume. The reaction mixture was made up to 25µl with distilled water The reaction profile included a two-step denaturing process, which included 4 cycles and 30 cycles respectively. Each thermocycle consisted of three steps: denaturation, annealing and extension. The reaction profile used is summarised in Table 2.3 below.

Table 2.3 PCR Protocol for Poly-GTG typing

Step	Temperature (°C)	Time (minutes)
1) Denaturation	95	2
2) Annealing	36	2
3) Extension	72	2
4) Denaturation	95	1
5) Primer Annealing	50	1
6) Extension	72	2
7)Final extension	72	10
8) Holding temperature	4	-

The reactions were performed in a Perkin Elmer (California, USA) Model 24600 PCR machine.

Amplification products were analyzed by electrophoresis (section 2.2) Two microlitres of tracking dye was added to 6 µl of PCR reaction mix in a final volume of 10 µl. The samples were then set to electrophorese for approximately 18 hours in a 2% (w/v) agarose gel submerged in 1xTAE (Appendix A). The slow electrophoresis was to allow for better resolution.

2.4 RESULTS

2.4.1 Antibiotic susceptibility

Thirteen clinical isolates of *A. baumannii* were included in this study. Disc susceptibility testing was used to determine the antibiotic resistance profile, as shown in Table 2.4 and MICs were determined according to the NCCLS criteria. This testing showed that 10 of these strains were resistant to the combination of piperacillin/tazobactem (MIC >32µg/ml), while another strain exhibited only intermediate resistance (MIC<32µg/ml) as seen in Table 2.4 below. During the same period, a susceptible strain (MIC 0.16µg/µl) of *A.baumannii* was isolated and it was used as a control for comparison. In addition to these 12 strains, another clinical strain of *A.baumannii* isolated outside of the time period was used as an out-group for the typing studies.

Table 2.4 Antibiogram patterns of *A.baumannii* strains used in the study

Isolate number	Antibiotic resistance profile	Pip-Tazo ^{R}
1	COAM,AMOX,CTX,CXM,CAZ,PPL,PTZ	Resistant -R
2	CAZ,PPL,PTZ,AMOX,COAM,CTX,CXM	R
3	COAM,CTX,CXM,AMOX,CAZ,PPL, PTZ	R
4	CXM,AMOX,CTX,COAM,CAZ,PPL,PTZ	R
5	COAM,CXM,CTX,AMOX,CAZ,FEP,PPL, PTZ	R
6	COAM,AMOX,CTX,CXM, CN,CAZ,PPL,PTZ	R
7	CAZ,FEP,PPL,PTZ,COAM, AMOX,CTX,CXM	R
8	CAZ,FEP,PPL,PTZ,AMOX,CTX, COAM,CXM	R
9	CTX,CXM,COAM,AMOX, CAZ,FEP,PPL,PTZ	R
10	COAM,AMOX, CTX,CXM,CAZ,FEP, PPL,PTZ	R
S	SXT, sensitive to :CTX,CXM, ,AMOX, CAZ,FEP,PPL,PTZ	S-sensitive
I	C, CN, AK, PTZ, sensitive to : CTX,CXM, ,AMOX	I-intermediate
Outgroup	CAZ,FEP,PPL,PTZ,AMOX,CTX, COAM,CXM	R

Abbreviations: AK, amikacin; **AMOX**, amoxicillin; **CN**, gentamicin; **COAM**, coamoxyclav; **CTX**, cefotaxime; **CAZ**, ceftazidime; **CXM**, cefuroxime; **PPL**, piperacillin; **PTZ**, piperacillin-tazobactam

2.4.3 POLY-GTG TYPING

2.4.3.1 Suitability of DNA Template for PCR (16S rDNA PCR)

Prior to carrying out PCR experiments for poly-GTG typing and other PCR based methods [Chapter 3], the suitability of extracted genomic DNA for PCR application was investigated using 16S rRNA PCR. Using universal primers specific for the coding region of the 16S rDNAa a single, 1,500-bp fragment was amplified from all of the samples [Fig. 2.4], indicating the suitability of the template for PCR application and the absence of any inhibitors in the reaction mix.

2.4.3 16sDNA PCR- Testing DNA integrity for PCR assay

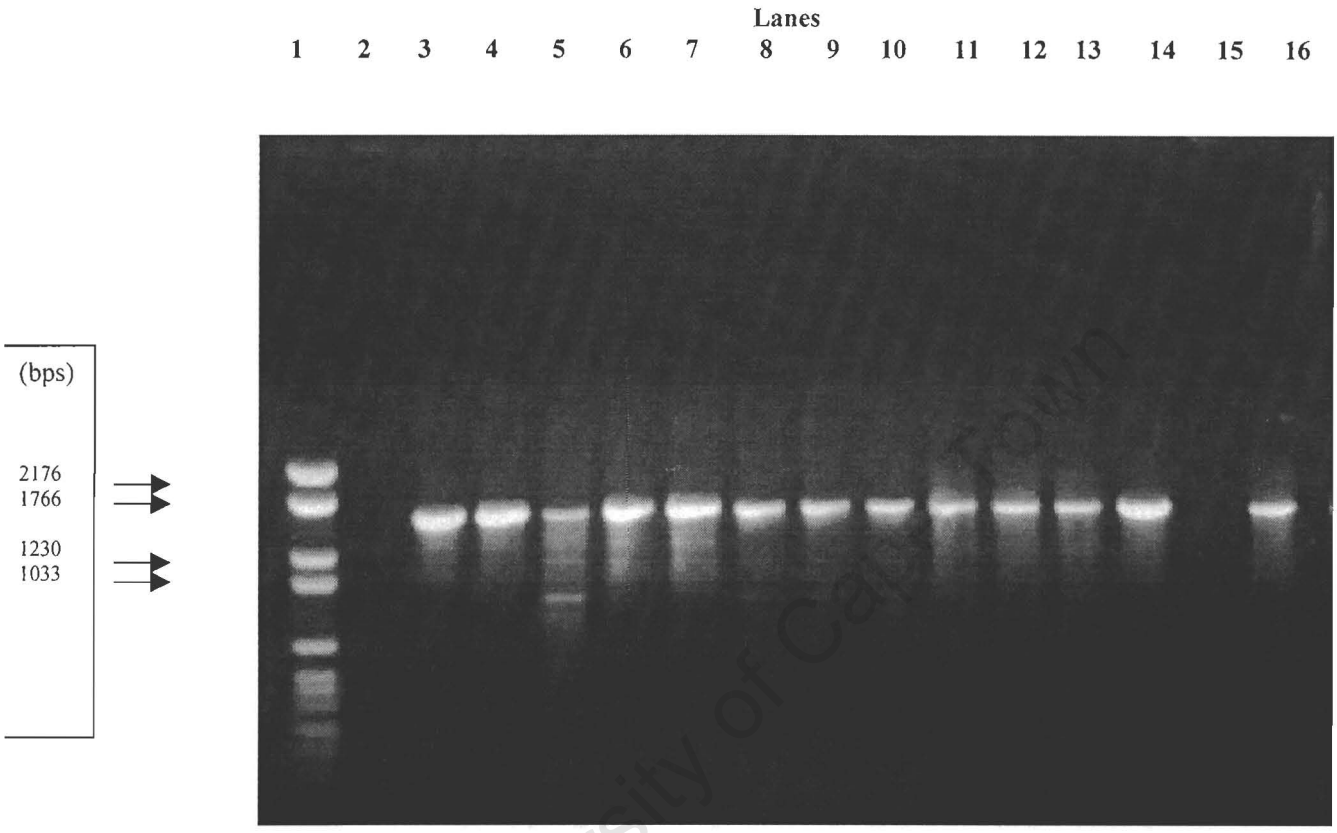

Fig 2.1 Amplicons from A.baumannii strains following 16S rDNA PCR:
Lane 1, DNA marker VI (Boehringer Mannheim); lane 2, empty;
Lanes 3-12, resistant strains 1-10; Lane 13, Intermediate resistance strain;
Lane 14, Sensitive strain; Lane 15, empty; Lane 16, outgroup strain.

2.4.3.2 Poly-GTG typing of *A.baumannii* strains

Poly-GTG typing was used to investigate the epidemiology of the clinical strains of *A.baumannii*. Thirteen strains were used in this study, 11 formed part of a cluster and one sensitive strain was used as a reference. Another strain of *A.baumannii* was taken from a different time period, to serve as an outgroup.

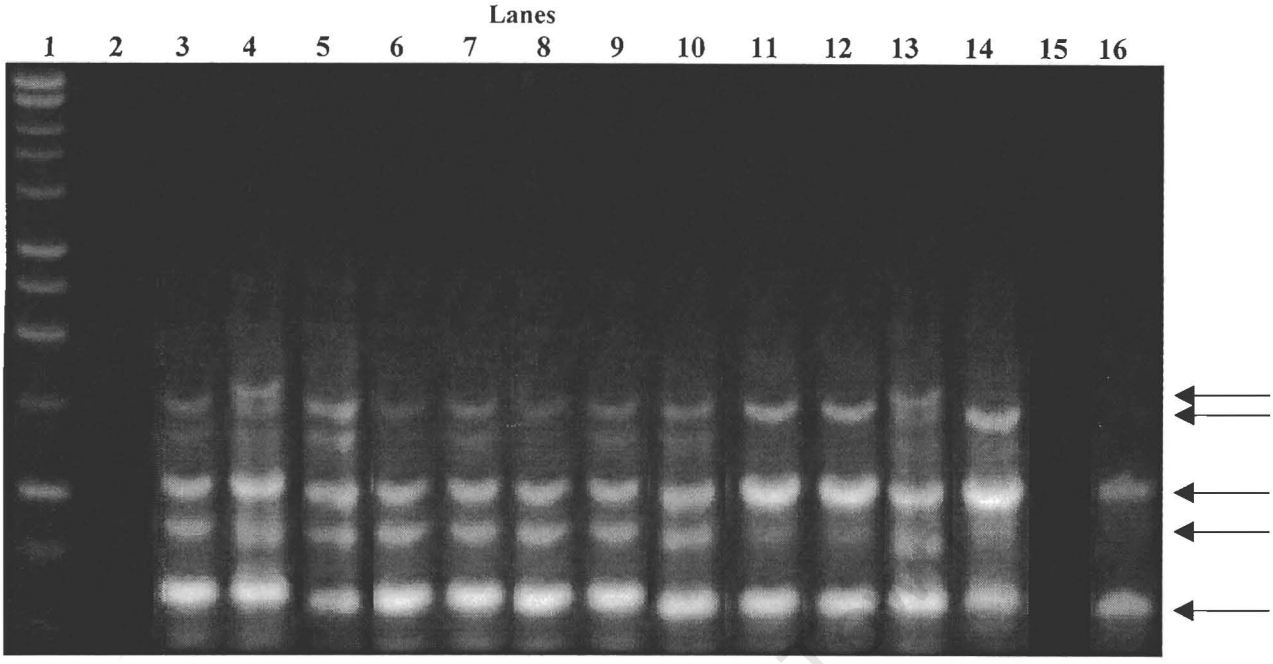

Fig 2.2 Amplicons from A.baumannii strains following polo-GTG typing:
Lane 1, DNA marker VI (Boehringer Mannheim); lane 2, empty;
Lanes 3-12, resistant strains 1-10; Lane 13, Intermediate resistance strain;
Lane 14, Sensitive strain; Lane 15, empty; Lane 16, outgroup strain.

Two profiles were observed (Fig.2.2); one type of banding pattern, consisting of 5-6 bands was identified in the sensitive, intermediate and the 10 resistant *A. baumannii* strains. A different banding pattern consisting of 2 bands was observed in the outgroup.

The poly-GTG typing was repeated in order to ensure consistent results of the experiment and also the accuracy of the first set of results. The two results correlated well and showed the relatedness and disparities between these clinical strains.

2.4 DISCUSSION

The use of the drug combination of piperacillin and tazobactam was implemented at GSH for the treatment of *A. baumannii* infections, in 1996. Since then, in less than 2 years, this combination ceased to be as effective, with *A.baumannii* developing resistance to this combination as well as a number of other β lactam antibiotics. Table 2.6 below shows the

incidence of *A. baumannii* strains in a two-year period, isolated from Groote Schuur Hospital. The drug combination of piperacillin/tazobactam was used until July 2000, when imipenem became the drug of choice. It is interesting to note the concomitant decrease in resistant strains following a change in the drug regimen.

Time period	Sensitive	Intermediate	Resistant
1 July –31 December 1999	370	102	156
1 January- 30 June 2000	338	191	139
1 July- 31 December 2000	228	109	70
1 January- 31 July 2001	181	132	40

Table 2.5 Incidence of piperacillin/tazobactem resistant strains over a two year period

A cluster of eleven strains were chosen in the period of January -February 2000, which exhibited resistance to this combination as well as to the other cephalosporins such as cefuroxime and ceftazidime. This extended spectrum of resistance was a cause for concern for future drug regimes. As the strains were isolated from disparate wards [Table 2.1], this suggested some form of resistance transfer, which did infer solely plasmid involvement. The isolation of strains from the disparate wards and sites, suggests that it was the organism carrying the resistance (whether chromosomally or extra-chromosomally) that spread through the hospital. It is most likely that the organism spread on the hands of uninfected hospital staff (human carriage) (Allan and Green, 1994) and also possibly spread via contamination of inanimate sources such as ventilator equipment, taps and beds (Mirren et al., 1990).

This outbreak appeared to be self-limiting, and as with *A.baumannii* infections, ceased to be a problem with the implementation of a new drug regime. The next course of action against *A.baumannii* infections was the carbepenems, initially imipenem and now meropenem, which is the current drug of choice at GSH for *A.baumannii* infections. It may be only a matter of time before this becomes ineffectual. Imipenem resistant Acinetobacters have been described as early as 1993 (Paton et al., 1993). More carbapenemases have being detected and earlier on this year, Yiu *et al* (2001) isolated IMP-4 from an *A. baumannii* strain. IMP-4 is a novel carbapenemase, which is active

mainly against imipenem but to a certain extent meropenem as well. This strain was isolated in Japan, where this is also the drug of choice.

Having observed a growing resistance amongst the species, it then becomes important to understand its epidemiology. Previously, in a clinical setting it had been observed that the prevalence of *Acinetobacter* spp. infections was quite low- with more cases being reported for colonization rather than infection (Dijkshoorn *et al*, 1993). Despite this low demand for diagnostic testing, it becomes important to type strains during outbreak or endemic episodes in order to discern between epidemic and sporadic strains. It is also useful in tracing the source of infection (Arbeit, 1995). In addition, typing also contributes to the understanding of the pattern of spread of infection and may be useful in influencing strategies for prevention of spread (Koeleman *et al*., 1998). It was decided that the strains should be typed in order to better understand their epidemiology.

Previously, a number of different typing techniques were shown to be useful in investigating the epidemiology of *Acinetobacter* spp. PFGE was shown to be a good discriminatory technique when compared against plasmid profiling and whole cell protein profiling (Seifert *et al*., 1994). RAPD, specifically, the use of ERIC primers also demonstrated good discrimination between outbreak strains of *A.baumannii* (Cimolai and Trombley, 1997). However, these techniques are fairly expensive, and the reliability of demonstrated using poly-GTG typing in our department (Matsheka, 2000), suggested that this tool could be used to investigate the origins of the strains and so made it the typing tool of choice for this project.

The technique worked well enough to observe a distinct profile. The GTG-profile obtained was very interesting. The strains; both the resistant and sensitive strains appeared to have a similar profile [Fig. 2.1.A lanes 1-12]. It can be clearly seen that all the resistant strains from the same group shared a similar profile. The same is true for the sensitive strain suggesting that they are part of the same cluster and share a common origin and hints at the fact that the resistance developed or was acquired later. As Koeleman *et al*., (1998) demonstrated, the common profiles could be taken as an

indication of a common origin. The outgroup (strain 13) does not share the same profile, suggesting that the strains that were initially isolated were in fact part of an outbreak. The parent strain/ progenitor was most likely susceptible to the β lactams, but in a short period of time, either acquired or developed resistance. Further investigation into the mechanisms of resistance of these strains will be further discussed in detail in the subsequent chapters.

CHAPTER 3

BETA LACTAMASES

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3.4 DISCUSSION

CHAPTER 3

DETECTION OF BETA LACTAMASES

3.1 INTRODUCTION

Bacterial resistance to the beta (β) lactam family of antibiotics is mediated in a number of ways. These include the alteration of PBPs, decreased permeability to the drug or inactivation of the β lactam drug by enzymes (Edberg, 1983). The most frequently described mechanism of resistance, is the enzymatic inactivation of β lactams (Page, 1991) by β-lactamases. A number of these β-lactamases have been described in *Acinetobacter*. As the focus of this chapter is the β-lactamases, an overall description of the various enzymes that are thought to play a role in resistance, will follow.

It was necessary, with the emergence of β-lactamase producing antibiotic resistant bacteria, to find some alternative way of allowing the antibiotic to work. One of the strategies developed to combat the action of β-lactamases was the development of compounds that would inhibit β–lactamases. To date, three compounds have been developed for clinical use; clavulanate, sulbactam and tazobactam (Livermore, 1993). The mode of action of these inhibitors is similar; they all bind irreversibly to the β lactamase, rendering it ineffective by means of suicide inactivation (Livermore, 1993). These three inhibitors have been used, in combination with newer β lactam antibiotics to treat infections resulting from bacteria producing β–lactamases.

3.1.1 Classification of the β–lactamases

Over the years a number of schemes have been proposed to classify the various β lactamases. Many of these schemes have drawbacks; as the selection criteria becomes more complex resulting in complex classifications.

One of the more commonly used classification schemes is the Ambler scheme (1980). It classifies the β–lactamases in four groups, a-d. This scheme is based on structure; groups a, b, c and d are evolutionarily distinguishable groups of enzymes and contain a serine residue at the active site. The class A enzymes include the plasmid encoded TEM and SHV enzymes. The chromosomally encoded enzymes fall into the class c classification, while class d describes those enzymes that preferentially hydrolyse cloxacillin (oxacillin) as well as penicillin. Class b, is unique in that the active site of the enzyme carries a zinc ion and these enzymes are referred to as metallo-enzymes. One of the characteristics of these class b enzymes is that they are able to hydrolyse the carbapenems.

For the purposes of this thesis, I will confine myself to the Ambler classification scheme, even though that of Bush (1996) is the most recent and most comprehensive to date.

A number of β–lactamases have been described in *Acinetobacter* spp. [Chapter 1]

As various enzymes such as AmpC, TEM, SHV and PER have been shown to mediate resistance to the β-lactam family of antibiotics in *Acinetobacter*, it is likely that they also play a role in the antibiotic resistance observed in the clinical isolates of *A. baumannii* from Groote Schuur Hospital. Thus, our investigations sought to determine whether the resistance was mediated by either of the enzymes, AmpC, TEM, SHV, PER-1, or any other β-lactamases. To investigate the role of β-lactamases in the clinical isolates in this study a number of assays were carried out including Iso-electric focussing (IEF), PCR, and DNA: DNA hybridisation, transformation studies and sequencing.

3.2 MATERIALS AND METHODS

3.2.1 STRAINS

The 12 clinical isolates of *A. baumannii* that formed part of the epidemiological study [Table 2.1] formed part of the β-lactamase study as well. As the epidemiological study

suggested that the isolates were related [Chapter 2], some of the assays were carried out using only one resistant strain (strain 2) and the sensitive strain

3.2.2 Antibiotic susceptibility testing

As previously mentioned, isolates were identified as *A. baumannii* by the Clinical Microbiology diagnostic laboratory of Groote Schuur, using standard morphological and biochemical tests.

Antibiotic sensitivity testing was determined by the disc diffusion test. Disc susceptibility testing was carried out according to the National Committee for clinical Laboratory Standards criteria (National Committee for Clinical Laboratory Standards criteria, 1998a). The resistance profile has been previously described [Table 2.5].

3.2.3 Detection of Extended Spectrum β Lactamases

This test was also carried out in the Clinical Microbiology Diagnostic Laboratory at Groote Schuur Hospital. The presence of extended spectrum β lactamases (ESBLs) is determined by means of the double disc diffusion test. This is the standard method for detecting the presence of ESBLs in bacteria, as dictated by the National Committee for clinical Laboratory Standards criteria (National Committee for Clinical Laboratory Standards criteria, 1998a). It is known that the ESBLs are inhibited to varying degrees by clavulanate, tazobactam and sulbactam. A disc containing one of the cephalosporins (often ceftazidime) is placed proximal to a disc containing clavulanate on agar inoculated with clavulanate. If an ESBL is produced by the isolate an increased zone of inhibition will occur between the 2 antibiotic discs, giving rise to a dumbbell shaped zone. This occurs because the clavulanate inhibits the action of the ESBL and the cephalosporin can then act in its absence.

3.2.4 Iso Electric Focussing

The β-lactamase content was primarily determined by Iso-electric focussing (IEF). IEF was initially described by Matthew *et al.*, 1976. It is a method of separation, in which proteins align themselves as sharp bands at the specific iso-electric points (pI) in an electrophoretically produced pH gradient. (Matthew *et al.*, 1976). The method almost always gives good resolution because forces that act against diffusion cause the focussing and therefore the proteins are concentrated during their separation. In addition to using this technique for the separation of any proteins, it has also been used successfully for the analysis of β-lactamases and is now the standard technique for detecting the presence and identifying the iso-electric focussing points of β-lactamases present in any given bacterial strain. The visualisation of the position and activity of β-lactamases is facilitated by the use of a chromogenic cephalosporin substrate, nitrocefin, whose degradation and subsequent colour change allows the detection of the position and activity (O'Callaghan *et al.*, 1972).

3.2.4.1 β-lactamase extraction

The extraction of β-lactamases was carried out using the protocol described by Matthew *et al.* (1976). A single colony of each culture was inoculated overnight into 5 ml 2YT (Appendix A) at 37°C with shaking. The strains were then sub-cultured 1/100 into 2YT (Appendix A) and subject to the same growth conditions. The culture was kept on ice for all the subsequent manipulations. The cells were harvested in a centrifuge (J2-21) for 10 minutes at 4°C. The harvested cells were then washed in 100mls of cold 10mM phosphate buffer (Appendix A), pH 7.2 once and re-centrifuged. This time the harvested cells were resuspended in 5ml of 10mM phosphate buffer. The chilled suspension was then passed through a French Pressure Cell, three times at 9000 psi. This treatment by the French Pressure cell releases intracellular enzymes as well as other intracellular proteins. The cell extract was then centrifuged for 10 minutes at 1000g to remove the unbroken cells and the supernatant was used for the subsequent β-lactamase assays. The β-lactamases would be distinguished from the other intracellular proteins by their

colourimetric reaction with nitrocefin, which is a substrate for the enzymes (O'Callaghan *et al.*, 1972; Matthew *et al.*, 1975)

The proteins were separated using an acrylamide gel system. Two polyacrylamide gel (PAG) systems were used for the IEF assays at different times: a **horizontal system** and a **vertical system**.

3.2.4.2 Horizontal PAG-IEF

Horizontal PAG-IEF was initially carried out at the University of the Western Cape Biochemistry Department, under the supervision of Dr. Graham Bradley. The protocol for horizontal PAG-IEF was described by Matthew *et al.* (1975). The PAG-IEF was performed in pre-cast gel slabs (120x 120 x 0.55mm) in an LKB 2117 Multiphor II Unit. The gel contained 10% acrylamide (v/v) (30 :1; acrylamide: bis-acrylamide) and 2% ampholytes (pH 3-10). A 1M sodium hydroxide (NaOH) solution was used as the electrolyte at the cathode and 1M phosphoric acid (H_3PO_4) as the electrolyte at the anode. The gel was cooled to ~10°C prior to the loading of the samples and maintained at this temperature throughout the focussing, with a Laua thermostatically controlled waterbath/cooling system. After pre-focussing for 20 minutes at 400V, 3 mA and 6 W, the protein extracts (at a concentration of ~ 20µg per reaction volume), and protein samples were loaded onto the gel. The necessary dilutions were made in 10mM phosphate buffer. No tracking dye was added to the samples in this assay. The gel was focussed at 500V, 6mA and 10W for 1 hour, after which the voltage was increased to 600 V at the same wattage and current and the gel allowed to continue for 90 minutes. Following the focussing, the gel was stained with nitrocefin, (concentration of 0.5mg/ml) to assay for β-lactamase activity (O'Callaghan *et al.*, 1972). The colour of intact yellow nitrocefin becomes pink when the β-lactam bond is broken, so the focussed bands with β-lactamase activity appeared as pink bands against a yellow background. This colour change was taken as an indication of the presence and activity of β-lactamases.

3.2.4.3 Vertical PAG-IEF

Vertical PAG-IEF was carried out at the Department of Medical Microbiology at the University of Cape Town. The protocol for this system was described by Robertson *et al.* (1987). This method was employed used due to the availability of apparatus and low cost, when compared with the horizontal system. A mini-gel system was used, and a gel of dimensions 90x70x 1.5mm was cast. The gel was comprised of 5% acrylamide (acrlyamide: bis-acrlyamide; 30:1), 10% glycerol and 2% amphloytes (pH 3-10). The components were de-gassed (by allowing to stand at room temperature for 15 minutes) and then 50µl of 10% ammonium persulphate (APS) and 20µl of TEMED were added. The gels were allowed to polymerise for an hour prior to loading. As with the pre-cast gels, no tracking dye was added. In this system, the cathode solution used was at a lower concentration, 25mM NaOH and the anode solution was 20mM acetic acid. The solutions were pre-cooled to 4°C prior to electrophoresis. Protein standards and samples were mixed with equal volumes of 60% glycerol (v/v) and 4% ampholytes (v/v) (pH 3-10). Electrophoresis was performed at a constant 200V for 1.5 hours at 4°C, then allowed to proceed at 400V overnight at 4°C. After electrophoresis, the gel was rinsed briefly in distilled water before being subjected to the nitrocefin assay, as described for the horizontal system.

3.2.5 EXTRACTION OF DNA

3.2.5.1 Extraction of Genomic DNA

Two methods were used for the extraction of genomic DNA: CTAB extraction as described by Ausubel and Sambrooke, and the boiling method. The method of extraction using CTAB has been described previously [2.3.2]. The second method, boiling, was described by Grimberg *et al.*, (1989). A single colony was suspended in 100µl of distilled water. The genomic DNA was released following a 5-minute boiling step. Cellular debris was removed by centrifugation in a microfuge (Eppendorf Centrifuge 5415C) for 2

minutes at 14 000 rpm. The DNA extracted via the boiling method was used primarily for PCR assays.

3.2.5.2 Plasmid DNA extraction

Plasmid DNA was extracted using two methods: small scale and large scale. The method employed was determined by the requirements of the assay. Small scale extractions were carried out when the small amounts DNA were required for checking recombinant clones. Large scale preparations were carried out when high quality and high yields of DNA were required, for example for cloning and sequencing reactions.

3.2.5.2.1 Small-scale plasmid preparation

Plasmid DNA was extracted from cultures using the method described by Ish-Horwicz and Burke (1981), and Sambrooke *et al.* (1989). A single colony was inoculated separately into 5ml of 2YT broth cultures, containing 25μg/ml ampicillin (Ranbaxy, South Africa) and incubated overnight at 37°C with shaking. A volume of 1.5ml of each culture was harvested following centrifugation at 14 000 rpm for 5 minutes in a microfuge (Eppendorf Centrifuge 5215C). The cells were resuspended completely in 200μl of Solution 1 (25mM Tris-HCl pH 8.0, 50mM EDTA, 1% w/v glucose;Appendix A). A volume of 400μl of solution 2 (0.2M NaOH, 1% w/v SDS;Appendix A) was added to denature the proteins and the resuspension was mixed until a viscous solution formed. The mixture was kept on ice for 5 minutes. Precipitation of cellular protein and chromosomal DNA was achieved by the addition of 300μl of solution 3 (Appendix A)to the viscous solution, and kept on ice for a further 5 minutes. A curdy white precipitate that formed was collected by centrifugation in a microfuge (Eppendorf Centrifuge 5215C) and discarded. The plasmid DNA was precipitated by the addition of 0.6 volumes of isopropanol, left at room temperature for 10 minutes and then centrifuged for a further 10 minutes at 14 000 rpm in a microfuge. The plasmid DNA was washed in 70% ethanol, air-dried and re-suspended in either TE (Appendix A) or distilled water.

3.2.5.2.2 Large-scale plasmid preparation

When large quantities of purified plasmid DNA were required, a commercial kit, Nucleobond Kit (Machery Nagel, Germany) was used. The extraction of DNA with this kit, works on the same principle as the alkaline lysis method employed in small scale extraction, with a few modifications to some of the solutions (appendix A).The plasmid DNA was extracted from a larger volume (100mls) of 2YT. After extraction of the DNA following the manufacturer's instructions, the lysate was filtered through 3M Whatmann paper to remove cellular debris. Purification and isolation of DNA was achieved through the use of an ion exchange silica cartridge. The lysate was loaded onto the cartridge and washed with a number of buffers, (N3). Plasmid DNA was eluted by the use of N5 which was facilitated by a change in pH (Appendix A) and precipitated with 0.6 volumes of isopropanol. Plasmid DNA was washed in 70% ethanol, air-dried and resuspended in TE (Appendix A) or dilstilled water.

3.2.6 Restriction endonuclease digestion

The presence of an insert in a putative recombinant plasmid was determined with restriction endonuclease digestion. Depending on the vector, different enzymes were used for releasing the insert; *Hind* III for pBGS8 (Appendix C) recombinants and *EcoR* I for pGEMT-easy (Appendix C) recombinants. The enzymes of choice had a site in the multiple cloning site of the vector (Appendix C). The enzymes were used at an activity of 20U/ reaction volume, and digestion was allowed to proceed at 37°C for 2 hours. The resulting DNA fragments were separated by AGE (Section 2.3.4) in a 1% (w/v) agarose gel in the presence of a commercial marker to determine size.

3.2.7 Agarose gel electrophoresis

Agarose gel electrohoresis (AGE) was carried out as mentioned previously (section 2.3.4)

3.2.8 Polymerase Chain Reaction

Polymerase chain reaction (PCR) assays were used to detect the presence of β–lactamase genes.

3.2.8.1 PCR for the detection of *ampC*

The reaction components and thermocycle profile were originally described by Bou and Martinez-Beltran (2000). The primers were directed against sites internal to the *ampC* gene. A 50µl amplification mix was comprised of 20mM Tris-HCl, 100mM KCl, 2.0 mM $MgCl_2$, 200µM dNTP, 50pmol of each primer, P1 (5'-taaacaccacatatgttccg) (GibCo Ltd.) which anneals to position 1129-49, P2 (5'- acttacttcaactcgcg) (GibCo Ltd.) which anneals to position 670-690, and 500ng of chromosomal DNA. The reaction was facilitated by 2.5U of *Taq* polymerase. TaKaRa supplied all the reaction components, except the primers and the chromosomal DNA. The thermocycle profile is summarised in Table 3.1 below.

Table 3.1: Thermocycle profile for the *ampC* PCR assay

Reaction Step	Temperature (°C)	Time (minutes)
Initial denaturation	94	4
30 cycles of the following		
Denaturation	94	1
Annealing	50	1
Extension	72	2
Final extension	72	10

The reactions were carried out in a Perkin-Elmer (California, USA) Model 24 600. A volume of 5-10µl of reaction mix and 2 µl of tracking dye was made up to 20µl with distilled water . The PCR products were visualised by AGE (section 2.3.4) .

3.2.8.2 PCR for the detection of SHV related genes

Primers were designed to anneal to the conserved sequences of all SHV-related genes (Whitelaw, 1999). A 50µl reaction volume was comprised of 10mM Tris-HCl (pH 8.3), 50mM KCl, 2.5mM $MgCl_2$, 25mM dNTPs, 10pmol of each primer; SHV-A: (5`-CTG GCG GCTACACGCCAGC) and SHV B: (5`- TGCGCTCTGCTTTGTTA), 500 ng of genomic DNA and 2.5U of *Taq* polymerase.

SHV A anneals to bases 154-171 and SHV B anneals to 852-868 respectively of SHV-1 gene. The primers were synthesized by an oligonucleotide synthesizer at GibCO Ltd.

The thermocycle profile is summarised in the table below

Table 3.2: Thermocycle profile of SHV PCR

	Temperature (°C)	Time (seconds)
Number of cycles: 30		
Denaturation	94	30
Annealing	55	30
Extension	72	50

3.2.8.3 PCR for the detection of TEM-related genes

PCR was used once again, this time, to detect the presence of TEM genes in *A. baumannii*. The primers used for the assay were designed by Canica *et al.*, (1997) to amplify a TEM- type β lactamases. A 50µl reaction volume was comprised of 10mM Tris-HCl (pH 8.3), 50mM KCl, 2.5mM $MgCl_2$, 25mM dNTPs, 10pmol of each primer; FIN (5'-ATTCTTAAGACGAAAGGGC) and DEB (5'-

ATGAGTAAACTTGGTCTGAC), 500 ng of genomic DNA and 2.5U of *Taq* polymerase.

This PCR assay was carried out on the 10 clinically resistant strains, the intermediate resistant strain and the sensitive strain of *A. baumannii*. The PCR conditions were previously described (Belaaouaj *et al.*, 1994).

The TEM PCR product was further investigated and this was facilitated by cloning the amplification product and subjecting it to automated sequencing

3.2.9 DNA:DNA hybridisation

DNA:DNA hybridisation assays were carried out to confirm the presence of the AmpC β-lactamase. The absence of a signal following Southern blot hybridisation, led to the use of a slot blot apparatus to obtain a signal. The following is a description of Southern blot hybridisation using a commercial kit, the ECL direct nucleic acid labeling and detection system (Amersham International; Buckinghamshire, UK)

3.2.9.1 Preparation of blot

Five micrograms of genomic DNA were digested at 37°C using 20 units (U) of Hind III (Boehringer Mannheim; Mannheim, Germany) in 1x buffer B (Boehringer Mannheim; Mannheim, Germany). The reaction mix was made up to a final volume of 20µl with distilled water. The reaction was allowed to proceed at for approximately 6 hours to ensure complete digestion of the DNA. The digested DNA was electrophoresed overnight in a 1 % (w/v) agarose gel in 1xTAE (Appendix A). The digested DNA was to be used as a template for DNA:DNA hybridisation experiments. The DNA was transferred to a Hybond N+ membrane (pre-wet with 10xSSC), resting on filter paper. The transfer apparatus was set up according to the manufacturer's instructions and allowed to proceed overnight. The DNA was then cross-linked to the membrane using UV light for 30

seconds at a wavelength of 254nm in a Hoefer Scientific Instruments (California, USA) UV cross-linker. The membrane was then subjected to blocking and hybridisation using a labelled probe.

3.2.9.2 Preparation of probe

A PCR amplification product was electrophoresed (section 2.3.4) and the fragment was excised, and the DNA was eluted from the agarose using phenol. The agarose was cut into very small pieces and placed in a microfuge tube. An equal volume of phenol was added to solubilise the agarose and release the DNA (Sambrook *et al.*, 1989; Seth 1984) and mixed by vortexing the tube. The tube was placed at -70°C for 10 minutes and then centrifuged at 14 000 rpm for 10 minutes in a microfuge (Eppendorf Centrifuge 5415C). After centrifugation, the upper aqueous phase containing the DNA was removed and the DNA was further purified by extraction with phenol-chloroform-isoamylalcohol as described by Sambrook *et al* (1989). An equal volume of phenol-chloroform-iso-amyl was added and after mixing the solution was centrifuged as before to remove any residual agarose and phenol. The DNA in the resulting supernatant was concentrated by precipitation with 1/10 volume of 4M LiCl (Appendix A) and 3 volumes of 100% ethanol. After placing the tube at –70°C for approximately 10 minutes to allow for precipitation, the DNA was collected by centrifugation as before. The DNA pellet was then washed in 70% ethanol , dried at 37°C for 10-15 minutes and then resuspended in 20µl of distilled water.

The DNA was left to resuspend for an hour, and following quantitation, 500 ng DNA was labeled using the ECL direct nucleic acid labeling and detection system (Amersham International; Buckinghamshire, UK) according to the manufacturer's instructions. The DNA was denatured by boiling for 5 minutes, snap cooled for a further 5 minutes to prevent renaturation and labeled with horse radish peroxidase (which is responsible for the loose attachment formed between the label and the DNA) and complexed with a positively charged polymer. Addition of glutaraldehyde resulted in the formation of chemical cross-links between the DNA and the peroxidase–polymer complex so that the

enzyme was covalently attached to the DNA. The labeled fragment was used in hybridisation experiments.

3.2.9.3 DNA:DNA Hybridisation

A volume of hybridisation buffer (Appendix A) equivalent to 0.125 ml/cm2 of membrane was used for the assay. A blocking agent which, was designed to pre-coat all the sites of the membrane to which the probe could bind non-specifically, was also added to the buffer in a final concentration of 5% (w/v). NaCl was also added to a final concentration of 0.5M. This blocking step was carried out in a Hybridiser HB 1D oven (Techne; Cambridge, UK) and was allowed to continue at 42°C for approximately 1 hour, during which time the non-specific binding sites on the membrane would be blocked. Thereafter the probe was added to the buffer and the hybridisation was allowed to continue overnight at 42°C. During this time, the probe was expected to hybridise to like genomic DNA immobilised on the Hybond N+ membrane .

3.2.9.4 Preparation of membrane and signal detection

After hybridisation the membrane was washed to remove unbound excess probe. Firstly the membrane was rinsed in 50-100ml of 5xSSC at 55°C for 10 minutes. Thereafter it was subject to two 5 minute washes in 100ml of primary wash buffer (without urea) (Appendix A). Washing the membrane in a low concentration of SSC and at 55°C increased the stringency of the wash, thereby creating conditions, which would allow for the hybridisation of the highest specificity between the probe and the target DNA. The membrane was subject to a further two washes, at room temperature (RT) on a Stovall Low Porfil Roller (Stovall Life Science, Inc.; North Carolina, USA) for 5 minutes in approximately 100 ml of in a secondary buffer (2xSSC).

Signal generation and detection was performed according to the manufacturer's instructions supplied with the ECL kit. Detection reagents 1 and 2 were mixed in equal volumes to give sufficient solution to cover the membrane. On completion of the post-

hybridisation washes, excess buffer was drained from the membrane, which was then flooded with the detection reagent mix and incubated at room temperature for 1 minute. Excess detection reagent was drained from the membrane, which was then sealed in a plastic sleeve. The reaction, which proceeds in the sleeve, is one where solution 1 decays to hydrogen peroxide, the substrate for peroxidase, with which the probe was labeled. The reduction of hydrogen peroxide by the peroxidase is coupled to a light producing reaction catalysed by detection reagent 2, which contains luminol, and produces blue light on oxidation. An enhancer prolongs and increases the light output, which can be detected on a blue-light sensitive film. The membrane was exposed to an X-ray film (AGFA CP-BU; Mortsel, Belgium) for 2 hours and the autoradiograph was developed.

3.2.9.5 Slot Blot DNA:DNA hybridisation

This assay was carried out to determine the presence of the *ampC* gene following the absence of a signal using Southern blot hybridisation.. Three different concentrations of genomic DNA from each of the *Acinetobacter* isolates was transferred to a Hybond ™-N + (Amersham International; Buckinghamshire, UK) using the manifold II Slot-BLOT apparatus (Schleicher and Scheull; Germany); 500 ng, 1μg and 5 μg respectively. Each sample was made up to a final volume of 50μl with distilled water.

The DNA was then denatured at 95°C for 10 minutes and placed on ice to prevent renaturation. Fifty microlitres of transfer buffer, 20XSSC (Appendix A), which provides the ionic strength necessary for the binding of DNA to the nylon membrane (Sambrook *et al.*, 1989), was then added to the denatured DNA. The slot blot apparatus was then assembled according to the manufacturer's instructions with the Hybond –N+ membrane (pre-wet with 10xSSC) resting on filter paper (also prewet with 10XSSC). The samples were then carefully loaded into the separate slots and a vacuum was applied until transfer to the membrane was complete. The apparatus was then dismantled and the Hybond membrane with the DNA was removed.

To denature the DNA, the membrane was placed on filter paper soaked in a solution of 1.5M NaCl and 0.5M NaOH for 5 minutes. Neutralisation was achieved by soaking it in a solution of 0.5M Tris- pH 7.2, 1.5M NaCl and 1mM EDTA for 5 minutes. After allowing the membrane to air dry, the DNA was cross-linked to the membrane using the UV light for 30 seconds at a wavelength of 254nm in a Hoefer Scientific Instruments (California, USA) UV cross-linker. The membrane was stored in a sealed plastic sleeve at 4°C until required. Detection of a signal was determined as previously described.

3.2.10 TRANSFORMATION STUDIES

3.2.10.1 COMPETENT CELL PREPARATION

In 1970, Mandel and Higa showed that bacteria treated with ice-cold solutions of $CaCl_2$ and then heated briefly could be transfected with bacteriophage λ DNA. Most current methods of bacterial transformation are based on these observations. The treatment induces a transient state of "competence" in the recipient bacteria during which they can take up DNA from various sources (Mandel and Higa, 1970; Sambrook *et al.*, 1989). Inoue *et al.*, (1990) described an alternative method for preparation of competent cells based on modifications of a protocol initially described by Hanahan (1983) and Okayama *et al.*, (1987). This method gave very high transformation efficiencies when compared to the calcium chloride method. Both methods were employed to obtain cells of the highest competency.

3.2.10.1.1 Calcium chloride preparation of competent *E.coli* cells

E.coli DH5α cells were made competent using the method of $CaCl_2$-shock procedure based on the method described by Dagert and Erlich (1979). A single colony of the bacterium was inoculated into 5ml of 2xYT broth (Appendix A) and grown at 37°C overnight with shaking. This starter culture was diluted 1/100 in 100 ml of 2YT broth culture and cultured into early logarithmic phase, corresponding to an optical density of +- 0.3. The cells were harvested by centrifugation in a Beckman Model J2-21 for 5

minutes at 4°C and were kept at 4°C during all the subsequent steps. Cells were then re-suspended in 50ml of ice-cold 0.1M $Cacl_2$ (Appendix A) and kept on ice for an hour. The cells were harvested as before and then re-suspended in 10ml of ice –cold 0.1 M $CaCl_2$, thereby inducing this transient state of competence. Ice cold, sterile glycerol was added to the cells to a final concentration of 10% (v/v), mixed and held on ice for a further 20 minutes. Aliquots of competent cells were stored at -70°C until required in transformation experiments.

3.2.10.1.2 Transformation of calcium chloride competent cells

A volume of 100µl of competent cells were thawed at room temperature and once thawed transferred immediately to an ice water bath at ~ 4°C. Approximately 100-500 ng of ligation mix was added to the competent cells, and this mix was kept on ice for 30 minutes. The cells were heat shocked at 42°C for 120 seconds, and returned to the ice bath. Following heat shock 900µl of 2xYT (Appendix A) was added. The transformation mix was then incubated at 37°C for 1 hour.

This method gave competent cells with a high efficiency, in the order of 10^{-6} however, sometimes not efficient enough to obtain the desired recombinants. 1

3.2.10.1.3 Preparation of cells of a high transformation efficiency

The protocol followed for obtaining the high efficiency cells was described by Inoue *et al.*, (1990). Between 10-12 colonies were inoculated from 2YT(Appendix A) agar into 250ml SOB (Appendix A), in a 1 liter flask (90% aeration). The cells were allowed to grow at room temperature (approximately 20°C) with shaking until an OD_{600} value of approximately 0.6 was obtained. A period of 24 hours was often required to obtain this OD_{600} value. Once the required OD (0.6) had been achieved, the flask was placed on ice for 10 minutes. The culture was then transferred to a chilled centrifuge tube and subjected to centrifugation at 3000rpm for 10 minutes at 4°C.

The cell pellet was resuspended in 40ml of ice-cold transformation buffer TB (Appendix A). The resuspension was placed on ice for another 10 minutes and then centrifuged as before. The cells were once again resuspended in one quarter the volume of TB. To this resuspension was added 700µl of DMSO (at a final concentration of 7%). A volume of ~ 11ml was kept on ice for a further 10 minutes. A volume of 300µl of cells were then aliquotted out and snap frozen in liquid nitrogen before being stored at -70°C until required.

The transformation of these cells was a little different to the method utilizing calcium chloride.

An aliquot of cells (~100µl) was thawed at room temperature and once thawed transferred immediately to a water bath. Approximately 100-500 ng of ligation mix or plasmid DNA were added to the experimental controls, and this mix was placed on ice for 30 minutes. The cells were heat shocked at 42°C for only 30 seconds, and returned to the ice bath. At this point, 800µl of SOC (Appendix A) was added. The transformation mix was then incubated at 37°C with shaking for 1 hour.
This method gave competent cells with a very high efficiency, in the order of 10^{-7} at least 2-fold higher than the competence efficiency of the DH5α cells. This was the method most often used to obtain competent cells.

3.2.10.2 Preparation of the insert

The insert, usually an amplification product, was purified by extraction with phenol following electrophoresis on a 1% (w/v) agarose gel at 80V for approximately 1 hour. This insert would be ligated into a commercially available vector. At times, digested genomic DNA (~ 1µg) would be used as an insert.

3.2.10.3 Preparation of vector

The amplified PCR product was cloned using two vectors, as previously mentioned. A commercially available vector, was used for the cloning of PCR products; to ensure a blunt-ended ligation, pGEM-T-easy vector (Amersham International; Buckinghamshire, UK) was used. This ligation method was carried out according to the manufacturer's instructions.

The vector was already supplied t-tailed (as described previously). 500ng of PCR product was added to 2 µl of vector in the ratio 2:1, in 1x buffer and 10 units (U) of T4-DNA polymerase.

3.2.10.4 Cloning strategies

Two cloning strategies were employed for the transformation studies: enriched cloning and shotgun cloning.

The first method, confines the insert size to a limited range; close to the fragment size of interest. An amount of ~ 1µg of genomic DNA was digested with Hind III for 6 hours and then electrophoresed on an 1% (w/v) agarose gel overnight in 1xTAE. The electrophoresed product was visualized using a Kodak DC 120. DNA (8->10kb) agarose was excised and extracted from the agarose with phenol. This eluted DNA was re-suspended in 10 µl of distilled water.

The shotgun cloning method is less defined. In fact, this cloning process utilises all the fragments generated from the digest. The digested DNA is not subject to electrophoresis. Instead, the digested DNA is extracted using phenol and resuspended in 10µl of distilled water.

3.2.10.5 Ligation

Using a ratio of vector : insert of 1:3, ligation reactions were set up. Two separate vectors were employed, pBGS8 (Spratt *et al.*, 1986) and pGEMT-easy. In both cases the ratio used was as described above. Approximately 300 ng of insert was used, 1 U of T4 DNA ligase, in the appropriate buffer was made up to 20µl, with distilled water. The reaction was set to run for approximately 16 hours at 16°C. The entire volume of 10µl of the ligated products were transformed into 100µl of competent *E.coli* DH5α cells.

3.2.10.6 Transformation of competent cells

Transformation studies were carried out using a modification of the protocols of Cohen *et al.*, (1972) and Dagert and Erlich (1979). Twenty microlitres of ligation mix (section 3.) was added to 100µl of competent E.coli DH5α cells and kept on ice for approximately 30 minutes to enable the DNA to bind to the cell surface. To facilitate the uptake of DNA by the bacterial cells (transformation), the transformation mix was heat –shocked at 42°C for 120 seconds. To allow for expression of the resistance markers carried by the vector and/ or the insert (kanamycin resistance in pBSG18 or ampicillin in pGEM-T-easy), 0.9ml of 2xYT was added to the transformed cells and they were incubated at 37°C for an hour. The transformation mix was plated out onto the appropriate selective media and incubated overnight at 37°C.

3.2.10.7 Selection of transformants and screening for putative recombinants

When selecting for recombinants containing a specific amplification product, 200µl of the transformation mix was plated out onto 2YT agar (Appendix A) containing either 33µg/ml of kanamycin or 50 µg/ml of ampicillin (Ranbaxy, South Africa), 100µ/ml of IPTG (Appendix A) and 200µg/ml of X-gal (Appendix A). Kanamycin or ampicillin was

vector of choice. X-gal was added as a colour indicator of β-galactosidase (β-gal) activity, which is induced by IPTG. The pUC based vectors (of which both pBGS18 and pGEM-T-easy are part) carry the amino-terminal (α-peptide) of β-gal. When this plasmid is introduced into bacteria containing the carboxy terminal (e.g. DH5α) the two portions can associate (α-complementation) to form an active enzyme. X-gal is hydrolysed by β-gal and releases a blue non-diffusable dye, giving rise to blue colonies on media containing X-gal. On the other hand, if the sequence encoding the α-peptide is interrupted by cloning into the multiple cloning site (MCS) (Appendix A),α-complementation cannot occur and colonies will be white. Thus, putative recombinants will appear as white colonies on media containing X-gal, ampicillin/kanamycin and IPTG.

Positive selection was used to isolate transformants resulting from the enriched cloning experiment by plating the transformation mixes on media containing ampicillin at a concentration of 25μg/ml)

3.2.11 DNA sequencing

DNA sequencing data was generated by the dideoxy chain termination method (Sanger *et al* 1977) using a rapid automated fluorescence in an automatic DNA sequencer (373A, Applied Biosystems, Perkin Elmer, California, USA). This was carried out at the department of Chemical Pathology UCT or Department of Biochemistry, University of Stellenbosch.

3.3 RESULTS

3.3.1 DETECTION OF β-LACTAMASES

3.3.1.1 IEF assays

When the focussed gel was treated with nitrocefin, the β-lactamase bands appeared gradually and by the time the weakest bands were visible the stronger bands had diffused. The pink bands associated with β-lactamase activity could not be fixed and the faint pink bands against a yellow background could not always be easily distinguished. . Serial photographs were taken to ensure a complete record of β lactamase activity. This was done using the Kodak DC 120 system, under normal light.

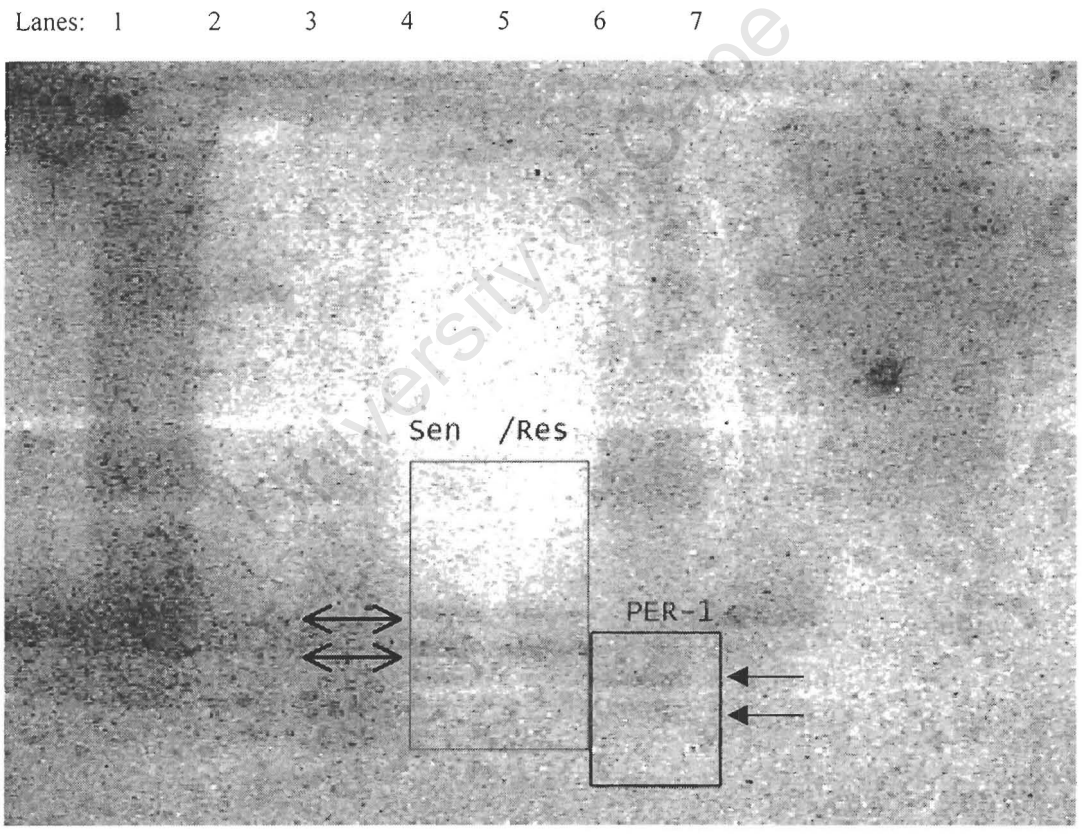

Figure 3.1: IEF PAG, showing the β-lactamases in the sensitive, resistant and control PER-1 strains.

Lane 3 carried the control TEM- containing strains. As indicated by the double-headed arrows, the doublet bands in both the sensitive strain (lane 4) and resistant strain (lane 5) of *A. baumannii* focussed at the same point (~pI 5.4) as the β-lactamases in the TEM control strain However, the doublet band, which was detected in the PER-1 (pI5.3) control strain (lane 6), was not observed in either of the test strains.

We were unable to visualise any β lactamases in the pI range of AmpC. It is most likely that the activity was confined to the top of the gel and as the pI exclusion limit was 10 and as the pI of AmpC is 9.4, it was most likely that this band could not be focussed using this gel.

3.3.1.2 DETECTION OF SHV

SHV PCR assay

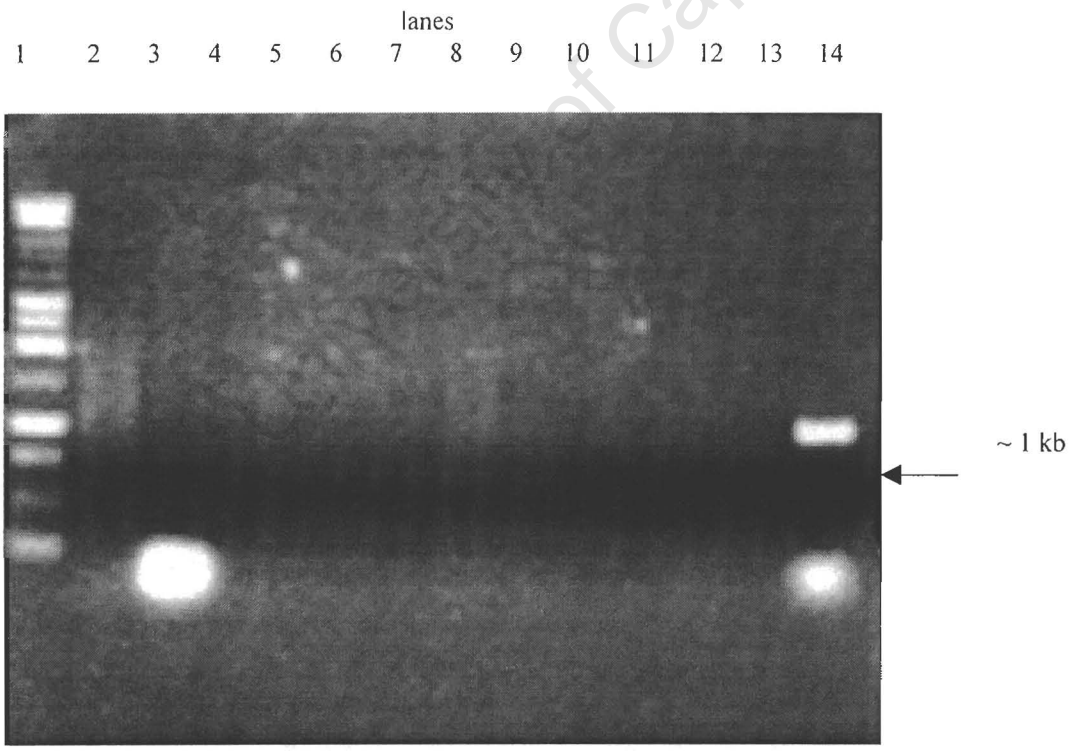

Fig 3.2: Amplification products following SHV PCR.

Lane 1, 1 kb marker; Lanes 2-11, resistant strains 1-10

Lane 12, intermediate resistance strain; Lane 13, sensitive strain; Lane 14, positive control- *K. pneumonia*

PCR was used to detect the presence of SHV-type β lactamases. The PCR assay resulted in no products from any of the 12 strains tested However, a amplicon of the correct size (~1kb) was obtained from the positive control of *K.pneomniae*, suggesting that the PCR assay worked[Fig 3.2]. The absence of any product suggested that this gene was not present in the clinical isolates of *A. baumannii*. To confirm these results, the PCR assay was repeated at the same time as a 16S rDNA PCR assay (see section 2.4.3.1). Amplicons were obtained for the 16S rDNA PCR indicating the suitability of the DNA for PCR, while at the same time no products were obtained for the SHV PCR. This assay served to suggest that the *A. baumannii* strains did not carry the SHV gene.

3.3.1.3 DETECTION OF TEM

TEM PCR assay

A PCR assay was carried out to determine the presence of TEM-type β lactamases. The amplicons from the initial PCR assay for the TEM-1 gene is shown in Fig. 3.5 below.

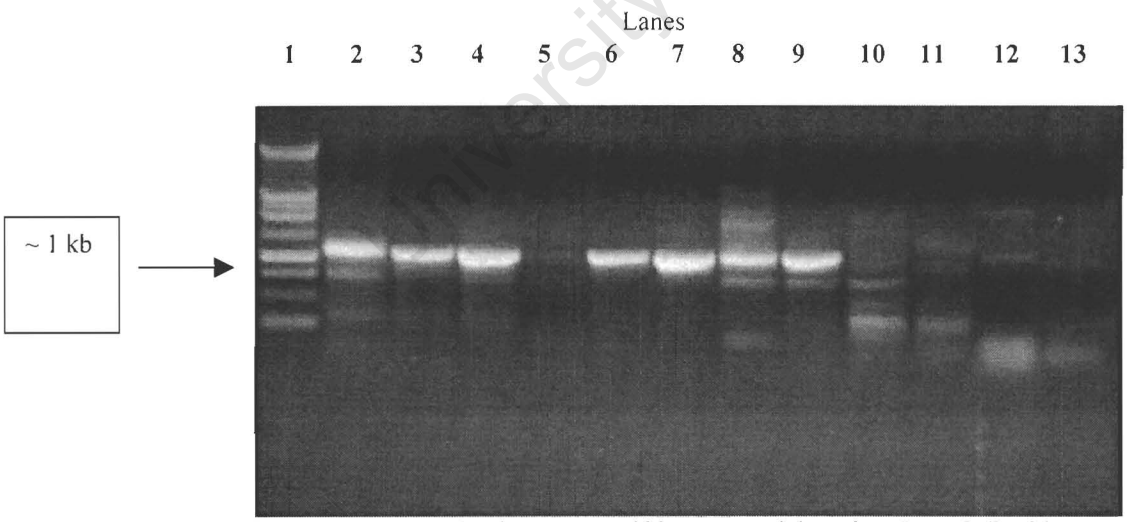

Fig. 3.5: PCR amplicons of TEM-1: Lane 1: I kb commercial marker; Lane 2: Positive control Lanes 3-12: strains 1-10; Lane 13: sensitive strain

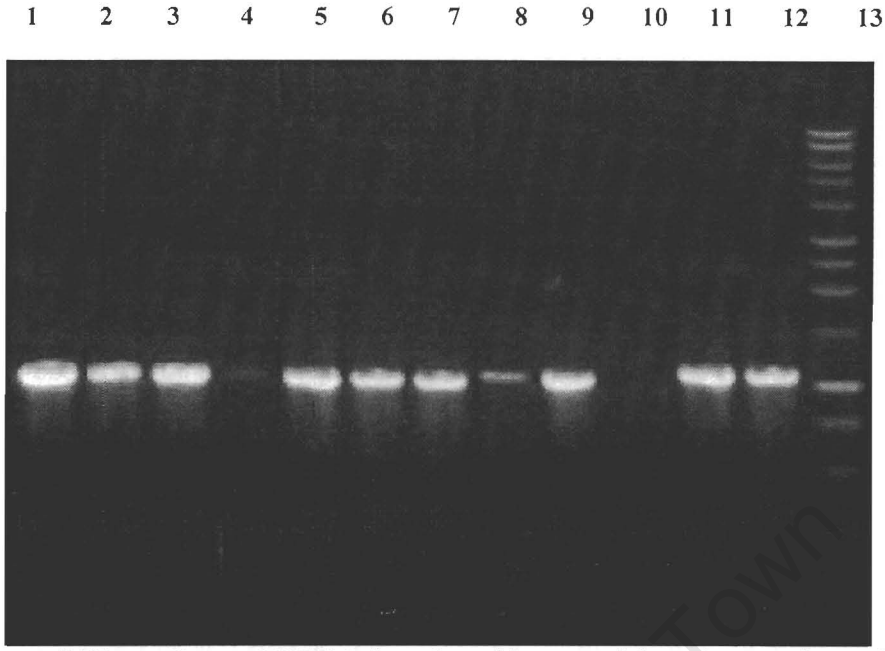

Fig 3.6: Repeat PCR amplicons of TEM-1: Lane 1, positive control; lanes 2-11, strains 1-10; Lane 12, sensitive strain; lane 13 , 1 kb commercial marker

Fig. 3.5 shows the intital TEM PCR assay. Amplification products were not obtained from all the strains tested in this preliminary assay, and so was repeated three times. Fig. 3.6 shows the PCR amplicons from the subsequent assays. No products were obtained from strains 3 and 9, respectively, consistently for the two subsequent assays. However, the TEM-type β-lactamase was detected in all the other strains.

Cloning of TEM-1 type β lactamase

In order to determine the nature of the TEM β-lactamase and to determine its susceptibility profile, the amplification product was cloned. The TEM PCR amplicon was cloned into a vector, pGETM-T easy, which is a T-tailed vector The cloned products were selected on ampicillin containing media (25 μg/ml). The putative transformatants were subject to digestion with *EcoR* I (see section 3.2.4) and AGE to ensure that the selection was of the transformants of choice. This cloned product was tested for ESBL activity, and none was detected.

SEQUENCING of TEM-type β lactamase

In order to determine the nature of the TEM gene , the TEM PCR amplicon from strain 2 was submitted for automated sequencing. The data is shown below.

```
1     ACGTTACCAT GCTTAATCAG TGAGGCACCT ATCTCAGCGA TCTGTCTATT TCGTTCATCC
61    ATAGTTGCCT GACTCCCCGT CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC
121   CCCAGTGCTG CAATGATACC GCGAGATCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA
181   AACCAGCCAG CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTTATC CGCCTCCATC
241   CAGTCTATTA ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA TAGTTTGCGC
301   AACGTTGTTG CCATTGCTGC AGGCATCGTG GTGTCACGCT CGTCGTTTGG TATGGCTTCA
361   TTCAGCTCCG GTTCCCAACG ATCAAGGCGG GTTACATGAT CCCCCATGTT GTGCAAAAAA
421   GCGGTTAGCT CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA
481   CTCATGGTTA TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT AAGATGCTTT
541   TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAT AGTGTATGCG GCGACCGAGT
601   TGCTCTTGCC CGGCGTCAAC ACGGGATAAT ACCGCACCAC ATAGCAGAAA CTTTAAAAGT
661   GCTCATCATT GGAAAACGTT CTTCCGGGGC GAAAACTCTC AAGGATCTTA CCGCTGTTGA
721   GATCCAGCTC GATGTAACCC ACTCGTGCAC CCAACTGATC TTCAGCATCT TTTACTTTCA
781   CCAGCGTTTC TGGGGTGAGC AAAACCAGGA AGGCAAAATG CCGCAAAAAA GGGAATAAGG
841   GCGACACGGG AAAGGTGAAT ACTCATACTC TTCCTTTTTC AATATTATGG AAACATTTAT
901   CAGGGGTTTT G
```

Fig. 3.7: DNA sequence of TEM-1 type β lactamase

The sequence data above is that of the TEM-1 type β lactamase gene amplified by PCR. The sequence data was submitted to a database and it was found to be identical (100% nucleotide identity) in terms of nucleotide sequence to a TEM 1F gene described in *E. coli* (Leflon-Guibout *et al.* 2000).

However, despite the 100% identity at a nucleotide level, it was important to determine changes at the amino acid level. Moreover, alterations in amino acid sequence in the active site of the structural gene, would be important in determining the gene's spectrum of activity. Fig 3.7 below shows the translated amino acid sequence of the TEM type PCR amplicon.

```
        MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1       MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP

61      EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
61      EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL

121     CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
121     CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM

181     PAAMATTLRKLLTGELLTLASRQHLIDWMEAEKVAG
        ||||||||||||||||||||||*|||||||*||||
181     PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAG
```

Fig. 3.9: Amino acid analysis of TEM-1 type with published sequence of TEM-1F (Italicised)

TEM promoter analysis

TEM promoter analysis showed it to be regulated by a P4 type promoter- which is involved in up-regulation. The amino acid changes in the structural gene have not been previously described. Analysis of the structural gene and comparison to the structural gene of TEM 1F as well as other β lactamases, showed that there are in fact amino acid changes at codons 205 and 213. At codon 205, H→Q and at codon 213, E→D.

3.3.1.4 DETECTION OF AMPC

AmpC PCR Assay

To detect the presence of the *ampC* gene, PCR assays were carried out. The results are show in Fig.3.10 below.

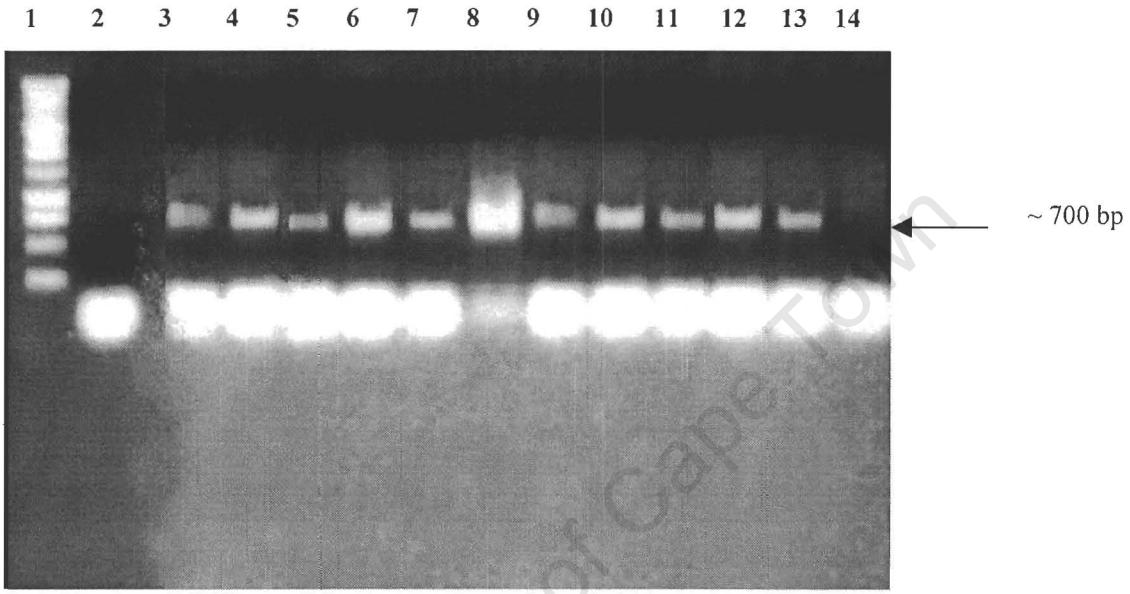

Fig 3.10: AmpC PCR amplification products: Lane 1, 1 kb commercial marker, Lane 2, negative control; lanes 3-12, strains 1-10; lane 13, intermediate resistance strain; Lane 14, sensitive strain.

A distinct band of ~700bp fragment was detected in all the resistant strains and the intermediate resistant strain [Fig 3.10] but not in the sensitive strain. However, on close inspection a very faint band was observed in the sensitive strain. To determine whether this was real or merely "spill-over" from the previous lane the PCR assay was repeated and an obvious band was not detected in the sensitive strain.

This was not the expected result as the AmpC was thought to be encoded in the genome, and intrinsic to the species, hence should be present in all the strains. To confirm these findings and to check this hypothesis an alternative was sought; DNA:DNA hybridisation assays were carried out..

Sequencing

The AmpC PCR amplicon was subjected to automated sequencing. The sequence data identified it as part of the published sequence of the *ampC* gene in *A. baumannii*.

Southern Hybridisation

As the PCR assay was unsuccessful in detecting the *ampC* gene in all the strains hybridisation assays were carried out. DNA:DNA hybridisation assays using a slot blot apparatus showed that the probe (the AmpC PCR amplicon- which was a fragment internal to the *ampC* section 3.1) hybridised to 5µg of genomic DNA in the sensitive strain (Fig. 3.11)

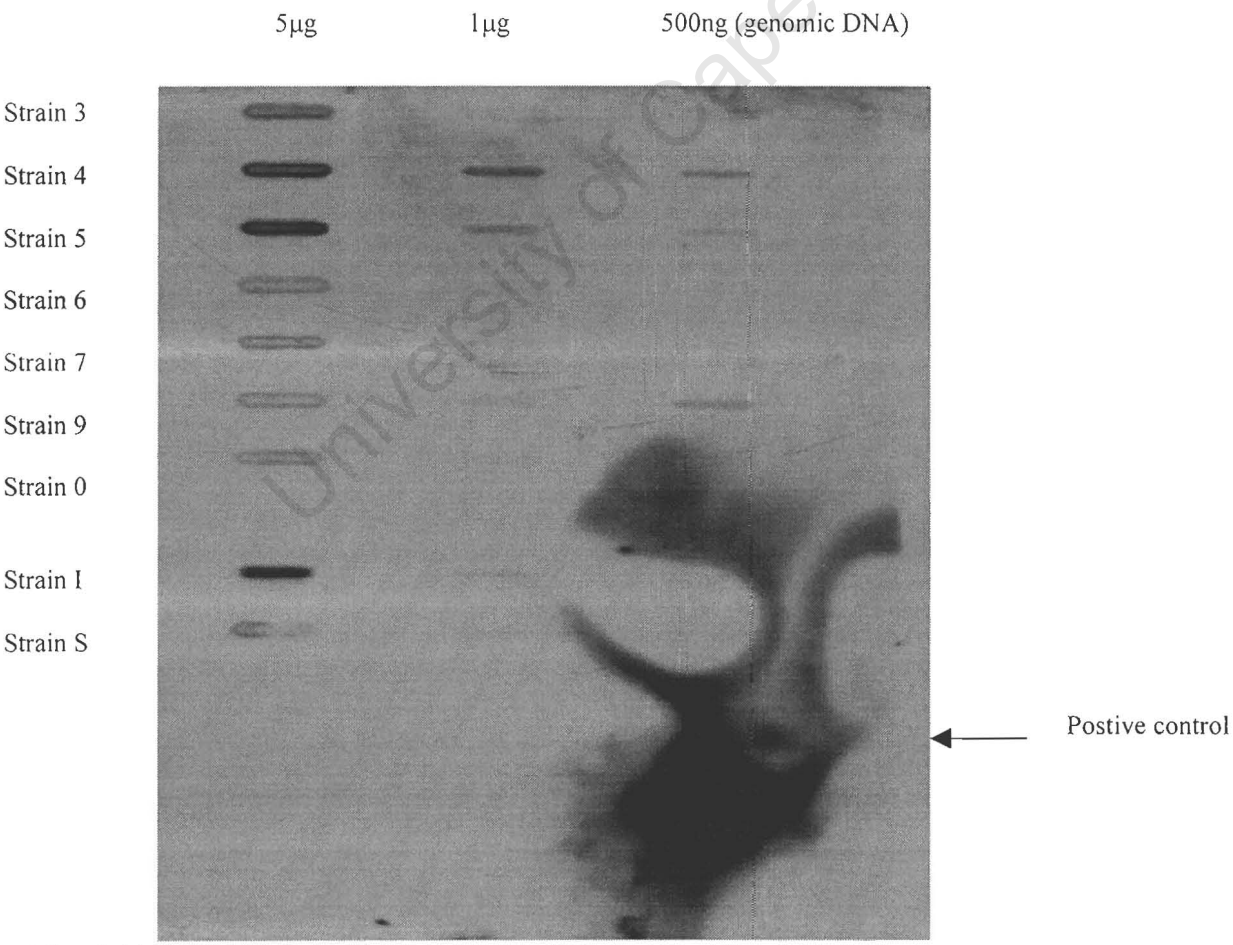

Fig. 3.11: Slot blot DNA hybridisation of *A. baumannii* strains.

This assay was carried out to detect the presence of the AmpC gene in the sensitive strain, and as controls a number of other strains were used as part of the assay. Signals were obtained from resistant strains 3-10, the intermediate resistant strain and the sensitive strain. As seen in Fig 3.11 above, a signal was obtained in the sensitive strain suggesting the presence of the *ampC* gene. Following the success of the slot blot hybridisation, Southern blot hybridisation was repeated using the ECL kit. An amount of 5μg of genomic DNA was digested with 20U of Hind III at 37°C for 6 hours. This template was transferred to a membrane and was probed with a labelled PCR amplicon of the *ampC* gene. No signal was obtained from a no-DNA control. In addition to this, the probe hybridised to the three tested strains; the resistant strain 2, the intermediate resistant strain and the sensitive strain, suggesting the presence of the *ampC* gene in these strains of *A. baumannii*. This data suggests the intrinsic nature of this gene in *A. baumannii*. (Fig 3.12)

The digested products can be seen in Fig. 3.2A. and the autoradiograph in Fig 3.2B below. In the resistant strain, the probe hybridised to a large fragment, of an approximate size of 10kb. In the intermediate resistant strain, the probe hybridised to a fragment >10kb. In the senstive strain, the probe hybridised to a fragment of a similar size to that of the resistant strain.

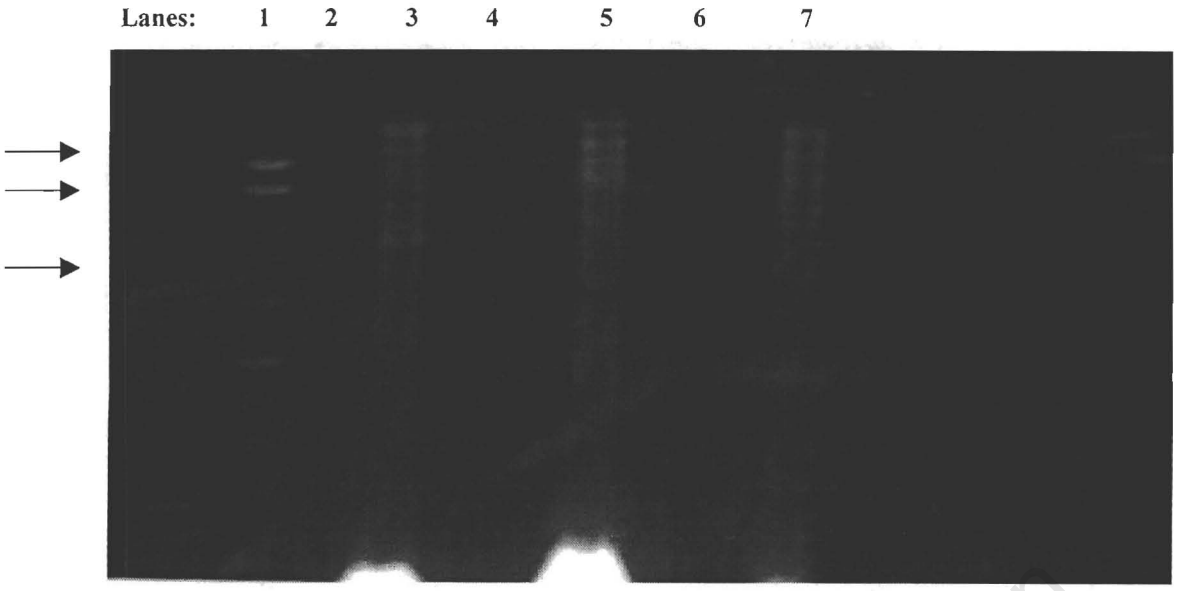

Figure 3.5 A: Genomic DNA template for DNA:DNA hybridisation.

Lane 1, 1 kb commercial marker (Boehringer Mannheim); Lane 2,empty;

Lane 3, 1µg of genomic DNA from resistant strain digested with Hind III; lane 4, empty;

Lane 5, 1µg of genomic DNA from intermediate resistance strain digested with Hind III; lane 6, empty;

Lane 7, 1µg of genomic DNA from sensitive strain digested with Hind III.

Figure 3.5B: Autoradiograph of DNA:DNA hybridisation.

Lane 3, AmpC probe hybridised to resistant strain

Lane 5, AmpC probe hybridised to the intermediate resistance strain

Lane 7, AmpC probe hybridised to sensitive strain

CLONING OF THE AMPC GENE

Once the presence of the *ampC* gene was determined in the strains of interest, it became necessary to clone the fragment carrying the gene in order to study its antibiotic susceptibility profile. Cloning of the fragment of interest (carrying the *ampC* gene) was carried out, based on the hybridisation results. Digested genomic DNA was used to isolate the fragment of interest and they were ligated into pBGS8. The ligated products were successfully transformed into highly competent cells (competence efficiency 10^7). The putative transformants were selected on media containing 25μg/ml of ampicillin. These clones were checked by digestion with *Hind* III, to ensure the fragment that was cloned was of the correct size; yielding fragments corresponding to the correct insert and the vector. This data is shown in Fig 3.3A below.

The PCR amplicon of a fragment internal to the *ampC* gene that was used previously for the Southern blot hybridisation assay was once again used as a probe for checking the cloned products. The subsequent sub-cloning assays were not succesful and the investigation was not continued further.

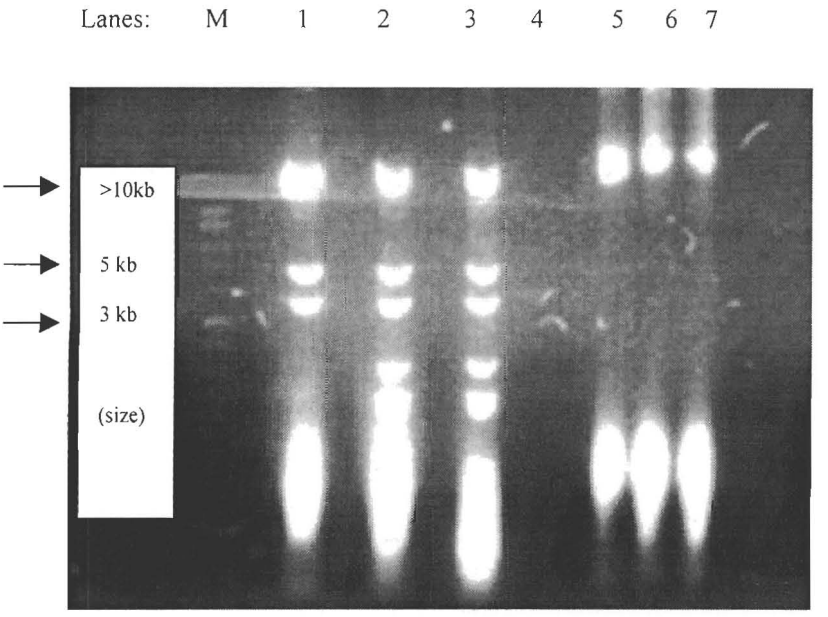

Figure 3.6A: Putative transformants digested with Hind III.
Lane M, 1kb marker
Lane 1, clone 1 digested
Lane 2, clone 2 digested
Lane 3, clone 3 digested
Lane 5, clone 1
Lane 6, clone 2
Lane 7, clone 3; lanes 5,6 and 7 are undigested controls.

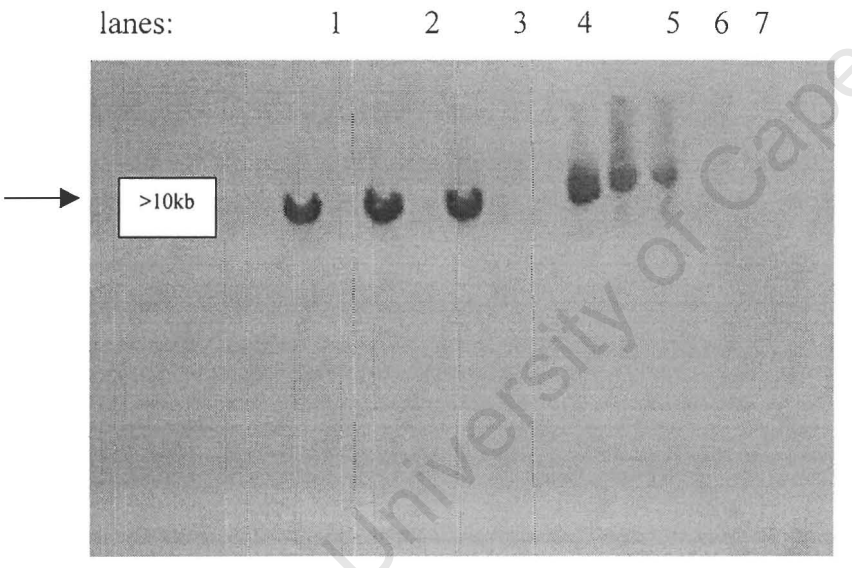

Figure 3.6B: Autoradiograph of AmpC probe hybridisation to putative transformants.
Lane 1, AmpC hybridised to clone 1
Lane 2, AmpC probe hybridised to clone 2
Lane 3, AmpC probe hybridised to clone 3
Lanes 5,6 and 7;AmpC probe hybridised to undigested clones.

Fig.3.6A shows the digested clones, which were to be used as templates for a DNA:DNA hybridisation assay. The digest generated 3 fragments, corresponding to >10kb, 5kb and 3.5 kb. Multiple inserts could be indicative of incomplete digestion or posiibly contcatamers of the fragment. The third arrow from the top, indicates the position of the 3.6kb fragment which corresponds to the vector, pBGS18 (see appendix C). The possibility remained that the *ampC* gene could be located on either the >10kb fragment or the 5kb fragment. The digested genomic DNA template was probed with the AmpC PCR product and the hybridisation results are shown in Fig 3.6B. The hybridisation results

indicate the presence of the *ampC* gene in all three of these clones. A single colony of clone 1, was inoculated into glycerol (20% v/v) and 2YT (80%v/v) for storage. This clone was designated E.coli (p101).

Antibiotic susceptibility testing of *E. coli* (p101)

Antibiotic sensitivity testing was carried out on *E. coli* (p101). The results are shown in the table below.

Table 3.1 Summary of Antibiotic testing on p101 (AmpC clone)

Antibiotic	Parent Strain *A.baumannii* (strain 2) (MIC)	*E. coli* (p101) (MIC)
Co-amoxyclav	No zone (R)	64μg/ml
Amoxycillin	No zone (R)	No zone (R)
Piperacillin	No zone (R)	No zone (R)
Piperacillin-Tazobactem	32μg/ml	No zone (R)
Cefuroxime	No zone (R)	8μg/ml
Ceftriaxone	No zone (R)	0.32 μg/ml

From Table 3.1, it can be seen that the parent strain is resistant to all the antibiotics tested with the pip-tazo combination having an MIC of 32μg/ml. On the other hand, for *E.coli* (p101), unlike the parent, co-amoxyclav had a MIC of 64μg/ml, and while resistant to amoxycillin, piperacillin and piperacillin-tazobactem, exhibited sensitivity toward cefuroxime and ceftriaxone.

3.4 DISCUSSION

The common profiles obtained following GTG- typing (section 2.4.4), justified using only one resistant strain as a representative of the population of resistant strains. Strain 2 was chosen as a representative of the resistant strains. β lactamases extracted from strain 2, a sensitive strain and a PER-1 control strain were subjected to IEF assays. The IEF data only served to confirm the presence of TEM-1 type β lactamases (which focussed at pI5.4) and the absence of PER-1 β lactamases in the test isolates of *A. baumannii*. The AmpC β lactamase was not visualised possibly due to the pI exclusion limit of the PAG, even though its presence was confirmed by PCR and Southern hybridisation.

The SHV β lactamase gene was not detected in any of the 12 clinical strains following PCR assays. This assay was repeated twice in order to confirm the absence of this β lactamase. The consistently negative results suggests that this β lactamase is not present in the clinical isolates.

A TEM-type β lactamase gene that was detected in all 10/12 clinical strains of *A. baumannii* following PCR assays, was shown to be expressed off a P4-type promoter (Chen and Clowes, 1984). In fact this promoter is involved in upregulation, which could help explain the some of the antibiotic resistance observed in the clinical strains, such as the resistance to the co-amoxyclav. In addition to this, 2 new mutations were found in the structural region of the gene at codons 205 and 213, where His→Gln and Glu →Asp respectively.

The sequence of the TEM1 gene isolated from the clinically resistant strain was shown to be identical with respect to the nucleotide sequence of already described TEM 1F, which confers resistance to amoxyclav.

The combined MIC data from the AmpC and TEM genes respectively could not account for the resistance to the extended spectrum cephalosporins, and hence it was decided to that some other mechanism might be responsible and so we went in search of some kind of protein-based antibiotic resistance mechanism.

The absence of β lactamases, which could account for the extended spectrum of activity , led us to look for alternative mechanisms by which this resistance could be mediated. Based on the literature on porins and transport systems described in other Gram-negative bacteria, the next course of action was to investigate these proteins in out clinical isolates of *A.baumannii*.

As previously mentioned, the presence of an AmpC β lactamase was determined by PCR, and its identity confirmed by sequencing. The absence of the AmpC in the sensitive strain could most likely be attributed to contamination of the experiment. It is also possible that no product was obtained using the sensitive genomic DNA as a template due the inaccessibility of the gene due to the topology of the template. Thus the absence of a product following the PCR assays led to investigation using DNA:DNA hybridisation. The AmpC probe was found to hybridise to fragments in the resistant, intermediate and sensitive strains, respectively. Hybridisation of the probe to the resistant strain DNA appears to have resulted in a doublet. There are two possibilities; I) The genomic DNA template was not completely digested or II) that there are 2 copies of the AmpC gene in the resistant strains. The investigation was not carried further at this point.

The fragment on which the AmpC was detected was cloned in order to determine its susceptibility profile. However, the cloning of the gene involved a rather large insert, which was difficult to manipulate, and subsequent sub-cloning was not carried out. The antibiotic susceptibility testing of this cloned gene showed that it conferred resistance to the combination of amoxicillin-clavulanate, amoxicilin, piperacillin, pip-tazo and to a limited extent cefuroxime (8μg/ml) but remains susceptible to ceftriaxone (0.32 μg/ml). However, the parent strain, strain 2, also exhibits resistance to cefuroxime and ceftriaxone (Table 3.12)

CHAPTER 4

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4.2.4 SDS-polyacrylamide gel electrophoresis
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CHAPTER 4

INTRODUCTION

4.1 Outer Membrane Permeability

Limited studies have been carried out with respect to the outer membrane protein content of *Acinetobacter* spp. Included in this limited list are a number of investigations into the possibility of some proteins which act as porins (Clark, 1991 ; Jyothisri *et al.*, 1999). These have been discussed previously [Section 1.9] and will be mentioned here briefly, as part of the introduction. This limited data will be subsequently discussed as two separate topics: outer membrane proteins (porins) and pumps.

The major mechanisms that contribute to the overall resistance of the strains appear to be the β-lactamases, as discussed in the previous chapter [Chapter 3]. Despite the contribution of the β lactamases, it has often been stated that the overall resistance displayed by *Acinetobacter* is the result of the total contribution of both enzymes and the protein-based mechanisms (secondary mechanisms).

The outer membrane permeability has been discussed previously (Sato and Nakae, 1991) and proposed as another contributing factor. A number of investigations have been made into the possibility of proteins, specifically outer membrane proteins playing a part in resistance mechanisms. Sato and Nakae (1991) have extensively discussed the outer membrane permeability of *Acinetobacter calcoaceticus (A. calcoaceticus)* and its implication in antibiotic resistance. A number of observations were made, one of which was the permeability of *A.calcoaceticus* to cephalosporins. The permeability was compared to *P. aeruginosa* and was shown to be 3-fold lower and a number of conclusions were made:

1) the size of the outer membrane of the pore is small in Acinetobacter
2) *Acinetobacter* produces only a limited amount of porin
3) a combination of the two above

In addition to this, the investigators concluded that the porin content was less than 5% of the total outer membrane component, as compared to *E.coli* which is reported to have a ~60% porin content (Rosenbushch, 1974). Limited data is available on these outer membrane proteins. Another interesting fact to note was that the diffusion rates of carbapenems through the outer membrane of *A.calcoaceticus* was about 2% of that of *E.coli* (Sato and Nakae, 1991). All in all this evidence suggests that the inefficient permeability in *A. calcoaceticus* is due to the low number of small sized porins. The authors concluded by saying that they did not rule out the possibility of the presence of any other unidentified porins

Outer membrane permeability or the lack thereof has been a topic of intense discussion over the past few years, and it has been thought to be an additional contributing factor to the growing resistance Gram–negative bacteria. The roles of pumps and porins have been extensively studied in other Gram-negative bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*) (Poole *et al.*, 1993; Nikaido *et al.*, 1991). Work carried out by Zgurskaya and Nikaido (2000) have shown how pumps have contributed to *P. aeruginosa*'s multiple antibiotic resistance profile.

Clark (1991) described another outer membrane protein in *A. baumannii*, which was designated 33-36 kDa protein. Clark (1991) discussed the reduction in imipenem resistance with the concomitant decrease in the expression of a 33-36 kDa protein.

The impermeability of the outer membrane of *A. baumannii* to certain classes of antibiotics is attributed to the changes in the protein content of the OM (Jyothisri *et al.*, 1999). These investigators isolated, purified and characterized a major 40 kDa protein which they designated Amp Ab from *A. baumannii*. This appeared to be the predominant outer membrane protein isolated from their clinical strain in an Indian hospital. The major band in the OM profile was a 40 kDa protein which was visualized on a polyacrylamide gel. The authors compared this outer membrane protein to OmpF of *E.coli*, which is a well studied porin, whose loss leads to concomitant antibiotic resistance (Pattnaik *et al.*, 1997).

As has been shown, a limited number of outer membrane proteins (OMPs) have been observed and described in *Acinetobacter* spp. These OMPs have also to a lesser extent been implicated in antibtiotic resistance. Although pumps have been well described in Gram-negative bacteria such as *P. aeruginosa*, to date, only one example of an efflux pump in *Acinetobacter* has been published (Lambert, 2001). This efflux pump was thought to play a role in the aminoglycoside resistance in a clinical isolate of A. baumannii.

4.2 MATERIALS AND METHODS

4.2.1 Strains

The activity of the various β-lactamases described in the previous chapter could not account for the resistance profile observed in the clinical strains 1-10 and the intermediate resistant strain of *A. baumannii*. As enzyme-based mechanisms could not account for the resistance, alternative mechanisms were investigated. The data on porins and porin functions and transport systems in other Gram-negative bacteria led us to investigate the role of these proteins, if any, in the *A. baumannii* strains.

Resistant strain 2, the intermediate resistant strain and the sensitive strain were used for further analysis. This decision was based on two factors; (i) the isolation of outer membrane proteins is a time consuming and laborious process and (ii) similar/identical profiles were observed in the ten resistant strains [Section 2.5] following poly- $(GTG)_5$ typing. It was decided that strain 2 would be used as a representative of the resistant strains for the protein studies. Strain 2, in conjunction with the intermediate-resistance strain and the sensitive strain formed the basis of the outer membrane protein analysis.

4.2.2 Outer membrane protein extraction

The purification techniques for outer membrane proteins specifically, porins are based on the fact that they are SDS-resistant trimers; they are resistant to the action of detergents and remain associated with the peptidoglycan. The method used for extracting the outer membrane proteins (OMPs) from *A.baumannii* was based on protocols for the extraction of OMPs of *E. coli* or *S. typhimurium* (Nikaido *et al.*, 1994). The method was initially described by Tokunaga *et al.*, () and modified slightly by Nikaido *et al.*, (1994). It was this modified method that was used for the extraction of outer membrane proteins of *A. baumannii*.

A single colony from each of the three strains was inoculated into 5 ml 2xYT (Appendix A) broth cultures, 25μg/ml of ampicillin was added (Appendix A) and the cultures were incubated aerobically at 37°C with shaking for 16-20 hours. The following day, each of the three strains were sub-cultured 1/100 into 1000ml of fresh 2YT broth (Appendix A), 25μg/ml of ampicillin was added, and the cultures were subjected to a further aerobic incubation at 37°C with shaking. The following day, the cells were harvested in a Beckmann centrifuge (J2-21) at 5000rpm for 10 minutes at 4°C. The harvested cells were then washed once in 100ml cold 50mM Tris-HCl buffer (Appendix A), pH 7.2, and centrifuged again. The harvested cells were resuspended in 5ml of 50mM Tris-HCl, pH 7.5. The chilled suspension was then passed through a French Pressure Cell, three times at 9000 psi to break the cells to isolate the proteins. The cell extract was then centrifuged for 10 minutes at 1000g to remove the unbroken cells. The supernatant was centrifuged in an Ultra-Centrifuge at 100, 000 g for one hour to pellet the cell envelope. Both the inner and outer cytoplasmic membranes as well as the peptidoglycan layer were contained in this layer.

The preparation of the peptidoglycan proteins required further manipulation of this fraction. The cell-envelope fraction was resuspended in 5 ml of 1% Sarkosyl, 10mM Tris-HCl, pH7.5 (Appendix A). This suspension was incubated at 32°C for 30 minutes

and then subjected to ultra-centrifugation at 100,000 g for 30 minutes at 20 °C. This step was repeated three times. The pellet that was obtained was then resuspended in 1% sarkosyl, 10mM Tris-HCl (appendix A) and stored at -20 °C until required.

4.2.3 Protein Concentration Determination

Protein concentrations were determined using the Bio-Rad DC protein assay kit (Amersham). This colourimetric assay is based on the protocol described by Lowry *et al.* (1951)

The standards for the assay were 0.25mg/ml, 0.5mg/ml, 0.75mg/ml and 1.0mg/ml. These standards were prepared by dilution of the Bovine Serum Albumen (BSA) supplied in the kit (at a concentration of 1.5mg/ml) in the appropriate buffer.

As part of each assay, 100µl of solution A' (20µl of solution S/ml of solution A) was mixed with 20 µl of protein sample (for both the standards as well as the clinical extracts) and 850µl of solution B. The samples were incubated at room temperature for 15-20 minutes and the OD at 750nm was determined using an IR Spectrophotometer. The OD_{750} values of the Bio-Rad protein standards were plotted against the protein concentrations and a linear graph was plotted.

The concentrations of the outer membrane proteins (OMPs) from the three clinical strains were determined from this graph.

4.2.4 SDS-Polyacrylamide Gel Electrophoresis

The acrylamide gel systems were prepared according to Schagger and van Jogow (1987). The SDS buffer components are listed in Appendix A. A 10% (w/v) bis-acrylamide gel, of dimensions 13x16x0.2cm was prepared according to the following description. It comprised two sections; a resolving gel system and a stacking gel system. The resolving gel component was comprised of 10.5 ml of 30% (v/v) acrylamide solution (30%

acrylamide : 1 % bis- acrylamide solution), 10ml of resolving gel buffer, and made up to 25 ml with distilled water. This mixture was allowed to de-gas for 10 minutes before the polymerisation step. A volume of 50 µl of TEMED (Appendix A) and 40µl APS (15% w/v) (Appendix A), were added to allow for polymerisation of the gel. In order to ensure that the gel polymerized evenly, 1 ml of water saturated butanol was added to the top layer, and allowed polymerise for one hour. The stacking gel buffer system, comprised of 2.5ml of stacking gel buffer, 1.5 ml of 10% acrylamide solution and was made up to a final volume of 5 ml, with distilled water, de-gassed for 10 minutes as before, then polymerized with 10µl of TEMED and 20µl of APS (10%) (Appendix A). The stacking gel component was then poured over the polymerised resolving-gel component, the comb added and the gel allowed to polymerize for a further hour. Prior to loading, 1/10 the volume of bromophenol blue and 50% mercapto-ethanol were added to the protein samples, and were denatured at 95 ° C for 3 minutes. The protein standards used for these assays were contained in the Low Molecular Weight Calibration Kit (Pharmacia) (Appendix A). The marker and the samples were loaded into the wells using a Hamilton syringe. The gel was set up in a vertical system (Hoefer Scientific systems), and buffer was added to 5cm from the top. The gel system did not require a separate cathode and anode running buffer. After loading the samples onto the gel, the proteins were electrophoresed at 20mA (constant current) for approximately 120 minutes until the proteins had electrophoresed out of the stacking gel and into the resolving gel system. The running buffer consisted of 1%SDS, 7% Glycine, and 1.5% Tris (appendix A). Electrophoresis was then allowed to continue for a further 4-16 hours at 40mA. The duration of electrophoresis depended on the percentage of acrylamide in the gel; the higher the percentage of acrylamide in the gel, the longer the electrophoresis step.

Following electrophoresis the gels were removed from the plates and were stained with Coomassie Blue (Appendix A) overnight. The gels were then de-stained with 10% acetic acid, 20% ethanol, with fresh de-staining solution being added every hour on the hour, approximately 6 times. The gel was then rinsed in 10% acetic acid to remove the ethanol.

The acrylamide gels were subsequently photographed using a Kodak DC 120 system, before they were subjected to drying, using (Hoefer Scientific Systems). The gel was placed between two silicone sheets, laid into a frame and great care was taken to ensure the absence of air bubbles. The gel was dried under a vacuum pump at 50°C for approximately 60 minutes.

4.2.5 Matrix-assisted Laser Desorption Time-of-Flight Analysis (MalDi- TOF)

A number of protein bands were visualised in the polyacrylamide gels. Proteins were chosen for further analysis on the basis of their size. The sizes corresponded to that of described porins in other Gram-negative bacteria, in the region of 25-45 kDa. A number of different gels were poured at different times. This was because the proteins were obtained at varying intensities (concentrations) and a certain concentration limit was required for analysis. Each time, only a select number of proteins were analysed. The proteins were stored in destain at 4°C. When required these proteins were excised and sent for Matrix-assisted Laser Desorption Time-of-Flight Analysis (MalDi TOF) analysis.

The MALDI TOF analysis was carried out by Dr. Anke Fiedler at the Department of Biochemistry, University of Cape Town. MalDi TOF analysis was carried out on a bench-top PerSeptive Biosystems Voyager De (Delayed Extraction) Workstation Mass Spectrometer. The delayed extraction technology provides improved resolution of data and mass accuracy relative to each other. The MALDI sample is embedded in a low molecular weight (MW) ultra-violet (UV) absorbing matrix. The time-of-flight mass spectrometry works on the principle that if the ions are accelerated with the same potential, from a fixed point and at a fixed initial time, and are allowed to drift, the ions will separate according to their mass to charge ratios (m/z).

The data was obtained, when the protein sample was subjected to trypsin digestion to generate smaller sequences of amino acids (polypeptide fragments or PPFs). These PPFs, each with a unique molecular mass generated by this trypsin digest would be subject to a current and would separate according to their mass and charge. These fragments would

then be detected by a UV laser (λ = 337nm) and be represented as mass (m/z) peaks on a graph.

The MalDi TOF analysis was returned in a graphical format (See Fig. 4.5-4.10). A number of peaks representing the different masses following trypsin digestion were observed in each graph. These mass peaks were used to determine the function of protein with the assistance of MS-FIT software. This analysis was carried out *in silico* and found at http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm. The parameters of MS-FIT were optimised to obtain the closest matches to the unknown proteins. The mass peaks (or m/z data) obtained from each unknown protein were the variables of the analysis, while the rest of the parameters remained constantly optimised. MS-FIT had the advantage of having access to a number of different databases, including Gen-Pept, Swiss-Prot and NCBI. One could be certain that the unknown proteins were compared against an extensive database, hence ensuring a close match.

4.3 RESULTS

4.3.1 Protein Concentration Determination

Table 4.1: Protein standards and *A. baumannii* strains for the Lowry Assay (OD_{750})

Protein standards concentration (mg/ml)	OD_{750} (A units)
0.25	0.795
0.50	0.454
0.75	0.355
1.0	0.306
Sensitive strain	*0.375*
Intermediate resistance strain	*0.221*
Resistant strain	*0.205*

The OD_{750} values presented above, were used to plot a protein calibration graph shown below. From this calibration graph, the concentrations of the unknown protein samples were determined: R= 1.33mg/ml; I= 1.25mg/ml; S= 0.7mg/ml.

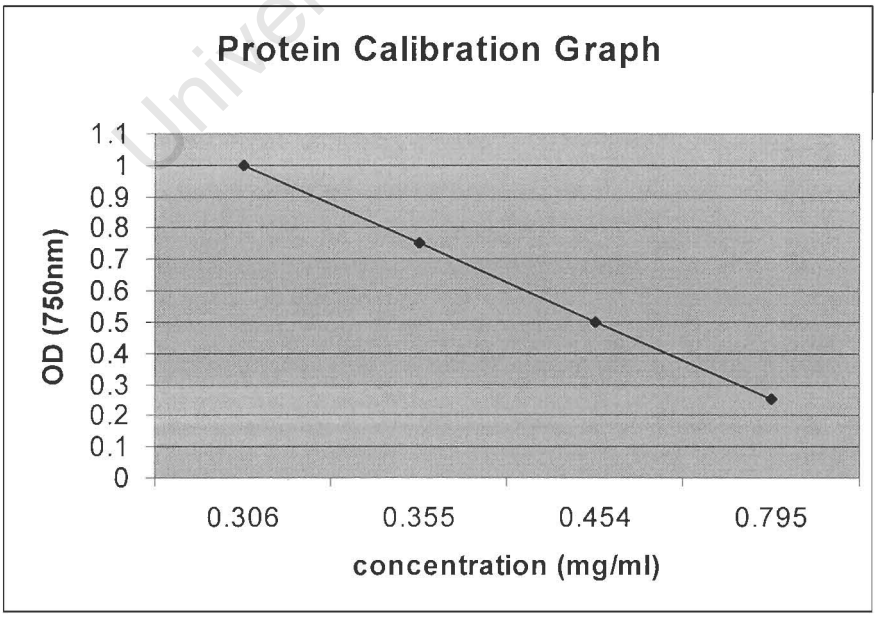

Figure 4.1: Calibration graph for protein concentration determination

The quantification of the proteins was a first step in this study. This ensured that the intensity of the protein bands in the polyacrylamide gels was an accurate reflection of the amount of protein present in each sample and hence a visual comparison was possible.

In all the subsequent acrylamide gels, the concentration of each protein sample loaded was the same (~ 1mg/ml). An approximate amount of 10μg of protein sample were loaded in each well.

4.3.2 Polyacrylamide gel electrophoresis

A number of different polyacrylamide gels (PAGs) were electrophoresed in the study. As mentioned previously, this was necessary because, at different time points, it became necessary to analyse different OMPs. Fig.4.1 shows one of the initial PAGs obtained.

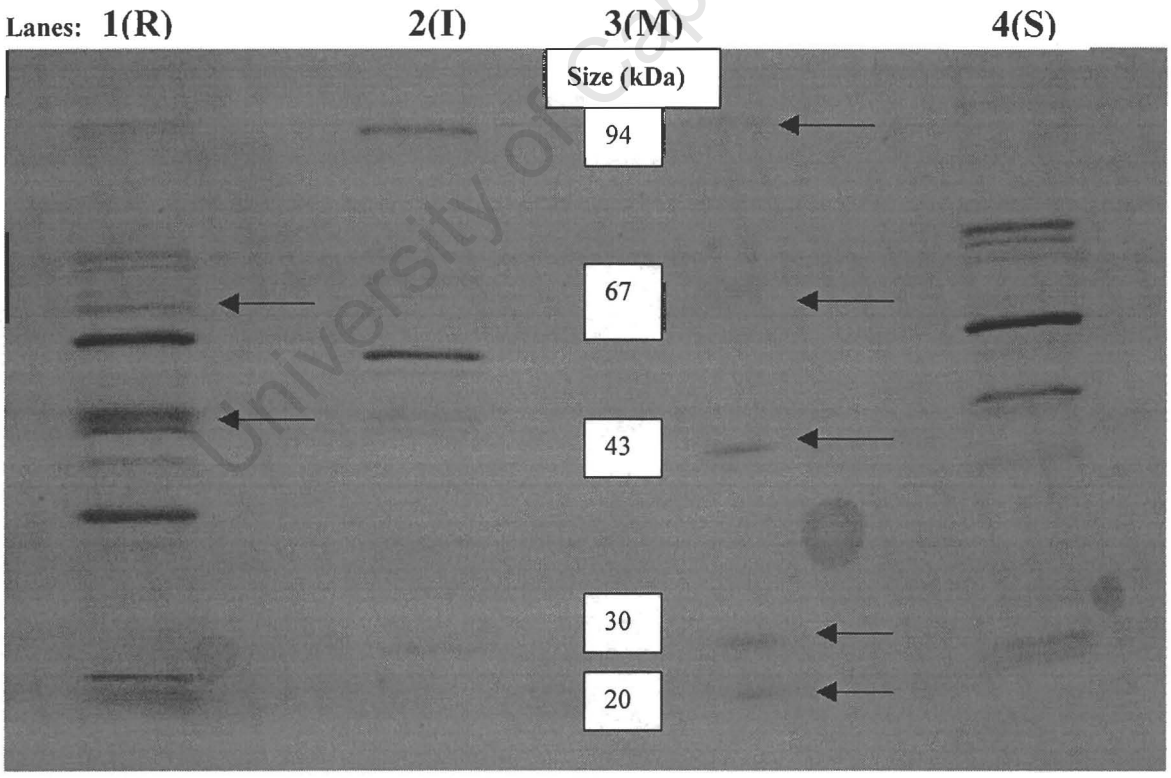

Figure 4.2: Polyacrylamide gels of OMPs from *A. baumannii*. Lane 1, **R**esistant strain 2; lane 2, **I**ntermediate resistance; lane 3, **M**arker; lane 4, **S**ensitive strain.

For the initial MALDI TOF analysis, the OMPs were chosen on the basis of their size. The size (kDa) was dependent on the size of previously described OMPs in other Gram-negative bacteria. A number of different OMPs were chosen from this limited range between 25-45 kDa.

In Fig. 4.1, the arrows in lane M, indicate the positions of the low molecular weight marker (LMW). A number of OMPs were observed in all the strains, however, only 2 were chosen from this PAG. A doublet was observed in the resistant strain, but not in the sensitive strain. In addition to this a 67 kDa OMP was seen only in the resistant strain and not in either of the other two strains. These two OMPs of sizes 67kDa and ~ 43kDa, were excised from the PAG and sent for MALDI analysis.

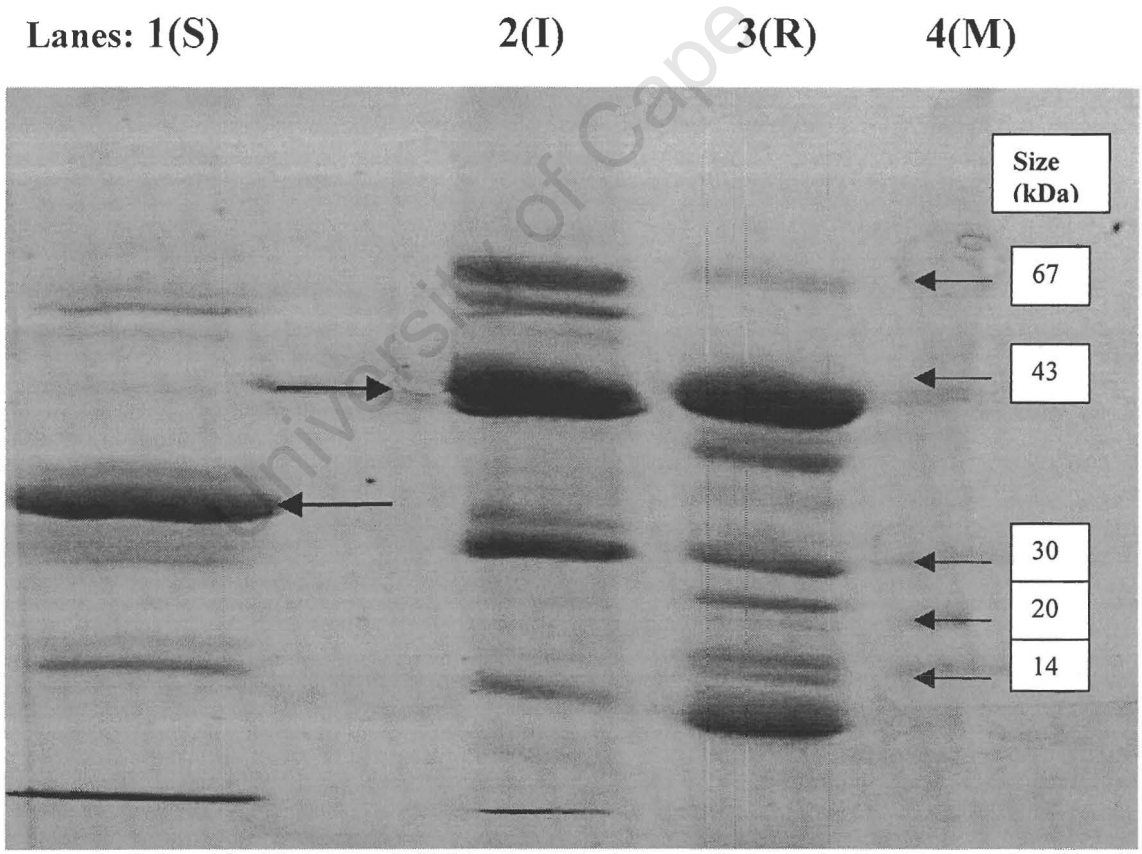

Figure 4.3: Polyacrylamide gels of OMPs from *A. baumannii.* Lane 1, Sensitive strain; lane 2, Intermediate resistance; lane 3, Resistant strain 2; lane 4, low molecular weight Marker.

In Fig.4.2, it can be seen that an increased concentration of proteins was loaded. A 40kDa OMP was excised from both the intermediate resistant and the resistant strains. Similarly, a 40kDa OMP was seen in the sensitive strain but to a lesser degree. An ~35 kDa OMP was taken from the sensitive strain for MALDI TOF analysis. From Fig. 4.2, it can be seen that this OMP does not appear to be in either of the intermediate or the resistant strains.

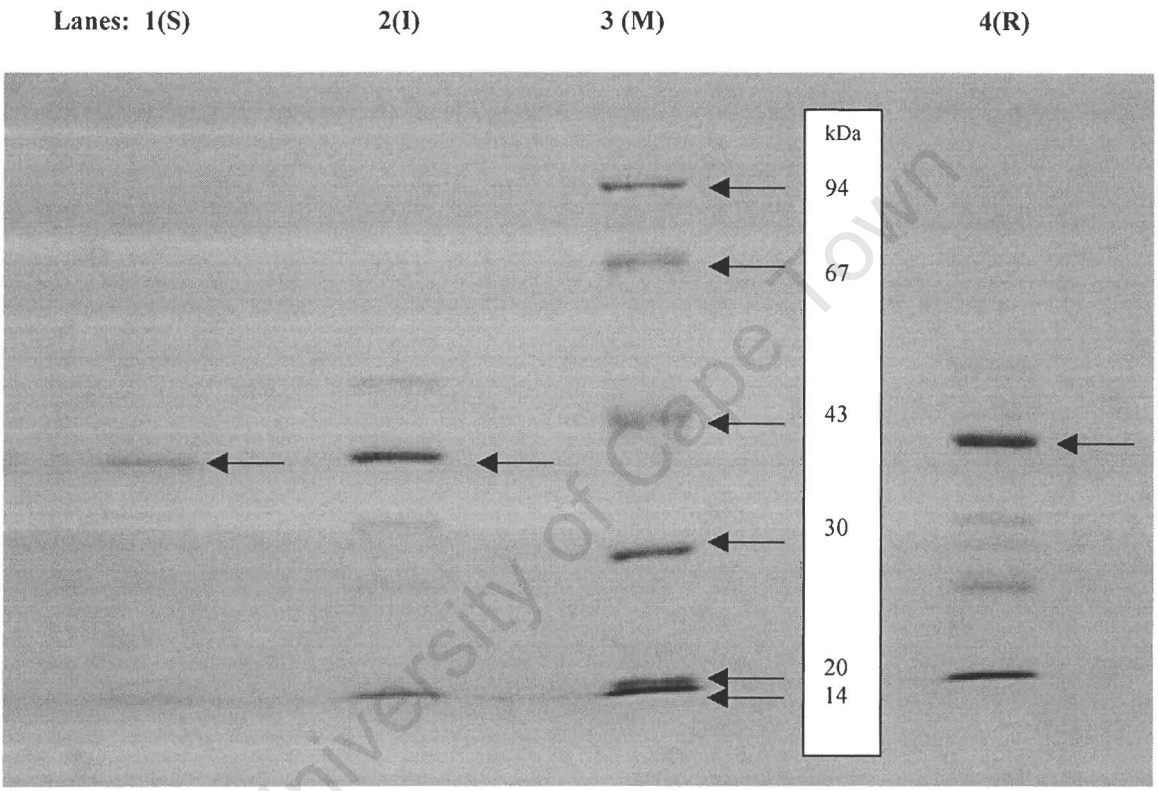

Figure 4.4: Polyacrylamide gel of OMPs from *A. baumannii* strains. Lane 1, sensitive strain OMPs; lane 2, intermediate resistance strain OMPs; lane 3, low molecular weight marker; lane 4, resistant strain OMPs. The three arrows in each of lanes 1, 2 and 3 indicate a ~40kDa OMP that was selected for MALDI TOF analysis.

An ~40kDa OMP was found to be present in all three clinical isolates. This OMP was excised and subjected to MALDI TOF analysis. Following MS-FIT analysis of the graphs, this 40kDa OMP was found to have the highest identity with OmpC from *Serratia marcescens*. However, due to its presence in all three strains it is unlikely to contribute to the antibiotic resistance profile of the clinical *A. baumannii* strains.

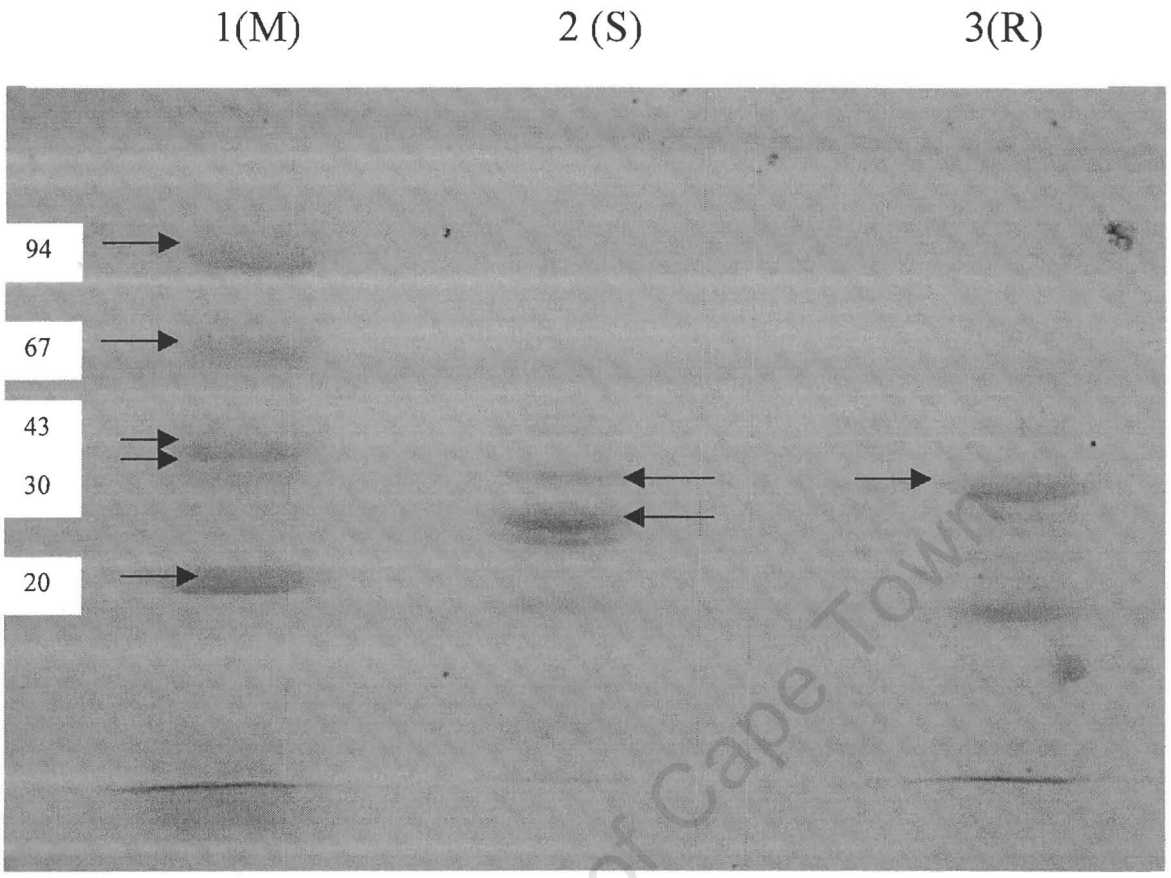

Figure 4.5: Polyacrylamide gels of OMPs from *A. baumannii* Lane 1, low molecular weight marker; lane 2, sensitive strain; lane 3, resistant strain.

A number of proteins were excised for further analysis from the gel shown in Fig 4.4. From the sensitive strain, as indicated by the arrows an OMP of size 25kDa and another of size 30 kDa were chosen. Similarly, a 30kDa OMP was also selected from the resistant strain.

The OMPs observed in all three clinical isolates of *A. baumannii* are summarised in Table 4.2. The graphs obtained from MalDi TOF analysis, which were used in MS-FIT to ascertain the functions of the various OMPs are shown in Figs. 4.5-4.12.

Table 4.2: Tabulated summary of the OMPs in clinical isolates of *A.baumannii*

Protein size (kDa)	Resistant strain	Intermediate strain	Sensitive strain	Protein designation
67	+	+		Protein component of a transport system
55	+	+		Protein component of a transport system
54	+			
53				
48			+	
46	+			
44	+			
43	+			
41				
40	+	+	+	OmpC of *Serratia marcescens*
38				
35			+	OmpC of *Nesseiria haemophilia*
33	+		+	
32	+			
30	+	+	+	
27	+			
25	+	+	+	
23			+	
20	+	+	+	

4.3.4 MALDI TOF Analysis

Protein sequencing was the initial choice for analysis of proteins. However, due to expense and difficulty of this technique, it was decided that MALDI TOF analysis was the next best option for protein analysis. This technique has a good return in identifying a protein in terms of its similarity to other proteins from other organisms. The search criteria were confined to bacteria only as some proteins may also be found in eukaryotes as well. This limitation increased the sensitivity of the tests, and so the data that was obtained could be relied upon.

In addition to this being the technique of choice, this is the first time analysis of this nature has been carried out on OMPs from *Acinetobacter* or even *A. baumannii* for that matter. The results obtained from this analysis in that respect, are therefore novel and unique.

The m/z data was inserted into a program, MS-FIT. This data was obtained from the graphs seen previously (Figs.4.5-4.12). MS-FIT had access to a number of databases, Swiss-Prot, NCBI, Gene-pro etc. The protein function was only assigned to those hits with a high PPF count and a high MOWSE score. The results were also cross-checked with different databases to ensure the accuracy of the results.

4.3.4.1 Resistant strain

A 40kDa protein was identified and isolated from the resistant strain. The MALDI analysis identified this protein as having the highest identity against a porin that was identified in *Serratia Marcencens*. This protein was designated as an outer membrane protein C (porin OMPC) expressed by the gene *ompC*. It was described as forming passive diffusion pores which allowed the passage of low molecular weight hydrophilic materials across the outer membrane. This OMPC sub-unit was described as an integral membrane protein that formed homo-trimers. In addition to this, the OMPC was classified under the PHOE family of porins. A 40kDa OMP had previously been

identified in *A. baumannii* (Jyotshiri et al., 1999) but it was not described as a porin, nor was any function assigned to it.

A 55kDa protein was identified in the resistant strain. The MALDI analysis on this protein, identified it as an ATP-binding protein as part of a hypothetical ABC transporter system. This is the first time that a 55kDa protein from *A. baumannii* has been subjected to MALDI analysis.

A 67kDa protein was identified in the resistant strain only, and gave the highest MOWSE score against a protein which had a function very similar to the 55kDa protein, in other words, it appeared to be part of an ATP-binding protein system.

4.3.4.2 Intermediate Resistance

A 40kDa protein was also identified in the intermediate resistance strain. This 40kDa protein had the same function as the protein identified in the resistant strain. A 55-kDa protein was also identified in the intermediate strain, as having the same functions as described for the resistant strain.

4.3.4.3 Sensitive Strain

Two proteins were identified in the sensitive strain; one of approximate size 40kDa and another 35kDa. The 40kDa protein was, as previously mentioned, identified as a porin, based on the high MOWSE score following MALDI analysis. The 35-kDa protein was also identified as a porin, this time, having the greatest identity with Neisseria haemophilia.

Protein Size (kDa)	Resistant Strain	Intermediate strain	Sensitive Strain
35	-	-	+
40	+	+	+
55	+	+	-
67	+	+	-

Table 4.5: A tabulated summary of the clinically significant OMPs identified in the clinical strains

DISCUSSION

Porins have been described in other Gram-negative bacteria and examples of these include OprD in *P. aeruginosa* (Srikumar *et al.*, 1997; Epp *et al.*, 2001), OmpF in *E.coli* and other porins, OMPK35 and OMPK36 in *K. pneumonia*. The regulation of OprD in *P. aeruginosa* is a well-studied example of a porin, where, the reduced expression of this protein results in the concomitant increase in imipenem resistance.

A 40kDa protein, which was identified as a porin, OmpC, from MALDI TOF analysis, is present in all three strains. What is interesting to note is that the porins allow the influx of molecules, possibly, hydrophilic antibiotics, and so it would be expected the porin only be expressed in the sensitive to antibiotic strain rather than in all three strains. It is then highly likely that this 40kDa porin does not play a role in the antibiotic resistance, to the beta lactam family of antibiotics anyway. This is the first time that this 40kDa OMP has been identified as a porin. Previous investigators only identified it in *A. baumannii*, and the authors did not conclusively determine a function for it.

However, Clark (1991) showed that a 33-36 kDa protein was involved in imipenem resistance. The decreased expression of this porin was associated with the concomitant increase in MICs to imipenem. Costa *et al.* (2000) also demonstrated imipenem resistance in a clinical isolate of *A. baumannii* that had lost an OMP band in the region 33-36 kDa.

In another study, the OMP profile of imipenem and meropenem resistant strains were compared to their sensitive counterparts, and the authors observed a decreased expression of a ~33kDa protein (Bou *et al.*, 2000). A 35-kDa protein that was identified as a porin was found in the sensitive strain only. The 35kDa porin was found to display the highest identity against a porin from *Neisseria meningitides* (Minetti *et al.*, 1998). Interestingly, *Acinetobacter* spp. were classified in the family of *Neisseriaciae* [see section 1.2] before the re-classification in the family *Moraxellaceae* [section 1.2]. This is first time that a protein of this particular size has been identified as a porin, in *A. baumannii*, following MALDI analysis.

Interestingly, then, the 35kDa OMP that was identified in the sensitive strain but not observed in either the resistant strain or the intermediate resistant strain could play a role in the antibiotic resistance. However, it is unlikely that this OMP plays a role in imipenem resistance since all the clinical isolates were susceptible to this antibiotic. The protein assay was carried out with the intention of determining a mechanism which could help explain the resistance to the extended spectrum cephalosporins, cefuroxime and cefotaxime observed in our clinical isolates of *A. baumannii*. Further analysis of this 35kDa OMP will be required in order to determine conclusively the correlation, if any, between this 35kDa OMP and the extended spectrum of resistance.

However, in *K. pneumoniae*, a porin, OmpK35, has been postulated to play a role in ceftazadime penetration (Rasheed *et al.*, 2000). This conclusion was based on kinetic data from their studies. This OMP is the same size as the 35kDa porin identified in our clinical isolate of *A. baumannii.* Siu (2001) argues against this hypothesis, however, further investigations will be required in order to determine a greater similarity between the two OMPs, and once again determine conclusively that OmpK35 is in fact involved in cephalosporins penetration.

ABC transporters have not been extensively described in prokaryotes. This complex tri-partite system has not often been detected either. In fact only isolated cases have been reported and that too only in *Streptococcus* spp.

However, both the 55kDa and 67kDa OMPs have been described as being part of some active transport system, specifically, a protein dependent ATP binding transport system. It is the first time that OMPs of this size have been described in *Acinetobacter* spp or even *A. baumannii*. Although, tri-partite transport systems have been well described in organisms such as *P. aeruginosa*, there has only been one such description of such a system in *Acinetobacter*. Magnet *et al.* (2001) describe the detection and inactivation of Ade efflux system, involved in the resistance to aminoglycosides. By insertional in activation of the *adeB* gene (encoding a resistance-nodulation-cell division (RND) protein) the authors were able to demonstrate its role in resistance. This protein being part of a three component system was not shown to confer a broad spectrum of resistance. However, the authors did not give any protein data, and hence a comparison based on size was not possible. It is likely that a similar system may be present in our clinical isolates of *A. baumannii* and this system may be involved in resistance to β lactams. It is unlikely that these two OMPs are part of an ABC transport system due to its rarity in eukaryotes. It is then likely that these OMPs then form some part of an active transport system or ATP dependent efflux. However, further investigations will be required in order to ascertain this hypothesis.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

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5.1 REVIEW

As mentioned previously, over the past few years, ***Acinetobacter*** *spp.*, specifically *A.baumannii* has emerged as a significant nosocomial pathogen (Bergogne- Berezin, 1990). A number of different factors come into play which contribute to its overall success as a pathogen and these include mechanisms such as β lactamases and small porins. Its intrinsic nature to be resistant and its ability to acquire resistance to a number of currently used antibiotics has made it a significant nosocomial pathogen. *Acinetobacter*'s ability to respond rapidly to the onslaught of antimicrobial therapy to which it is subject means that is one step ahead. In particular, the *Acinetobacter baumannii* species has showed its ability to be multi-resistant, in strains isolated from the hospitals. It becomes important to be able to better understand the underlying mechanisms that contribute to this resistance and therefore be able to treat the infections effectively.

The combination of choice at Groote Schuur for treating *Acinetobacter* infections, during the 1999 and the early part of 2000, was piperacillin-tazobactem. These two work in tandem to combat and halt growth of *A. baumannii* infections. Since its introduction about 4 years ago, this combination therapy has proven no longer effective. These resistant strains formed part of a clinical study which set out to:

i) to identify the different mechanisms that are contributing towards piperacillin tazobactem resistance in *Acinetobacter* baumannii

ii) to characterise these mechanisms and hence help better understand the resistance displayed by this organism

5.2 TYPING AND EPIDEMIOLOGY

The use of the drug combination of piperacillin and tazobactem was implemented at GSH in 1996. In less than 2 years, this combination ceased to be effective, with *A.baumannii* developing resistance to this combination as well as a number of other antibiotics including the cephalosporins. A cluster of eleven strains were chosen in the period of January and February 2000, which exhibited resistance to this combination as well as to the other cephalosporins, such as cefuroxime and ceftazidime. As the strains were isolated from disparate wards, this suggested some other form of resistance transfer, which did not solely infer plasmid involvement. The isolation of the resistant strains from disparate wards and sites suggests that the resistance was due to organism spread. It is most likely that the organism spread on the hands of uninfected hospital staff (human carriage). This data is supported by work carried out by Allan and Green, (1994) which showed that *Acinetobacter* infections were spread via hospital staff. It is also likely that the organism spread via contamination of inanimate sources such as ventilator equipment (which is shared throughout the hospital), taps and beds. This suggestion is supported by Mirren *et al*, (1990) who also showed that inanimate objects contributed to spread via contamination.

Some interesting observations were made on the statistics of piperacillin-tazobactem resistance over a two-year period. Firstly, there was a decrease in the incidence of resistant strains from December 1999-June 2000 (156→139 clinical isolates), suggesting that the resistance was self-limiting. Secondly from July 2000, a new drug regime was implemented to treat *A. baumannii* infections; the carbapenems. Following the implementation of this new regime, the incidence of resistance strains decreased even further. In fact from June 2000-July 2001, the incidence decreased from 139→40, an almost 4-fold drop. Therefore, this outbreak appeared to diminish, and as with *A. baumannii* infections, ceased to be a problem with the implementation of a new drug regime. Our findings correlate well with that of Siegman-Igra *et al.* (1993), where an outbreak of *A. baumannii* spontaneously reverted in the absence of antibiotic selective pressure. The current drug of choice at GSH for *A.baumannii* infections is meropenem. It

may be only a matter of time before this becomes ineffectual. Imipenem resistant *Acinetobacters* have been described as early as 1993 (Paton et al., 1993). More carbapenemases have being detected and earlier on this year, Yiu *et al* (2001) isolated IMP-4 from an *A. baumannii* strain in Japan, where this is also the drug of choice. IMP-4 is a novel carbapenemase, which is active mainly against imipenem but to a certain extent meropenem as well as the other penicillin derivative drugs.

Having observed a growing resistance amongst the species, it then becomes important to understand its epidemiology. Previously, in a clinical setting it had been observed that the prevalence of *Acinetobacter spp.* infections was quite low- with more cases being reported for colonization rather than infection (Dijkshoorn, 1993). Despite this low demand for diagnostic testing, it becomes important to type strains during outbreak or endemic episodes in order to discern between epidemic and sporadic strains.

Previously, a number of different typing techniques were shown to be useful in investigating the epidemiology of *Acinetobacter* spp. PFGE was shown to be a good discriminatory technique when compared against plasmid profiling and whole cell protein profiling (Seifert et al., 1994). RAPD, specifically, the use of ERIC primers also demonstrated good discrimination between outbreak strains of *A.baumannii* (Cimola and Tromblyet, 1997). The success demonstrated using Poly-GTG typing in our department and the reliability of this typing tool (Matsheka, 2000), recommended this is a first choice in to investigate the origins of the strains.

The technique worked well and the GTG-profiles obtained were very interesting. The strains; both the resistant and sensitive strains appeared to have a similar profile (Fig. 2.2. lanes 1-12; Fig. 2.3 lanes 2-13). The identical profiles, suggest that all the strains had a common origin or ancestor and hints at the fact that the resistance was in some way acquired or developed. The parent strain/ progenitor was most likely susceptible to the beta lactams, but in a short period of time, either acquired or developed a resistance. It can be seen from Fig 2.2 that isolate 13 has a different profile and this only serves to confirm that the other 12 strains formed part of an outbreak and therefore, this strain is

not related to them. These findings are novel and unique with respect to these *A. baumannii* strains. This is the first time that poly-GTG typing has been used at this level to study the epidemiology of *Acinetobacter* spp. Our findings have shown that this technique is in fact useful and its reproducibility is good.

The typing of these strains was only a first step to this study and further investigations into the mechanisms of resistance of these strains will be discussed in detail in the subsequent sections.

5.2 BETA LACTAMASE DETECTION

PCR was used initially for the detection of the *ampC* gene. The primers designed, amplified a portion of the structural region of the published sequence data. This ~ 650 bp fragment was found to be present in all the strains tested, except the sensitive strain. In fact, after altering certain parameters to optimise the PCR, we failed to amplify a product. The slot blot hybridisation studies indicated the presence of the *ampC* gene in all the clinical isolates, including the sensitive strain, leading us to conclude that the *ampC* gene was intrinsic to *A.baumannii* and also chromosomally based. The *ampC* gene has not yet been fully characterised, in fact to date, only one publication has been released (Bou et al., 2000).

A portion of the *ampC* gene amplified via PCR that was detected in the resistant strains was further analysed by sequencing.. The sequence data that, correlated well with the sequence information on the database. The test sequence displayed 90% identity to the published sequence leading us to the conclusion that we had in fact identified the *ampC* gene in *A.baumannii*.

The *ampC* was found to be on a very large fragment, >10 kb. This fragment was cloned into pBSG18, and the transformant was tested for its susceptibilities to a number of antibiotics. Analysis of this data showed that although the parent strain was resistant to

amoxycillin, piperacillin, piperacillin-tazobactem, co-amoxyclav, cefuroxime and ceftriaxone, *E.coli* (p101) was sensitive to cefuroxime (8μg/ml) and ceftriaxone (0.32μg/ml). This suggested that some other mechanism was responsible for the resistance to these two cephalosporins. These findings are different to those of Bou *et al,* (2000). Unlike in our clinical isolate, the AmpC detected in their clinical strain of *A. baumannii* conferred resistance to cefuroxime (>256μg/ml). They did not, however, test the activity against ceftriaxone.

The presence of the TEM-1 gene which has a P4 type promoter which contributes to the up-regulation, was also shown to have the identical sequence to TEM 1F. The structural region of the gene was shown to have 2 new previously un-described mutations, at codons 205 and 213, which corresponded to changes His→Gln and Glu →Asp respectively. This TEM, although conferring resistance to the penicillin derivative drugs was not found to display any ESBL activity. It is then likely that the mutations are silent; despite the fact that they are silent they are still significant, as this could be described as a new TEM β lactamase.

Based on the literature on porins and transport systems described in other gram-negative bacteria, the next course of action was to investigate these proteins in out clinical isolates of *A.baumannii*.

The combined MIC data from the *ampC* and TEM genes respectively could not account for the resistance to the extended spectrum cephalosporins, led us to look for alternative mechanisms by which this resistance could be mediated; protein-based antibiotic resistance mechanism.

5.4 OUTER MEMBRANE PROTEINS

Porins have been described in other Gram-negative bacteria and examples of these include OprD in *P. aeruginosa* (Srikumar *et al.*, 1997; Epp *et al.*, 2001), OmpF in *E.coli* and other porins, OMPK35 and OMPK36 in *K. pneumonia*. The regulation of OprD in *P. aeruginosa* is a well-studied example of a porin, where, the reduced expression of this protein results in the concomitant increase in imipenem resistance.

A 40kDa protein, which was identified as a porin, OmpC, from MALDI TOF analysis, is present in all three strains. What is interesting to note is that the porins allow the influx of molecules, possibly, hydrophilic antibiotics, and so it would be expected the porin only be expressed in the sensitive to antibiotic strain rather than in all three strains. It is then highly likely that this 40kDa porin does not play a role in the antibiotic resistance, to the beta lactam family of antibiotics anyway. This is the first time that this 40kDa OMP has been identified as a porin. Previous investigators only identified it in *A. baumannii*, and the authors did not conclusively determine a function for it.

Clark *et al*,(1991) however showed that a 33-36 kDa protein was involved in imipenem resistance in *A. baumannii*. The decreased expression of this porin was associated with the concomitant increase in MICs to imipenem. Costa *et al.* (2000) also demonstrated imipenem resistance in a clinical isolate of *A. baumannii* that had lost an OMP band in the region 33-36 kDa. In another study, the OMP profile of imipenem and meropenem resistant strains were compared to their sensitive counterparts, and the authors observed a decreased expression of a ~33kDa protein (Bou *et al.*, 2000). A 35-kDa protein that was identified as a porin was found in the sensitive strain only. The 35kDa porin was found to display the highest identity against a porin from *Neisseria meningitidis*. Interestingly, *Acinetobacter* spp. were classified in the family of *Neisseriaciae* before the re-classification in the family *Moraxellaceae*. . This is first time that a protein of this particular size has been identified as a porin, in *A. baumannii*, following MALDI analysis.

Interestingly, then, the 35kDa OMP that was identified in the sensitive strain but not observed in either the resistant strain or the intermediate resistant strain could play a role in the antibiotic resistance. However, it is unlikely that this OMP plays a role in imipenem resistance since all the clinical isolates were susceptible to this antibiotic. The protein assay was carried out with the intention of determining a mechanism which could help explain the resistance to the extended spectrum cephalosporins, cefuroxime and cefotaxime observed in our clinical isolates of *A. baumannii*. Further analysis of this 35kDa OMP will be required in order to determine conclusively the correlation, if any, between this 35kDa OMP and the extended spectrum of resistance.

In *K. pneumoniae*, however a porin, OmpK35, has been postulated to play a role in ceftazadime penetration (Rasheed et al., 2000). This postulate was based on kinetic data from their studies. This OMP is the same size as the 35kDa porin identified in our clinical isolate of *A. baumannii.* Further investigations, however, will be required in order to determine a greater similarity between the two OMPs, and determine conclusively that OmpK35 is in fact involved in cephalosporins penetration.

However, both the 55kDa and 67kDa OMPs have been described as being part of some active transport system, specifically, a protein dependent ATP binding transport system. It is the first time that OMPs of this size have been described in *Acinetobacter* spp or even *A. baumannii*. Although, tri-partite transport systems have been well described in organisms such as *P. aeruginosa*, there have been no descriptions of such systems in *Acinetobacter*. It is unlikely that these two OMPs are part of an ABC transport system due to its rarity in eukaryotes. It is then likely that these OMPs then form some part of an active transport system or ATP dependent efflux. However, further investigations will be required in order to ascertain this hypothesis.

5.5 CONCLUSIONS

Therefore, in conclusion, we can see how the antibiotic resistance profile is built up. We have found more than one underlying mechanism to help explain the resistance observed in the strains.

It is likely that the beta lactamases described above, TEM and AmpC together with the porins 35kDa and 40kDa, as well as putative transport systems, contribute to the overall resistance displayed by the clinical strains. Their has been limited work done on the contribution of these porins and pump-type systems in β lactam resistance.

Further investigations into the exact nature of the outer membrane proteins and more detailed analysis, such as protein sequencing will be required in order to conclusively invoke their role in the resistance. As *A. baumannii* emerges as a significant nosocomial pathogen, it becomes important to understand the mechanisms contributing to its pathogenicity and help curb its spread.

APPENDIX A

Buffers, Media and Solutions

Ampicillin (100 mg/ml)

Ampicillin	2 g
Distilled water	20 ml

The solution is filter sterilized and stored in aliquots at -20 °C. For selective plates ampicillin is added to the media at a final concentration of 100 μg/ml.

Chloroform-isoamylalcohol

The chloroform is mixed at a ratio of 24:1 with isoamylalcohol

EDTA (0.5 M)

EDTA	93.05 g
NaOH	10 g

The ingredients are dissolved in 400 ml of water and autoclaved.

Ethidium Bromide (EtBr)

Ethidium Bromide	0.1 g
Distilled Water	10 ml

Gel Tracking Dye (6×)

Bromophenol blue	25 mg
Sucrose	4 g
EDTA (0.5 M, pH 8.0)	0.4 ml
Distilled water	to 10 ml

Guanidium Thiocyanate Solution

Guanidium Thiocyanate	60 g
EDTA (0.5 M, pH 8.0)	20 ml
Distilled water	20 ml

The solution is heated to 65°C with mixing until dissolving. Solution is cooled and 5 ml of 10% (w/v) N-Lauryl-Sarcosine Sodium salt is added and made upto 100 ml with distilled water. Solution is filtered through a 0.45 μm pore.

IPTG (Isopropyl-β-D-thio-galactopyranoside)

IPTG	2 g
Distilled Water	8 ml

The solution is mixed and made up to 10 ml and frozen in 1 ml aliquots at -20°C. For selective plates, 50 μl IPTG is added to 100 ml agar.

Phenol

Commercial crystallized phenol	500 g
8-hydroxyquinoline	0.6 g
NaOH (2 M)	7.5 ml
Distilled water	130 ml
Tris-HCl (1 M, pH 7.6)	6 ml

The solution is liquefied at 40°C. The result is a solution of phenol in 10 mM Tris, pH 7.6.

Phosphate-Buffered Saline (PBS)

NaCl	6.8 g
Na_2HPO_4	3.5 g

Made upto 1000 ml, pH adjusted to 7.0.

Sodium Acetate (3 M)

Sodium acetate	204.05 g
Distilled water	400 ml

The pH is adjusted to 5.2 with glacial acetic acid and the volume made up to 500 ml. Stored at room temperature.

Sodium Hydroxide (10 N)

Sodium hydroxide	40 g
Distilled water	100 ml

Solution I

Tris-Cl (pH 8)	2.5 ml
EDTA (0.5 M, pH 8)	10 ml
Glucose (20% w/v)	5 ml
Distilled water	to 100 ml

Solution II

NaOH (10 N)	2 ml
SDS (20% w/V)	5 ml
Distilled water	to 100 ml

Solution III

Potassium acetate (5M)	60 ml
Glacial acetic acid	11.5 ml
Distilled water	to 100 ml

Store at 4°C.

SSC (10X)

$C_6H_5Na_3O_7.2H_2O$	0.15 M
NaCl	1.5 M

Tris-Acetate (TAE) Buffer (50×)

Tris	242 g
Glacial acetic acid	57.1 g
EDTA (0.5m, pH 8.0)	100 ml

The ingredients are mixed in distilled water to a final volume of 1000 ml, autoclaved and stored at room temperature.

To make 1X solution, the buffer is diluted 50 fold.

Tris-EDTA (TE) Buffer

Tris	10 mM
EDTA	1 mM

The pH is adjusted to 7.6.

X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside)

X-gal is dissolved in dimethyl sulfoxide (DMSO) at a concentration of 40 mg/ml. After the X-gal is dissolved, 1 volume of distilled water is added and the solution is stored at -20°C.

For selective plates, 0.5 ml X-gal solution is added to 100 ml agar.

2× YT Broth/Agar

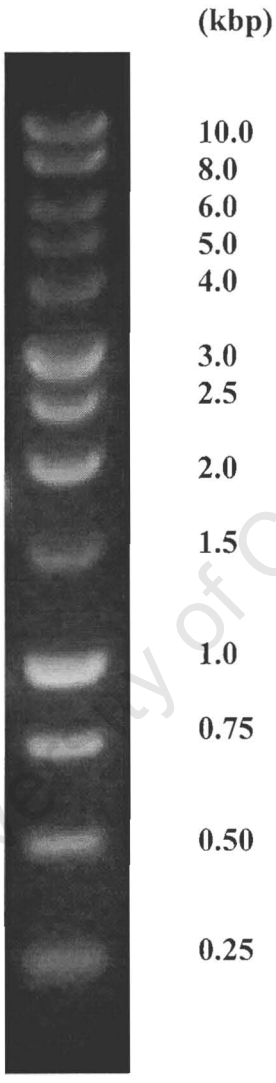
Tryptone	16 g
Yeast extract	10 g
NaCl	5 g

Made up to 1000 ml with distilled water and autoclaved

For agar, 15 g is added.

APPENDIX B

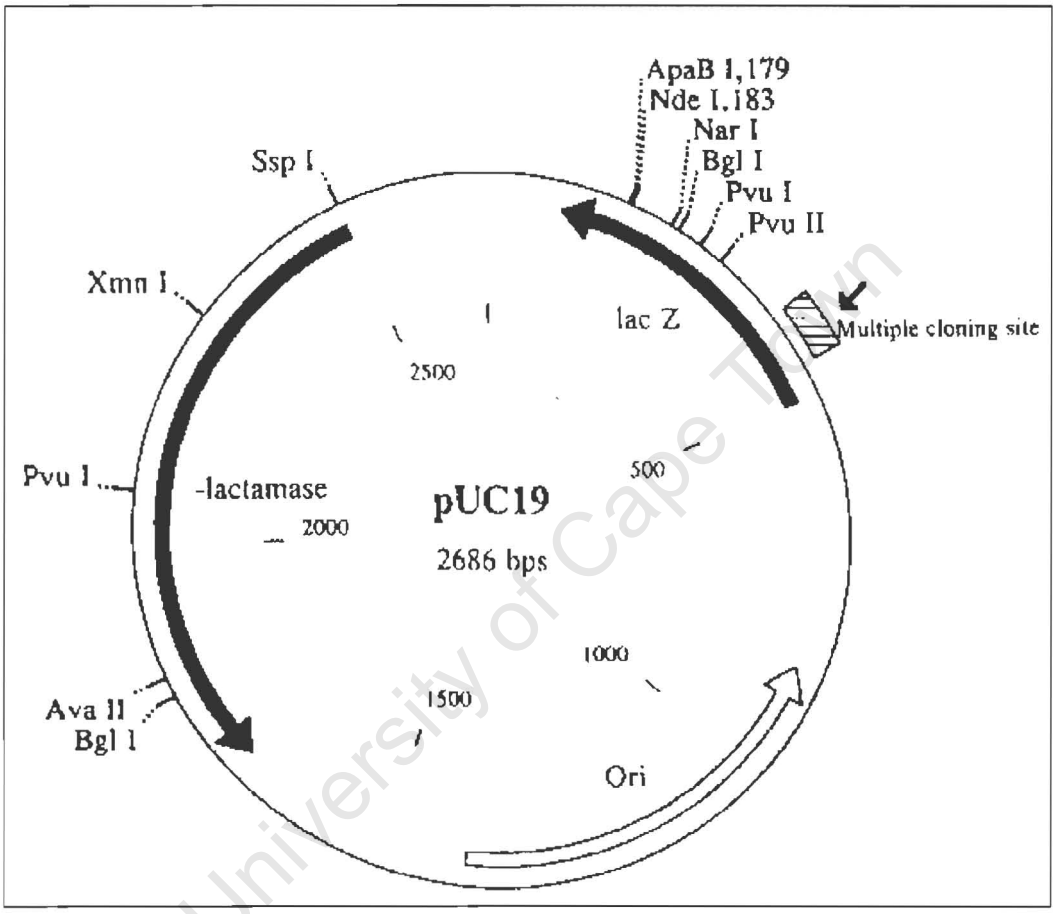
Molecular Weight Markers



Appendix B1: 1kb DNA Ladder (Promega).

APPENDIX C

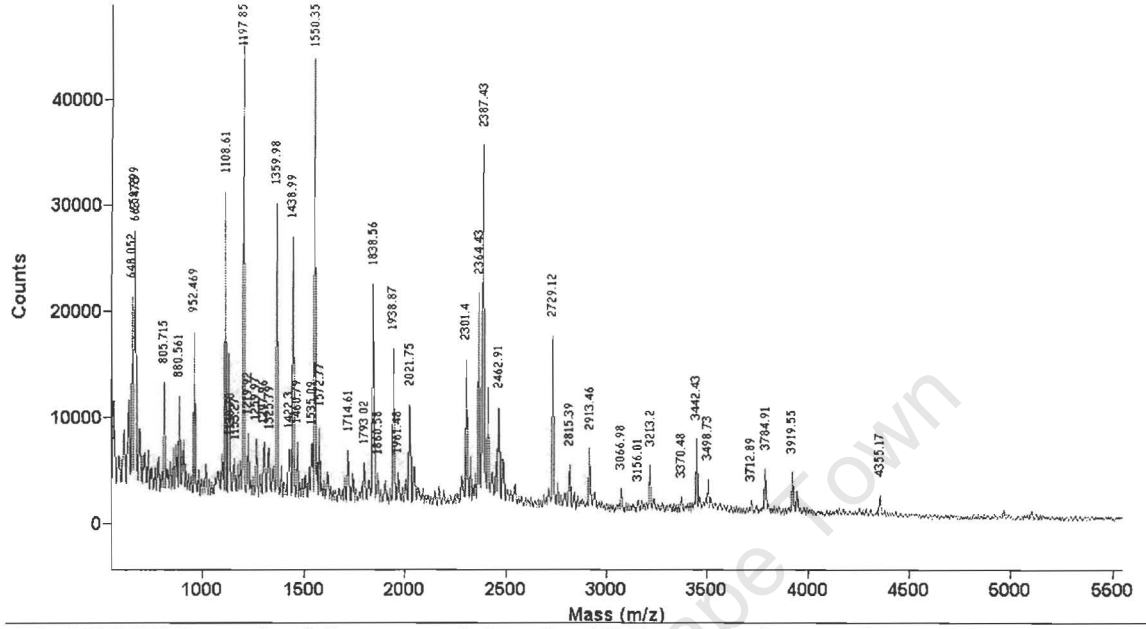
Maps of Plasmids



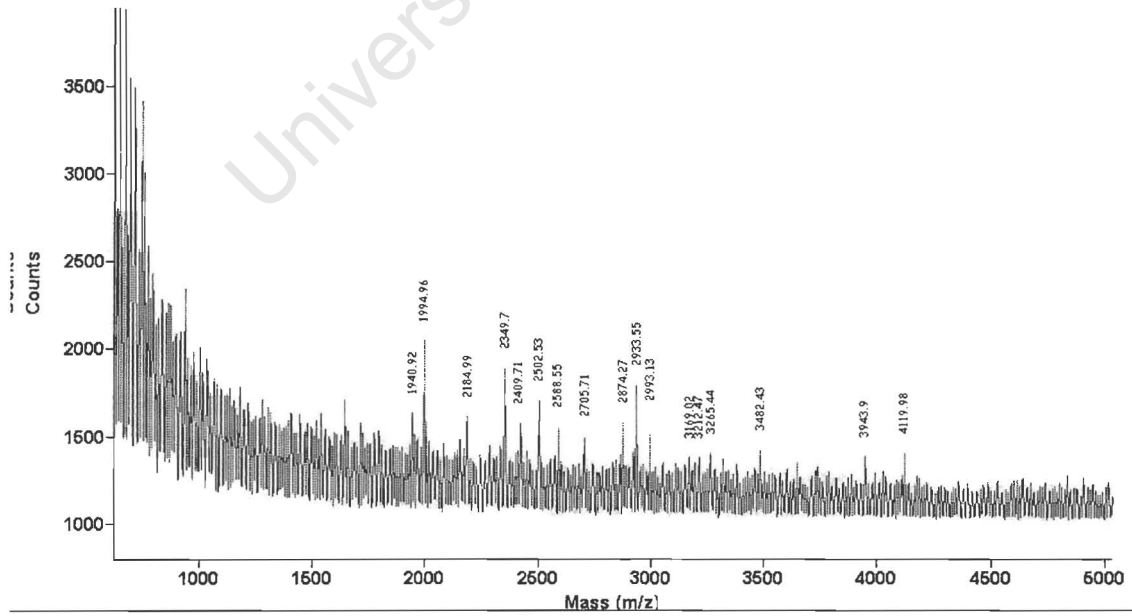
Appendix C1: pUC19

Appendix D

MALDI-TOF Graphs of Counts vs. mass (m/z)



MALDI-TOF graph of Counts vs. Mass for 43kDa OMP from *A. baumannii*



MALDI-TOF graph of Counts vs. Mass for 25kDa OMP from *A. baumannii*.

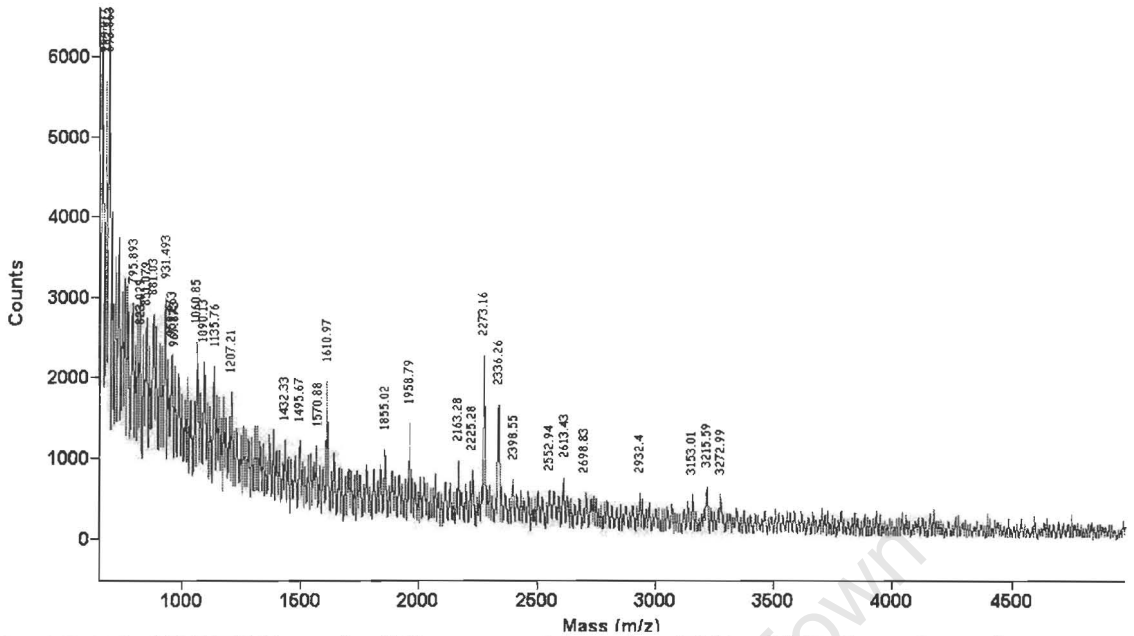
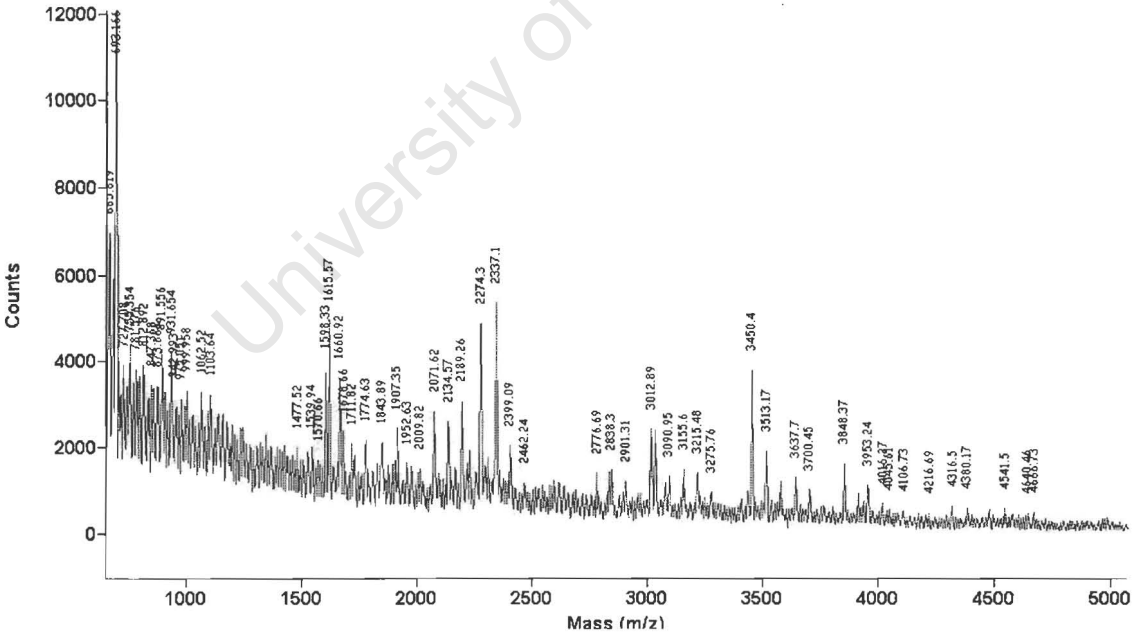
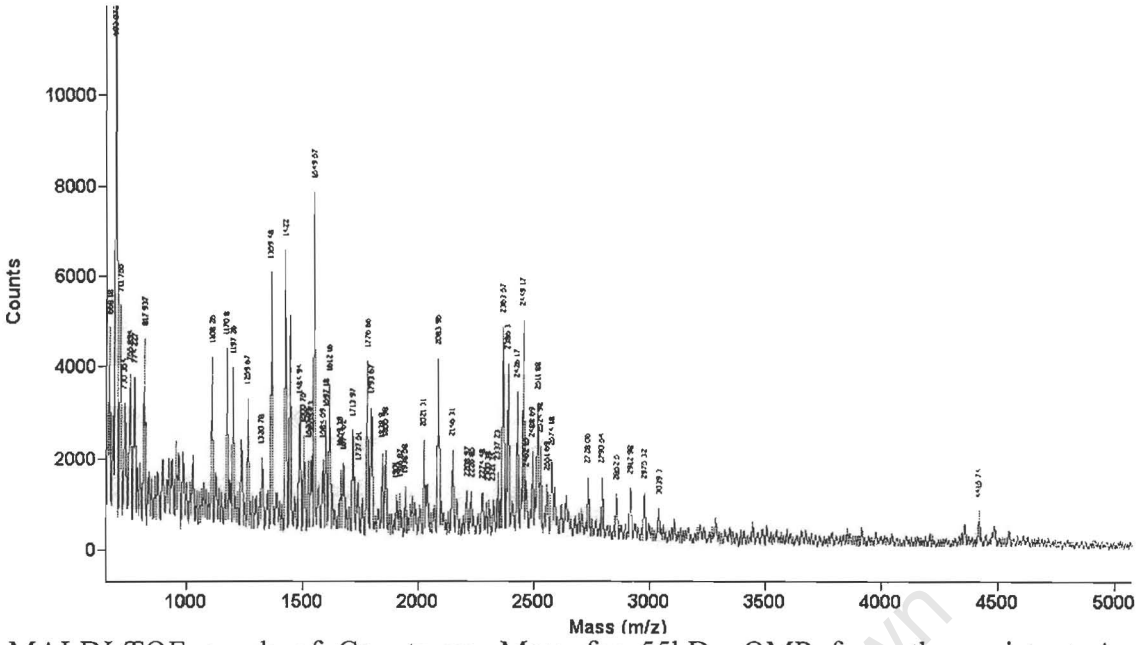


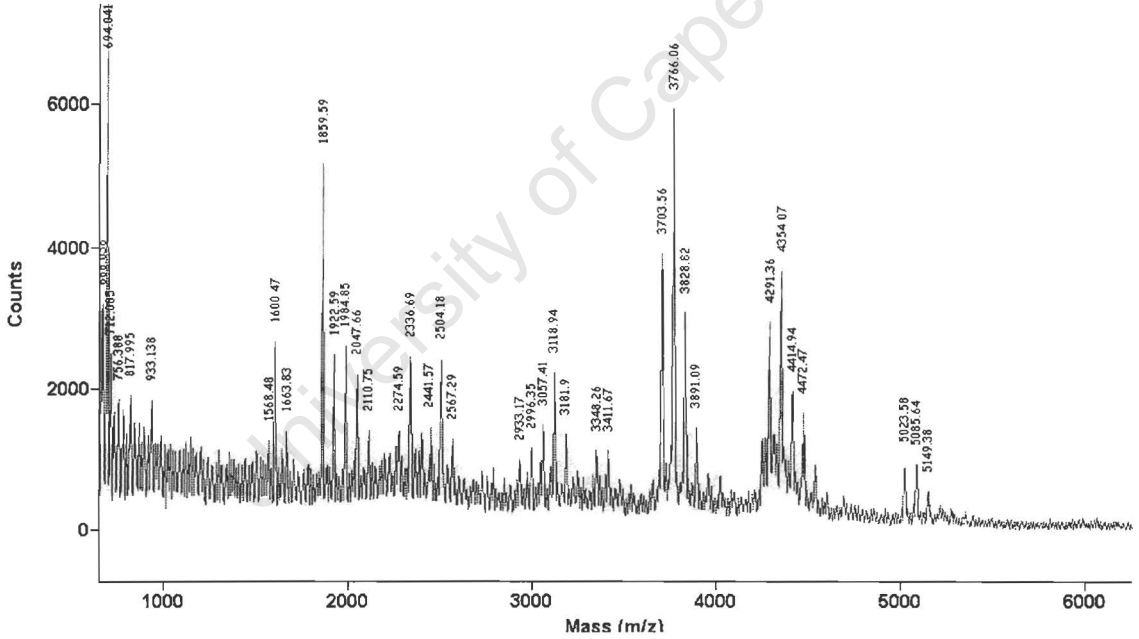
Fig 4.7: MALDI-TOF graph of Counts vs. Mass for 67kDa OMP from the resistant *A. baumannii*.



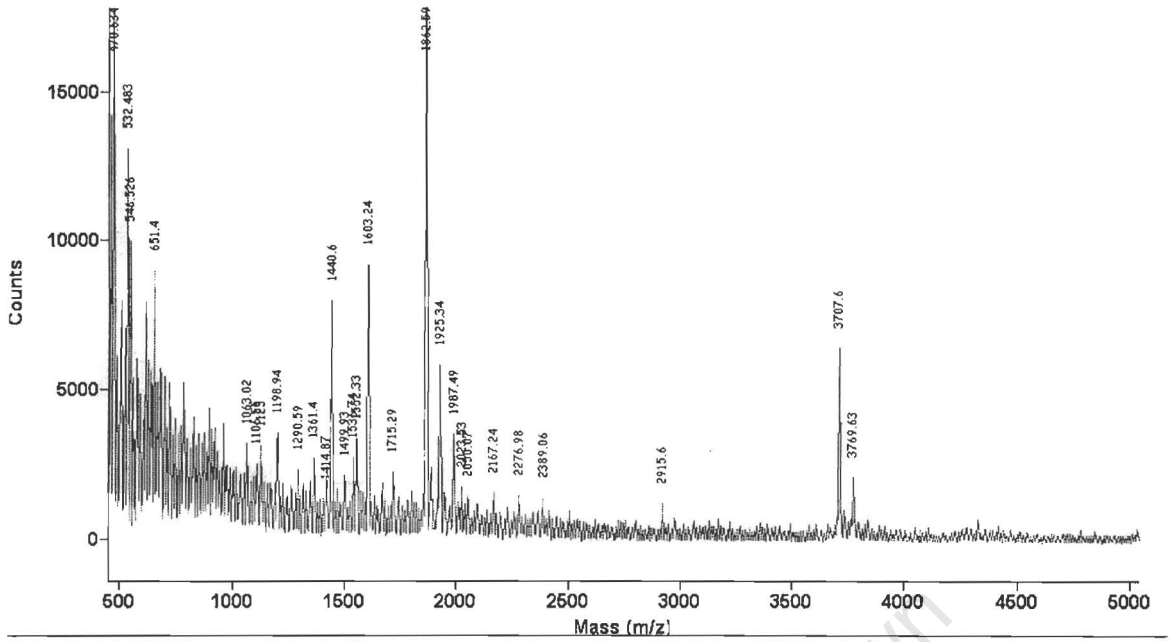
MALDI-TOF graph of Counts vs. Mass for 40kDa OMP from the resistant *A. baumannii*.



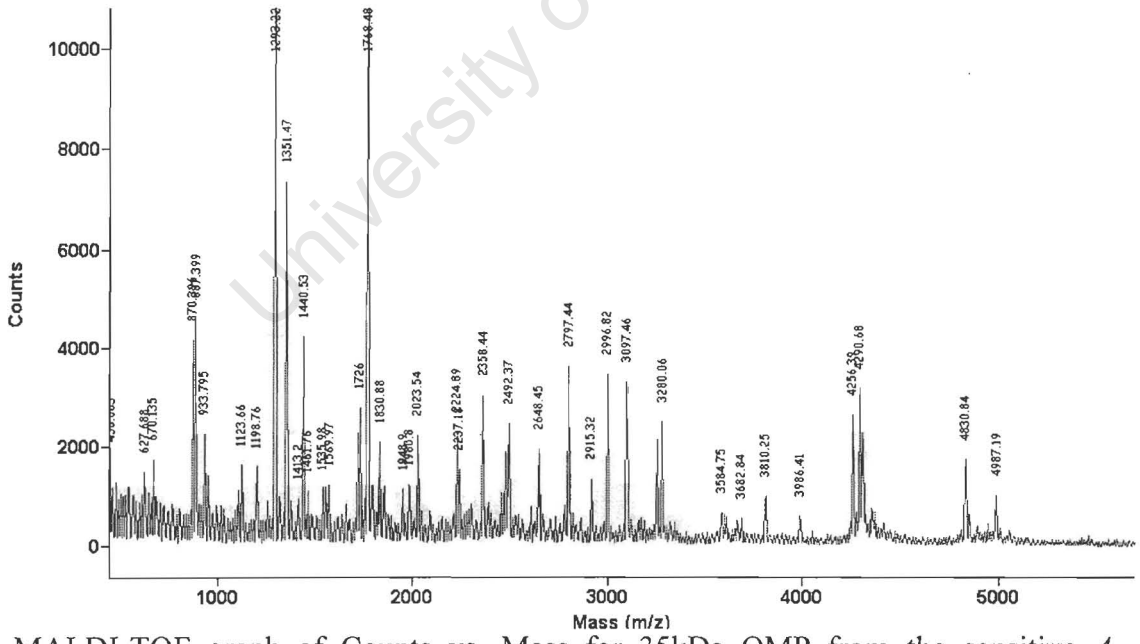
MALDI-TOF graph of Counts vs. Mass for 55kDa OMP from the resistant A. *baumannii*



MALDI-TOF graph of Counts vs. Mass for 30kDa OMP from the resistant *A. baumannii*.



MALDI-TOF graph of Counts vs. Mass for 33kDa OMP from the resistant *A. baumannii*.



MALDI-TOF graph of Counts vs. Mass for 35kDa OMP from the sensitive *A. baumannii*.

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