SUSCEPTIBILITY & SYNERGISM PROFILES OF MULTI-DRUG RESISTANT *PSEUDOMONAS AERUGINOSA* IN AN INTENSIVE CARE ENVIRONMENT

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SUMMARY

SUSCEPTIBILITY AND SYNERGISM PROFILES OF MULTI-DRUG RESISTANT *PSEUDOMONAS AERUGINOSA* IN AN INTENSIVE CARE ENVIRONMENT

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Pseudomonas aeruginosa is an aerobic gram-negative bacillus commonly found in soil, water, sewage and plants. Owing to the ability of this organism to inhabit almost any type of moist environment it is a real and common hazard in the nosocomial environment. *P. aeruginosa* is an opportunistic pathogen that can infect almost any part of the body.

An outbreak of multi-drug resistant *P. aeruginosa* occurred in the Pretoria Academic Hospital (PAH). These organisms tended to appear in the intensive care units due to selection pressure caused by the use of antibiotics.

Over a three-year period (March 1998 to September 2001) 100 isolates of multidrug-resistant *P. aeruginosa* were collected. These organisms were obtained from cultures from patients hospitalized in mainly intensive care units (ICUs) of the PAH. The identity of the isolates was confirmed using the 20NE API identification system. Thereafter the DNA of the multidrug-resistant *P. aeruginosa* isolates was purified.

Molecular studies were attempted to determine if one or more different clones of *P. aeruginosa* were involved. An arbitrarily-primed polymerase chain reaction (AP-PCR) method was used to determine the randomly amplified polymorphic (RAPD) DNA patterns of these isolates. Numerous PCR attempts failed and finally the minimum inhibitory concentrations (MICs) of all 100 isolates were determined using the agar dilution method according to National Committee of Clinical Laboratory Standards (NCCLS). Commonly used antipseudomonal drugs such as Amikacin, Ciprofloxacin, Aztreonam, Piperacillin, Tazobactam, Ceftazidime, Cefepime, Imipenem and Meropenem were used to test the isolates. The organisms were then divided into four different groups according to their specific susceptibility patterns. Data gathered over the study period was then compared to establish the resistance trends of these isolates. Various methods including Time-kill curves and double-disc methods were performed to determine possible synergy. A novel E-test method provided the best results in the end. It was found that Amikacin in combination with Meropenem followed by Cefepime yielded the best combination therapy options.

OPSOMMING

SENSITIWITEITS- EN SINERGISTIESE PROFIELE VAN VEELVULDIGE WEERSTANDBIEDENDE *PSEUDOMONAS AERUGINOSA* IN 'N INTENSIEWESORG OMGEWING

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Pseudomonas aeruginosa is 'n aërobiese, gram-negatiewe basil wat algemeen voorkom in grond, water, riool en plante. As gevolg van hierdie organisme se vermoë om in amper enige tipe vogtige omgewing te groei, skep dit 'n algemene probleem in hospitale. *P. aeruginosa* is 'n opportunistiese patogeen wat feitlik enige deel van die menslike liggaam kan infekteer. 'n Uitbraak van multi-weerstandbiedende *P. aeruginosa* het ontstaan in die Pretoria Akademiese Hospitaal (PAH). Hierdie organismes het hoofsaaklik voorgekom in die intensiewesorgeenhede. Hul teenwoordigheid kan moontlik toegeskryf word aan seleksie druk veroorsaak deur die gebruik van antibiotika.

'n Honderd multi-weerstandbiedende *P. aeruginosa* isolate was versamel oor 'n periode van drie jaar (Maart 1998 tot September 2001). Hierdie organismes is versamel van kulture afkomstig van pasiënte hoofsaaklik in intensiewesorgeenhede van die PAH. Die identiteit van hierdie isolate is bevestig m.b.v. die 20NE API sisteem (bioMerieux, La Balme les Grottes) waarna die DNS van hierdie multi-weerstandbiedende *P. aeruginosa* isolate gesuiwer is. 'n "Arbitrarily-primed" polimerase ketting reaksie (AP-PKR) metode was gebruik om die "randomly amplified" polimorfiese DNS (RAPD) patrone te bepaal.

Die doel hiervan was om te bepaal of een of meer verskillende klone van multi-weerstandbiedende *P.aeruginosa* teenwoordig was. Verskeie PKR pogings was onsuksesvol en uiteindelik is die minimum inhibitoriese konsentrasies (MIKs) van al honderd isolate bepaal deur die agarverdunningsmetode te gebruik soos voorgeskryf deur die NCCLS. Antibiotika wat algemeen vir behandeling van *P.aeruginosa* infeksies gebruik word is getoets naamlik: Amikasien, Siprofloksasien, Aztreonam, Piperasillien, Tazobaktam, Keftasidiem, Kefepiem, Imipenem en Meropenem. Hierdie organismes was daarna in vier verskillende groepe verdeel volgens elke spesifieke sensitiwiteitspatroon. Inligting versamel tydens hierdie studie was vergelyk om die neiging(s) van weerstandbiedendheid van hierdie isolate te bepaal. Verskeie metodes insluitend die "time-kill" kurwe metode en die dubbel-skyfie diffusie metode was uitgevoer om te toets vir moontlike sinergie. 'n Relatiewe nuwe E-toets metode het uiteindelik die beste resultate gelewer. Dit is gevind dat die kombinasies van Amikasien en Meropenem, gevolg deur Kefepiem die beste kombinasieterapie opsies is om te oorweeg vir die behandeling van multi-weerstandbiedende *P. aeruginosa* infeksies.

KEY WORDS

Virulence	The degree of an organism's capability to cause disease
Clone	A group of bacterial cells, which originate from only one cell by asexual reproduction. These cells are genetically identical to each other as well as to the mother cell
MIC	The minimum inhibitory concentration needed to inhibit growth of an organism
Sensitivity	The antibiotic used inhibits the organism's growth.
Resistance	The antibiotic used does not inhibit the organism's growth.
Multi-drug Resistance	Bacterium resistant to most drugs
Synergism	A greater or equal to a hundred-fold decrease in the number of colony forming units per milliliter (CFU)/ml after four to twenty-four hours of incubation with the combination, in comparison with the most active single drug
Antagonism	A greater or equal to hundred-fold increase in CFU/ml after twenty-four hours with the combination, as compared with the most active single drug
Indifference	A less than ten-fold change (increase or decrease) in killing at twenty-four hours with the combination, in comparison to the most active single antimicrobial alone

LIST OF ABBREVIATIONS

A-P	Arbitrarily-Primed
BHI	Brain Heart Infusion
CARB	Carbenicillin-type ESBL
CFU	Colony Forming Units
CVP	Central venous pressure
DMSO	Dimethylesulfoxide
DNA	Deoxyribonucleic Acid
dNTP	deoxyribonucleotide phosphate
EDTA	Ethylene-diamine-tetra acetic acid
ESBL	Extended-Spectrum β-Lactamases
GM-1	Ganglioside receptor on epithelial cell surface
HIV	Human Immunodeficiency Virus
ICU	Intensive Care Unit
kb	kilobase
MH	Mueller-Hinton
MIC	Minimum Inhibitory Concentration
min	minutes
NCCLS	National Committee of Clinical Laboratory Standards
OprD	Outer membrane porin D
OprM	Outer membrane porin M
ONPG	Ortho-nitro-phenyl-β-D-galactopiranocide
OXA	Oxacillinase-type ESBL
PAH	Pretoria Academic Hospital
PCR	Polymerase Chain Reaction
RAPD	Randomly Amplified Polymorphic DNA
sec	seconds
SHV	Ambler class A β-Lactamases
Ta	Annealing temperature
TAE	Tris-acetate-EDTA

Taq	DNA polymerase from *Thermus aquaticus*
TE	Tris-HCl-EDTA
TEM	Ambler class A β-Lactamases
Tm	Melting temperature
UV	Ultra Violet
V	Volts
$ZnCl_2$	Zinc chloride

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CHAPTER 1: INTRODUCTION

1.1 Identification

The genus *Pseudomonas* is a member of the family Pseudomonadaceae. These organisms can be straight or slightly curved motile gram-negative rods with polar flagella (1). They do not form spores, stalks or sheaths (2). *Pseudomonas aeruginosa* is aerobic with respiratory metabolism using oxygen as the terminal electron acceptor. In some cases nitrate can be used as an alternate electron acceptor, allowing growth to occur anaerobically. Growth occurs at temperatures ranging from 4°C or lower to 43°C, with optimal growth occurring at 37°C. These gram-negative bacilli are chemo-organotrophic, able to use other than one-carbon organic compounds as sole carbon and energy sources. *P. aeruginosa* is catalase positive, usually oxidase positive with the molar percentage of the G+C content of deoxyribonucleic acid (DNA) ranging from 58 to 71. Most, if not all, species of the *Pseudomonas* genus fail to grow in acidic conditions (pH4.5), and many species do not require organic growth factors (1).

Two main colony types of *P. aeruginosa* can be observed on common solid media. One is large and smooth with flat edges and an elevated centre ("fried egg" appearance). The other is small, rough and convex. Clinical specimens are, in general, good sources of the large colony type, while the smaller type is commonly obtained from natural sources. Variation of the large type to the small type is easy to observe, but the reverse variation is extremely rare. A third mucous colony type can often be obtained from respiratory as well as urinary tract secretions. Mucoid mutants of *P. aeruginosa* can be divided into two groups according to whether the mucous (alginate) is produced in chemically defined media (1).

This organism is commonly found in soil, water, sewage as well as in plants and it has the ability to inhabit almost any type of moist environment (3,2). Most strains produce pyocyanin, a water-soluble green phenazine pigment that imparts a greenish colour to the culture medium. *P. aeruginosa* has the following key characteristics:

- Oxidase positive
- Produces pyoverdin
- Ferments glucose
- Maltose variable
- Non-lactose fermenter
- Mannitol variable
- Arginine positive
- Lysine negative
- Reduces nitrates (NO_3^-) to nitrite (NO_2^-)
- Variable reduction of nitrates (NO_3^-) to gaseous nitrogen (N_2)
- Urea variable
- ONPG negative
- DNAse negative
- Acetamide positive
- Does not hydrolyze esculin
- Polymyxin sensitive
- Kanamycin resistant, but Carbenicillin sensitive (4).

Most strains of *P. aeruginosa* can be identified on the basis of a few morphological characteristics and chemical tests. Not only does the rapid identification of these common isolates provide the physician with immediate information, but it also relieves the laboratory of performing a battery of time-consuming and expensive secondary tests (4). Observing the presence of the following primary characteristics can identify more than 95% of *P. aeruginosa* strains recovered from clinical specimens:

- Large colonies, grape-like odour
- Pyocyanin production
- Oxidase positive within 10 sec. (4)

It is probable that the observation of pyocyanin might be the only characteristic required for identification of *P. aeruginosa* because no other non-fermenter synthesizes this pigment. However, some mucoid strains of *P. aeruginosa* from patients with cystic fibrosis may not produce pigment and, therefore, may be misidentified if pigment production is the only criteria used for identification of these aberrant strains (4). The colonies are large, may be mucoid or dry, and often spread. A few strains may produce pigment with other colours: pyorubin (red), pyomelanin (brown to black) and pyoverdin (yellow) (4). Using a long wavelength ultraviolet light source fluorescein pigment can be visualized by observing the growth on certain media. Media containing proteose peptone three and cations, such as magnesium or manganese, enhance fluorescein synthesis. King's medium B, Sellers' medium, and Mueller-Hinton (MH) agar are also suitable for demonstrating fluorescence. Fluorescence may be enhanced if cultures are incubated at 20-30°C rather than at 35-37°C (4).

The following additional characteristics are helpful in identifying non-pigment-producing strains of *P. aeruginosa*:

- Growth at 42°C
- Alkalinization of acetamide
- Denitrification of nitrates and nitrites
- Motile with polar, monotrichous flagellum (4)

1.2 Clinical importance

In the pseudomonas group *P. aeruginosa* is the most frequently recovered species from clinical specimens. Infections commonly occur at any site where moisture tends to accumulate namely tracheostomies, indwelling catheters, burns and the external ear (commonly known as swimmer's ear), as well as weeping cutaneous wounds. The exudation of blueish pus, with a grape-like odour, from the production of pyocyanin, is characteristic of such infections (4). Initial localized infections can often lead to invasion of the bloodstream resulting in bacteraemia (3). Infections due to this pathogen are especially prevalent among patients with cystic fibrosis, acute leukemia, organ transplants and intravenous drug addiction (4). Urinary tract infections caused by this organism can be detrimental and even fatal in immunocompromised hosts (4). This pathogen can often cause severe or even fatal pneumonia in cystic fibrosis patients (3). *P. aeruginosa* can also cause devastating eye infections (4). Pseudomonal keratitis, infection of corneal ulcers including endophthalmitis must be approached as a medical emergency that can be fulminant and threaten permanent loss of vision. Individual cases of endocarditis, meningitis and brain abscess as well as infections of bones and joints from haematogenous spread appear regularly in the literature. Most cases of endocarditis require valve replacement due to the difficulty of eradicating the infection (4).

P. aeruginosa produces several substances that are thought to enhance the colonization as well as infection of host tissue. These substances together with a variety of virulence factors make *P. aeruginosa* the most clinically significant bacterium among the non-fermenting bacilli (4). Virulence factors include the following:

• Alginate	Capsular polysaccharide that allows infecting bacteria to adhere to lung epithelial cell surfaces and form biofilms which, in turn, protects the bacteria from antibiotics as well as the body's immune system
• Pili	Surface appendages that allow the adherence of the organism to GM-1 ganglioside receptors on host epithelial cell surfaces
• Neuraminidase	Removes sialic acid residues from GM-1 ganglioside receptors, facilitating binding of pili.
• Lipopolysaccharide	Produces endotoxin, causes sepsis syndrome: fever, shock, oliguria, leukopenia or leukocytosis, disseminated intravascular coagulation as well as metabolic abnormalities
• Exotoxin A	Tissue destruction, inhibition of protein synthesis, interrupts cell activity as well as macrophage response
• Enterotoxin	Interrupts normal gastrointestinal activity leading to diarrhoea
• Exoenzyme S	Inhibits protein synthesis
• Phospholipase C	Destroys the cytoplasmic membrane, pulmonary surfactant and inactivates opsonins
• Elastase	Cleaves immunoglobulins, complement components as well as disrupts neutrophil activity
• Leukocidin	Inhibits neutrophil as well as lymphocyte function
• Pyocyanins	Suppress other bacteria, disrupt respiratory ciliary activity, cause oxidative damage to tissues especially oxygenated tissues such as lung tissue

[Adapted from table 5-5 (4)]

An unusual mucoid morphotype of *P. aeruginosa* is frequently recovered from respiratory secretions of cystic fibrosis patients who are chronically infected with *P. aeruginosa.* The mucoid morphotype is due to the production of large amounts of alginate (a polysaccharide) that surrounds the cell. The production of alginate is ultimately responsible for the poor prognosis as well as high mortality rates among patients with cystic fibrosis (4). Risk factors found to occur more frequently in patients infected with carbapenem-resistant *P. aeruginosa* include:

- Presence of intravascular catheters (5)
- Ventilator support (5)
- Prophylaxis with quinolones (5)
- Prior colonization with *P. aeruginosa* (5)
- Prolonged stay in hospital (5)
- Previous therapy with β-lactam antibiotics, including Imipenem (5)
- Solid-organ transplantation has also been found to be an independent risk factor (6)

A study confirmed the common view that exposure to Imipenem is a major risk factor for the isolation of *P. aeruginosa* with decreased susceptibility to this drug (6).

1.3 Multidrug resistance

Infections due to *P. aeruginosa* are often difficult to treat because of its virulence and because of a relatively limited choice of effective antimicrobial agents (6). Lower permeability of the outer membrane contributes to multi-drug resistance in *P. aeruginosa.* Yet even with the low permeability co-efficients measured, one can predict that many agents would reach half-equilibration across the outer membrane in less than a minute. This is a very short period in comparison with the doubling time of the organism. Thus, in addition to the low permeability, removal of the incoming antibiotic molecules via mechanisms such as degradation or modification is needed in order to produce a significant level of resistance (7). Resistance in *P. aeruginosa* can arise by various mechanisms including:

- Reduced uptake of aminoglycosides across the outer and cytoplasmic membranes or production of aminoglycoside-modifying enzymes
- Loss of the outer membrane porin D_2 resulting in Imipenem resistance
- Mutational derepression of AmpC chromosomal β-lactamases
- Acquisition of plasmid-transposon or class 1 integron-borne mediated β-lactamases
- Modification of DNA gyrase (in the case of quinolone resistance)
- Multidrug efflux pumps (8)

All *P. aeruginosa* strains have a chromosomal Class C (Bush group 1) ß-lactamase, AmpC, which is normally inducible but may be derepressed by mutation. This enzyme confers resistance to Ampicillin as well as first- and second-generation cephalosporins and, whether inducible or derepressed, gives slight protection against Imipenem. Although AmpC cannot itself confer Imipenem resistance, its presence is essential to the Imipenem resistance that arises in *P. aeruginosa* when porin D_2 is lost. Meropenem, a weaker inducer of AmpC than Imipenem, is almost stable to the enzyme and has the ability to even deactivate it (9).

Carbapenems (Imipenem and Meropenem) are often used as antibiotics of last resort against infections caused by gram-negative bacteria resistant to other ß-lactams. They evade hydrolysis by most ß-lactamases, except Ambler class B types. Acquired carbapenem resistance has only been reported regularly in *P. aeruginosa*, where it is mostly low level (MIC 8-16mg/L) and reflects reduced drug accumulation (10). *P. aeruginosa* has become Imipenem-resistant due to two known distinct mechanisms. The first and the more common consists of the interplay of two mechanisms: chromosomal ß-lactamase activity as well as decreased permeability due to the loss of a specific outer-membrane protein or porin called D_2. Loss of porin D_2 gives resistance to Imipenem as well as reduces Meropenem susceptibility, but it does not affect the minimum inhibitory concentrations (MICs) of other ß-lactams (10). The second consists of the expression of a rare plasmid-mediated metallo-ß-lactamase capable of hydrolyzing carbapenems very efficiently (6).

Active efflux is already a well-known major mechanism of antibiotic resistance. The presence of active efflux was noted even in the wild-type strains of *E. coli*. An active efflux of Chloramphenicol was also recently documented in *E. coli* showing convincingly that *E. coli* has an intrinsic, saturable efflux mechanism for fluoroquinolones. Thus, the finding of active efflux processes for several drugs in the wild-type *P. aeruginosa* is not surprising. Of note however, is that the active efflux processes, probably in synergy with the low permeability of its outer membrane barrier, can produce high levels of intrinsic resistance in wild-type *P.aeruginosa* (7).

CHAPTER 2: LITERATURE REVIEW

2.1 Incidence of *P.aeruginosa*

In a North American study performed from 1985-1990, Meropenem was found to be the most active antimicrobial drug. Isolates of *P. aeruginosa* were less frequently resistant to Meropenem (4.2%) than to Imipenem (12.5%) (11). Of the 147 Imipenem-resistant isolates 43.8% were susceptible to Meropenem, and 26.9% additional isolates were moderately susceptible to Meropenem. Of the forty-nine Meropenem-resistant (MIC ≥16µg/ml) isolates, 85.7% were also Imipenem-resistant. Meropenem MICs were lower than Imipenem and Ceftazidime. Carbapenem MICs of ≥16µg/ml for selected *P. aeruginosa* isolates were associated with loss of the 45kD outer-membrane protein and/or production of type I ß-lactamases. No metallo-ß-lactamases were detected (11).

From March to June 1995 fifteen microbiology laboratories throughout Italy participated in a survey (8). Two hundred consecutive, clinically significant *P. aeruginosa* isolates were collected from inpatients and were sent to the University of Catania for re-examination and antibiotic susceptibility testing. From a total of 1153 isolates received, 1005 were confirmed to be *P. aeruginosa*. These isolates were cultured from the following different sites of infection: respiratory tract (35.5%), urinary tract (22.7%), skin and soft tissue (19.5%), blood (3.9%), ear (2.8%) followed by 11.4% from other sites. Resistance of *P. aeruginosa* to penicillins, Ceftazidime, Ciprofloxacin and Amikacin appeared to be common in Italy. Approximately half of the isolates tested were resistant to one or more antibiotics tested. Meropenem showed the highest antibacterial activity while Ciprofloxacin had the lowest. Patterns of susceptibility were equally distributed in Italy. Resistance to all the antimicrobials was more prevalent in intensive care units (ICUs) than other hospital units. Findings of this surveillance study indicated that Meropenem retains a high level of activity against clinical isolates of *P. aeruginosa* (8).

Another study was done to determine the mechanisms of resistance on the same 1005 clinical isolates of which 325 strains were resistant to one or more ß-lactam antibiotics (12). Among these 183 (56.3%) strains revealed a broad-spectrum intrinsic resistance to carboxypenicillins and other ß-lactam antibiotics. Thirty five percent of these strains produced stably derepressed chromosomal ß-lactamases; 18.6% of strains expressed secondary plasmid-mediated ß-lactamases, particularly PSE-1; 14.5% produced secondary plasmid ß-lactamases together with stably derepressed chromosomal ß-lactamases, and finally 17.5% of strains were resistant only to Imipenem, probably because of the absence of the D_2 porin. These isolates were tested against Ceftazidime, Cefepime and Imipenem. The conclusion was drawn that Ceftazidime as well as Imipenem retained their activity against the majority of clinical isolates collected in Italy. Cefepime did not offer competitive advantages in terms of *in vitro* activity (12). In a study combining Amikacin, Meropenem, Ceftazidime, Cefepime and Imipenem, fifteen carbapenem-resistant *P. aeruginosa* isolates were tested. The results indicated that Cefepime, especially in association with Amikacin, displayed bactericidal properties against carbapenem-resistant strains that make it a suitable choice for treating infections caused by these organisms (13).

During 1995-1997 infections due to *P. aeruginosa* were analyzed in the Tohid Burn Centre in Teheran in order to estimate their frequency and antibiotic susceptibility (14). A total of 3365 bacterial strains were isolated from approximately 2122 patients admitted during this period. In 1993 a dramatic decrease from 90% susceptibility to 18% Ciprofloxacin susceptibility was observed after the introduction of Ciprofloxacin to the burn center. The increase in resistance to Ciprofloxacin, Amikacin and Gentamicin was attributed to the over-usage and unavailability of Ciprofloxacin and the high cost of preferred antimicrobial agents (14).

The circulation of multi-drug resistant *P. aeruginosa* was followed over a period of 15 months in different ICUs of the University hospital in Greifswald, North-eastern Germany. Agar diffusion tests were confirmed by E-tests. All the strains tested were resistant to β-lactams (Ceftazidime, Cefepime, Cefsulodine, Azlocillin and Tazobactam, including the carbapenems (Imipenem and Meropenem). These isolates were also resistant to Aztreonam, the aminoglycosides (Amikacin, Tobramycin, Gentamicin) and the quinilones (Ciprofloxacin, Ofloxacin). *In vitro* activity was demonstrated with Polymyxin B. *In vitro* synergy was detected between Amikacin and the following antibiotics: Cefepime, Ceftazidime and Tazobactam. The combinations that showed synergy in the primary screening were tested further using the agar dilution-agar diffusion test. The best results were obtained in combining Amikacin with Cefepime, independent of which agents were used in the agar dilution. Between October 1994 and January 1996 results concerning the synergic *in vitro* interactions of Ceftazidime and Amikacin corresponded to the findings of another study in which twenty-six *P. aeruginosa* isolates were gathered from three different hospitals in Athens (15,16)

2.2 Mechanisms of resistance

To date, three classes of antibiotic resistance have been described: intrinsic resistance, acquired resistance, and genetic resistance. Intrinsic resistance comprises those mechanisms that exist in the average strain of a given species, irrespective of antibiotic exposure such as low-level permeability of the outer membrane (17,18). Acquired resistance involves the induction of unstable resistance without any observable change in genotype due to exposure of a strain to a set of inducing conditions that can include antibiotic exposure. Such resistance will revert to full susceptibility when the inducing conditions are removed. Genetic resistance involves the stable acquisition of new genetic information, either through mutation of an existing gene product or control mechanism, or through acquisition of a drug resistance plasmid (17).

Progress has been made in increasing the range of ß-lactams available for treating *P. aeruginosa* infections, but resistance to these antibiotics can arise by various mechanisms, including mutational derepression of Class C chromosomal ß-lactamases, acquisition of secondary plasmid- or transposon-mediated ß-lactamases, and loss of D_2 porin. Multi-drug resistance has also been associated with multi-drug efflux (19).

2.2.1 Outer Membranes

The outer membrane of any bacteria has three general functions namely surface reception, maintenance of structural integrity and permeation of substrates diffusion pores or channels (20). Porins of gram-negative bacteria form water-filled channels that permit the diffusion of hydrophylic solutes across the outer membrane (21). Such an interacting complex may consist out of matrix- and lipoproteins based on observations and properties of these two proteins (20). These porins are generally divided into two classes: nonspecific porins which permit the general diffusion of hydrophylic molecules below a certain size, and specific porins which facilitate the diffusion of specific substrates by virtue of containing stereospecific binding sites (21). In addition to porin-mediated uptake of water soluble molecules, two extra uptake systems exist. Polycations, including aminoglycosides and polymyxins, use a self-promoted uptake system. Hydrophobic drugs cross directly through the outer membrane bilayer (22). The primary role of the outer membrane porin D_2 is in the passive uptake of basic amino acids across the outer membrane, but it forms pores that are also permeable to carbapenems (which mimic the natural substrates of these pores), though not to other ß-lactams. The loss of porin D_2 raises the Imipenem MICs from 1-2mg/L, as for typical *P. aeruginosa*, to 8-32mg/L, thus conferring clinical resistance. MICs of non-carbapenems are unaffected (22). Results from previous studies suggest that Meropenem has two pathways through the outer membrane of *P. aeruginosa*. If a mutant lacking either of the routes develops, Meropenem would be active against it, since it can use another pathway (25). It has been suggested that Meropenem can use the D_2 porin pathway to enter the pseudomonal cell but that, unlike Imipenem, it is recognized and ejected by MexB-mediated efflux, presumably due to its 2' heterocyclic side chain (26).

In fact, Meropenem is more active against D_2 porin-deficient mutants of *P. aeruginosa*. Therefore, the frequency of highly Meropenem-resistant mutants arising must be extremely low, because they result from two mutation events in *P. aeruginosa*, namely loss of porin D_2 and upregulation of MexAB-OprM efflux pump (22,23).

2.2.2 Extended-spectrum ß-lactamases

Production of β-lactamases is one of the most frequent cause of resistance to β-lactam antibiotics in gram-negative bacteria. *P. aeruginosa* produces a chromosome-encoded cephalosporinase which renders it naturally resistant to Ampicillin and Cephalothin. This could be one reason why secondary (or 'acquired') β-lactamases are less common in this species than in the *Enterobacteriaceae*. In *P. aeruginosa* these acquired β-lactamases are mostly plasmid-encoded such as Carbenicillinase-type (CARB), PSE-, or Oxacillinase-type (OXA) rather than TEM- or SHV-type enzymes (24,25). In 1965 the TEM-1 enzyme was first recorded in *E. coli* and has since spread to 20-60% of isolates of *Enterobacteriaceae* (26). Extended-spectrum β-lactamases (ESBLs) are more prevalent in *Klebsiella pneumoniae* than in any other enterobacterial species. Outbreaks of infections caused by ESBL-producing strains have been reported widely. The majority of ESBLs are mutant TEM and SHV enzymes. Over sixty ESBL variants have been described within these β-lactamase families. The plasmids determining ESBLs are mostly large (≥80 kb) and encode multiple resistances (27). OXA-type enzymes, less closely related to one another than the TEM- and SHV- type enzymes, have been assigned to four groups. OXA-13 is closest to the OXA-10-related group. It differs from OXA-7 and OXA-10 (formerly known as PSE-2) in seven and eight amino acids respectively.

It is essentially different from the extended-spectrum, Ceftazidime-resistance-conferring β-lactamases of this group : OXA-11, OXA-14, OXA-13-1 (formerly OXA-13) and OXA-19 in that it does not display the Gly-to-Asp change in position 157 (24). CARB-type enzyme and OXA-type enzyme genes are frequently carried by integrons or related genetic structures, which explains why β-lactam resistance is associated with resistance to various other antibiotics, including aminoglycosides (24). Several potent plasmid-mediated β-lactamases such as PER-1 (a class A ESBL) and extended-spectrum mutants of class D β-lactamases have been dscribed from *P. aeruginosa*. The latter including the OXA-11, -14, and –16 derivatives of OXA-10 β-lactamase, the OXA-15 deravitive of OXA-2 enzyme and the OXA-18 enzyme, which is closely related to the OXA-12 and AmpS chromosomal enzymes of *Aeromonas sobria* (28). PSE-1 and –4 are the most common secondary β-lactamases in *P. aeruginosa*. These enzymes are often regarded as plasmid-encoded, but in fact are mostly determined by chromosomal inserts, and their dissemination reflects clonal spread, not gene transfer (26).

A clinical isolate of *P. aeruginosa,* PAe391 resistant to β-lactam antibiotics, aminoglycosides, sulfonamides and mercuric ions was studied. It was found that novel variants of an aminoglycoside acetyltransferase gene, *aac*(6')-Ib_{10} and a β-lactamase gene, bla_{OXA-13} , was inserted in an integron structure in the form of cassettes. This integron appeared to be chromosome-borne. OXA-13, OXA-10, AND OXA-7 producing strains was efficiently inhibited by Imipenem (24). OXA-17, a OXA-10-related enzyme with serine replacing asparagine at position seventy-three, has also been described. This β-lactamase, like OXA-10, increased the MICs of Ampicillin, Piperacillin, Carbenicillin, Ceftriaxone, Cefoperazone and Aztreonam.

Additionally, and unlike OXA-10, OXA-17 conferred protection against Cefotaxime, Latamoxef, Cefsulodin, Cefepime and marginally, Ceftazidime (28).

GES-1 is an enzyme which expands the group of Ambler class A ESBLs. This enzyme is weakly related to the other Ambler class A β-lactamases, particularly to the plasmid-located ESBLs so far described in *Enterobacteriaceae*. As with most class A ESBLs, GES-1 was found in *K. pneumoniae*. The activity of GES-1 was inhibited by Imipenem but not by cephamycins. Protein sequence alignment shows that GES-1 had the highest sequence identity with L-2, a chromosome-borne class A cephalosporinase from *Stenotrophomonas maltophilia*, the narrow-spectrum carbenicillinase from *Proteus mirabilis* and YENT (chromosome-borne penicillinase) from *Yersinia enterocolitica* (29). Clavulanic acid-inhibited ESBLs conferring resistance to extended-spectrum cephalosporins have been reported in *P. aeruginosa*. The clavulanic acid-inhibited enzymes that have been characterized in this organism are the Ambler class A enzymes SHV2a, TEM-4, -24, -42, VEB-1-like enzymes and PER-1 as well as the class D oxacillinse OXA-18. ESBL-producing *P. aeruginosa* have been reported from rare isolates in Europe, Kuwait and South East Asia, whereas PER-1 is widespread in Turkey. A novel clavulanic acid-inhibited class A enzyme, GES-2, was recently (March–July 2000) isolated from an outbreak of *P. aeruginosa* strains in South Africa. This enzyme is peculiar due to the hydrolysis of extended-spectrum cephalosporins and, to a lesser extent, Imipenem. The report illustrated that an outbreak of *P. aeruginosa* with a β-lactamase-mediated resistance to Imipenem might be due, in part, to a strain producing a class A β-lactamase (GES-2) and a class B metallo-enzyme (e.g. IMP-like β-lactamase) (30).

2.2.3. AmpC ß-lactamases:

A multi-centre Italian survey was performed on 325 isolates from 1005 *P. aeruginosa* isolates used in a previous study (12). The mechanisms of resistance to ß-lactam antibiotics present in these isolates were investigated. Based on their reactions with Nitrocefin and inhibitors as well as their resistance to carbapenems the isolates tested were divided into five groups. The first group included 183 isolates that reacted very weakly with Nitrocefin. This reaction was inhibited completely by Cloxacillin but not by Clavulanate and probably reflects the production of Class C chromosomal ß-lactamase. One hundred and forty-nine of these strains were resistant to Carbenicillin and showed reduced susceptibility to Ceftazidime, penicillins, Ciprofloxacin and Amikacin. This could be considered the typical pattern of 'intrinsic resistance', which was probably caused by reduced permeability and/or multi-drug efflux. The second group (64 strains) rapidly hydrolyzed Nitrocefin and was completely inhibited by Cloxacillin but not by Clavulanate. This pattern was also observed for the derepressed control 2297-con, which is typical of hyperproduction of Class C chromosomal ß-lactamase. These isolates produced at least five-fold higher uninduced levels of ß-lactamase than the R20 strain, confirming derepression of the Ambler Class C chromosomal ß-lactamase. Almost all the strains in this group were resistant to Ceftazidime and to some extent Piperacillin. The level of Meropenem resistance was unrelated to the amount of ß-lactamase produced without induction. For strains in the third group crude cell extracts displayed strong hydrolysis of Nitrocefin and were inhibited by Clavulanate, but not by Cloxacillin. This inhibition profile and high resistance to carboxypenicillins as well as ureidopenicillins suggested the presence of secondary plasmid-mediated ß-lactamases. The PSE-1 type enzyme was the most common plasmid ß-lactamase detected (31).

The fourth group comprised 12 isolates that gave a strong reaction to Nitrocefin and were not inhibited by Cloxacillin or Clavulanate. Eight strains had PSE-1-type enzymes. Quantitative Nitrocefin assays confirmed that all the strains had the highest ß-lactamase specific activities and were partially derepressed for Class C chromosomal ß-lactamase. These microorganisms were characterized by a strong activity against penicillins and Ceftazidime. Finally, isolates resistant to carbapenems were placed in a separate group since Imipenem resistance in *P. aeruginosa* seems to be independent of resistance to penicillins and cephalosporins. Thirty-two strains were resistant either to Imipenem or Meropenem. Of these 24 were resistant to Imipenem and only eight to both carbapenems. It should be noted and emphasized that the occurrence of a mechanism does not prove it to be the only cause of resistance. It is possible that some isolates may pose further, undetected mechanisms (31).

Interplay between the chromosomal AmpC ß-lactamases and the Mex-AB-OprM efflux system was examined. Alterations in the susceptibility of *P. aeruginosa* that accompany defects in AmpC ß-lactamase production and the Mex-AB-OprM system were determined using isogenic mutants from a laboratory strain, PAO1. In PAO1 the efflux system removed Carbenicillin, Piperacillin, Cefoperazone, Aztreonam and Cefsulodin from the periplasm more effectively than the ß-lactamases. The ß-lactamases removed Amoxycillin, Cefmetazole, Flomoxef, Panipenem and Imipenem more effectively from the periplasm than the efflux system. Results show that either mechanism alone is almost sufficient to provide the wild-type level of resistance to Cefpodoxime, Cefuroxime, Ceftriaxone, Moxalactam, Meropenem and S-4661. Both mechanisms contribute equally and powerfully to the removal of these agents from the periplasm (32).

2.2.4. Metallo-ß-lactamases:

In the early 1990s an integron-borne *bla*$_{IMP}$ metallo-ß-lactamase gene made its appearance among *Enterobacteriaceae, Pseudomonas* and other nonfastidious, gram-negative nonfermenters isolated in several hospitals in Japan (33). IMP-1 is a plasmid-mediated metallo-ß-lactamase produced in the presence of the *bla*$_{IMP}$ gene. This plasmid belongs to ß-lactamase subgroup 3a in Bush's functional group and is able to hydrolyze a wide variety of ß-lactams including penicillins, cephems and carbapenems (34). IMP-1 is notable for it special character in that it is hardly blocked by ß-lactamase inhibitors such as Clavulanate, Sulbactam and Tazobactam (35). It was reported that the *bla*$_{IMP}$ gene-cassette of *Serratia marcescens* AK 9373 is located in the space between the integrase gene (*int*I3) and the aminoglycoside acetyltransferase gene [*aac*(6')I*b*]. This gene-cassette is transposed into other plasmids or the chromosomes of gram-negative bacteria by this integron element, which is mediated by large plasmids with wide host ranges. These findings signal the possibility of further dissemination of the *bla*$_{IMP}$ gene cassette into various gram-negative rods (35).

Since IMP-1 producers tend to demonstrate a wide range of resistance to various broad-spectrum ß-lactams including the oxyamino cephalosporins, cephamycins, and carbapenems, early recognition of IMP-1 producers is of utmost importance for rigorous infection control. Polymerase chain reaction (PCR) analyses usually give reliable results, but this method is of limited practical use for daily application in clinical laboratories due to the costs involved. Thus, there is a necessity for the development of a simple, inexpensive testing method for the screening of IMP-1 producers (36).

Such a method was developed based on double-disc diffusion using 2-mercaptopropionic acid, which gave the best results by blocking IMP-1 activity very strongly even at a low concentration. Ceftazidime appeared to be the most suitable substrate for this test (36).

A multi-drug-resistant *P. aeruginosa* strain (VR 143/97) with unusually high-level resistance to Imipenem (MIC=256µg/ml) was isolated from a surgical wound of a patient admitted to the ICU of the Verona University Hospital, Italy. A crude cell-free extract obtained from this strain exhibited Imipenem-hydrolizing activity. A bla_{IMP}-specific probe containing the 0.5kb *Hind*III fragment internal to the bla_{IMP} gene did not hybridize to strain VR 143/97 in a colony blot assay, indicating that a metallo-carbapenemase other than IMP-1 was produced by this strain. PCR analysis confirmed the presence of bla_{VIM} in all strains, including the genetically unrelated strain (37). In a BLAST search the bla_{VIM} gene was found to encode a polypeptide showing high similarity scores with other class B ß-lactamases. The VIM-1 enzyme produced in the presence of bla_{VIM} is structurally rather divergent from other class B ß-lactamases and represents a new member of this family. VIM-1 apparently shares the closest ancestry with the *B. cereus* Bc-II enzyme. Although bla_{IMP} and bla_{VIM} are both carried on mobile gene cassettes inserted into integrons, their highly divergent sequences along with differences in the G+C content as well as codon usage indicate an independent phylogeny of these two determinants and of their associated cassette frameworks (33). In VR-147/97 the bla_{VIM}-containing integron was located on the chromosome and is apparently not transferable by conjugation, at least to *E.coli* (33). A second carbapenemase-hydrolyzing ß-lactamase namely VIM-2 has been discovered in an isolate from the French Riviera region (Marseilles), which is only 300km from Verona, where VIM-1 had been isolated. A 39-year-old French woman was hospitalized for chronic myelogenous leukemia as well as pancytopenia before the performance of an allogeneic bone marrow transplantation (38).

The patient had fever and received a course of Imipenem and Amikacin. Despite this treatment, she died of septic shock 5 days later. The day after her death, blood cultures revealed a carbapenem-resistant *P. aeruginosa* isolate, COL-1 (38). The isolate was resistant to most ß-lactams, including ureidopenicillins, ureidopenicillin-ß-lactamase inhibitors, narrow-spectrum cephalosporins, Cefepime, Ceftazidime, Imipenem and Meropenem, but remained fully susceptible to Aztreonam (a monobactam). VIM-2 activity was higher against Imipenem than against Meropenem. Its activity was inhibited by EDTA and was restored in the presence of 2mM $ZnCl_2$. Thus, VIM-2 could be included in the functional group 3a of the Bush ß-lactamase classification. VIM-2 shared 90% amino acid identity with VIM-1, but it did not share VIM-1's peculiar property of increased hydrolysis of Meropenem than Imipenem (38).

2.2.5 MexAB-OprM Efflux

Active efflux pump systems have recently been recognized to play a major role in the intrinsic and required resistances of *P. aeruginosa* to antimicrobial drugs (39). The wide substrate specificity and constitutive expression of the efflux pump system encoded by the *mexA-mexB-oprM* operon in wild-type *P. aeruginosa* cells contributes significantly to the elevated resistance that this opportunistic pathogen naturally displays to a variety of antibiotics. Substrates for MexA-MexB-OprM include compounds as structurally diverse as ß-lactams, ß-lactamase inhibitors, quinolones, tetracyclines, Trimethoprim, Chloramphenicol, macrolides and Novobiocin. When overproduced, the efflux system allows bacterial cells to reach higher levels of resistance to the substrate antibiotics mentioned above. In the *nalB* multidrug-resistant mutant OCR1, overexpression of *mexA-mexB-oprM* has been found to be associated with a point mutation in *mexR*, the regulator gene of the efflux operon, leading to a predicted substitution of Trp for Arg at position sixty-nine in the encoded peptide MexR. Such an amino acid change has been proposed to alter the function of MexR *in vivo*. In addition, inactivation of *mexR* in several knockout mutants was found to result in overexpression of *mexA-mexB-oprM* and elevated multidrug resistance, although to a lower extent than in the *nalB* strain OCR1 (39). MexA is a fusion protein associated with the cytoplasmic membrane via the fatty acid residue and the peptide moiety extending to the periplasmic space physically linking the MexB and OprM components (22,40). The MexB protein is a broad-spectrum pump, located in the cytoplasmic membrane (22). It has been assumed to function as the substrate-exporting subunit across the cytoplasmic membrane (40). OprM is a pore-forming protein that provides an exit portal through the outer membrane (22).

MexA-MexB-OprM is a constitutive efflux system, which causes four- to six-fold increases in MICs of many ß-lactams when it is overexpressed. Studies done with OprM-deficient and OprM-overexpressing mutants showed that while the MICs of Meropenem were dramatically affected by the expression of OprM, Imipenem MICs remained unchanged (41). In patients treated with any antibiotic that acts as a substrate for this efflux system, resistance was selected for by overexpression of the pump. The mechanism of this overexpression was found to be a mutation in the MexR repressor gene, which resulted in derepression. In contrast to MexA-MexB-OprM, activation of MexE-MexF-OprN does not cause direct efflux of ß-lactams, but rather down-regulates porin D_2 expression (41). Upregulation of MexA-MexB-OprM raises the MICs of penicillins, cephalosporins, quinolones, Tetracycline and Chloramphenicol and Meropenem but not those of Imipenem (22,41). Selection of *nalB* mutants may occur *in vitro*, or during therapy with fluoroquinolones, penicillins and cephalosporins (22). It is postulated that MexA-MexB-OprM has a natural role in removing amphiphatic substances, which otherwise invade and disorganize the cytoplasmic membrane. The lack of activity against Imipenem may be due to this carbapenem lacking any lipophilic phenyl or heterocyclic side chain(s) (22). In the United Kingdom resistance to either carbapenems appears to be rare in *P. aeruginosa*. Present arguments relate only to the therapy of *P. aeruginosa* infections. Upregulation of MexA-MexB-OprM and loss of the D_2 porin may not be the sole routes to carbapenem resistance in *P. aeruginosa* (although they are considerably the most prevalent). In the differential selection of carbapenem resistance, the difference in response of *P. aeruginosa* to the stepwise selection of resistance using Imipenem and Meropenem should be considered. When a susceptible strain of *P. aeruginosa* is exposed to Imipenem or Meropenem at levels fourfold higher than the MIC, resistance is selected for with both drugs, as evidenced by an increase in MIC due to porin D_2 deficiency (41).

When these resistant mutants are further exposed to Imipenem, no further increase in MIC is observed. When the resistant mutants are further exposed to Meropenem, however, the MIC again increases and the strain is now positive for MexAB-OprM overexpression. This is of great clinical concern because activation of the MexAB-OprM efflux system brings with it not only Meropenem and ß-lactam resistance, but also resistance to quinolones, Tetracycline, Chloramphenicol and Trimethoprim (41). Using a *mexA-phoA* fusion, expression of the MexAB-oprM efflux genes was assessed as a function of growth in a variety of strains. The efflux operon was growth-phase regulated in both wild-type and *nalB* strains, being minimally expressed in lag phase and increasing in log to late log phase. MexR, the only known regulator of MexAB-OprM and target of mutation in *nalB* strains, was not involved in the growth-phase regulation (42). Upregulation of another efflux system, MexE-MexF-OprN is associated with raised MICs of both carbapenems and fluoroquinolones, although it is unclear whether this pump itself recognizes carbapenems or whether the association reflects co-regulation of MexE-MexF-OprN with porin D_2. This infrequent mechanism, mediated by the *nfxc* mutation, is sometimes selected by quinolones but rarely, if ever, by carbapenems (22). MexEF-OprN has properties such as providing resistance to fluoroquinolones, Chlorampenicol and Trimethoprim, lacking porin D_2 resulting in Imipenem resistance and showing hypersusceptibility to β-lactam antibiotics by an unknown mechanism (40). In a study to clarify the antibiotic selectivity of Mex-EF-OprN Tetracycline was identified as an excellent substrate for this efflux pump (40). Mutations in the *nfxB* and *nfxC* loci located near the *ilvB* (0 min) and *catA* (46 min) genes of the *P. aeruginosa* chromosome render the bacterium hypersusceptible to β-lactam antibiotics and resistant to fluoroquinolones, Chloramphenicol and other antibiotics (40).

This resistance is mainly attributable to the expression of the MexAB-OprM, MexCD-OprJ, and MexEF-OprN efflux pumps, respectively. Only the MexAB-OprM pump is expressed in most, if not all, strains of *P. aeruginosa* so far tested, including laboratory and clinical strains. Thus most *nfxB* and *nfxC* mutations occur in strains producing at least low levels of MexAB-OprM. This indicates that the resistance profile of these mutants might be a consequence of MexCD-OprJ, MexEF-OprN and low-level expression of MexAB-OprM (40). A study of the *nfxC* mutant revealed that this mutant expresses three efflux pumps simultaneously: MexAB-OprM, MexEF-OprN and an additional pump, Mex-EF-OprM (40). MexT has been established as the transcriptional activator of the *mexEF-oprN* efflux operon demonstrating its requirement for the expression of the *nfxC* multidrug resistance phenotype. The majority of LysR-type regulators is synthesized in a nonactive form and become activated upon binding of a cognate effector molecule(s). It was therefore assumed that in the *nfxC*-type mutants, the effector molecule of MexT is produced constitutively or in larger amounts than in the wild-type, thereby causing permanent activation of MexT and hence over-expression of the MexEF-OprN efflux system. Even in the absence of MexT effector molecules this plasmid-encoded transcriptional activator is able to activate *mexEF-oprN* transcription and confer *nfxC* multidrug resistance phenotype on the susceptible wild-type strain (43). Two new genes *mexXY* similar to *mexAB, mexCD* and *mexEF* and mediating multidrug resistance were cloned from the chromosome of *P. aeruginosa*. Elevated ethidium extrusion was observed with *E. coli* cells harboring the plasmid carrying *mexXY*. The MexXY system confers higher resistance to fluoroquinolones than the MexAB and MexCD systems. It was also found that the TolC of *E. coli* or OprM of *P. aeruginosa* was necessary for the functioning of the MexXY system. The substrate specificity of the MexXY system is similar to that of the MexAB system (44).

2.3 Treatment

Twenty-five clinical isolates of multi-drug resistant *P.aeruginosa* were tested and were inhibited by 2% citric acid in a broth dilution assay (45). Topical application of 2-3% citric acid to a wound, for three to seven times, successfully eliminated *P. aeruginosa* from the infection site. The use of this agent is recommended as an effective and economical approach to the control of multiple-drug resistant strains of *P. aeruginosa* causing superficial infections (45). A prospective study of 200 patients with *P. aeruginosa* bacteremia showed lower mortality with combination therapy (27%) than monotherapy (47%). Combinations of ß-lactams and high-dose aminoglycosides remain the first choice for therapy of *P.aeruginosa.* Quinolone-containing combinations are an alternative, while intravenous Colistin should be used as last resort (46).

PURPOSE OF THE STUDY

P. aeruginosa should be considered to be a very important nosocomial pathogen, which may have far-reaching effects. It is crucial to provide information on the current status of *P. aeruginosa* in hospitals in the Pretoria area, especially in view of the incidence of increasing multi-drug resistance in this organism. When used appropriately by doctors, health administrators and the commercial pharmaceutical industry, surveillance data can offer economic as well as health benefits to the health care system. Considering the background it is crucial to investigate the prevalence as well as susceptibility patterns of *P. aeruginosa* in hospitals.

THIS STUDY AIMS
TO DETERMINE THE FOLLOWING:

1. The prevalence of multi-drug resistant *P. aeruginosa* in ICUs of a hospital in the Pretoria area
2. If more than one clone of multi-drug resistant *P. aeruginosa* is involved
3. Susceptibility patterns of the different clones present
4. The specific trends of resistance from March 1998 to September 2000
5. Possible combination therapy options

CHAPTER 3: METHODS

Study design

A retrospective cross-sectional analysis was carried out on one hundred (n =100) multi-drug resistant clinical isolates of *P. aeruginosa*.

Statistical analysis:

Susceptibility and synergy values obtained for antibiotics alone and in combination with Amikacin were expressed as percentages (n = 50). Percentages were subjected to a two-tailed probability test incorporating 49 degrees of freedom, utilizing a microcomputer equipped with Statpac™ software, available over the Internet from http://www.statpac.com. Statistical significance was defined as $p < 0.05$.

3.1 PREVALENCE OF *P.AERUGINOSA*

Over a three and a half-year period (March 1998 until September 2001) isolates of *P. aeruginosa* were collected from the routine isolates of the PAH pathology laboratory in Pretoria. The PAH is a tertiary academic hospital with 929 beds. Isolates tested in this study were gathered from nine different ICUs (table 1). Relevant data such as age, sex, ward, date of isolation and source was documented. *P. aeruginosa* was isolated mostly from patients in ICUs, from specimens such as endotrachial aspirates, pus swabs, blood cultures, central venous pressure (CVP) catheter tips, urine, body fluids, tissue samples, bronchoalveolar lavage and sputum's. These isolates were then identified using the API20NE system. All *P. aeruginosa* isolates were then inoculated into stock culture media (in MacCartney bottles) and incubated overnight at room temperature. Stock cultures were stored at 4°C (see appendix A).

TABLE 1: Description of ICU wards in the PAH

ICU WARD	DESCRIPTION
23 ICU	Surgical
23A ICU	Paediatric surgical
11 ICU	Neurosurgical
12 ICU	Neurosurgical
60 ICU	Neurosurgical
51 ICU	Thoracic surgery
51A ICU	Thoracic surgery
54 ICU	Internal medicine
KALAFONG ICU	Adult, mixed surgical and internal medicine

3.2 DNA ISOLATION

Prior to DNA isolation an organism was plated out onto a MacConkey agar plate from the stock culture and incubated overnight at 37°C. The following day one colony from the overnight culture was picked off and then inoculated into 1.5ml of brain heart infusion (BHI) broth, which was again, incubated overnight at 37°C. Bacteria were removed from the culture media by centrifugation for four min at 13000-16000 g. After centrifugation, 600µl of nuclei lysis solution was added by gently pipeting until the cells were resuspended. This solution was then incubated at 80°C for five min. After the solution cooled down to room temperature 3µl of RNase solution was added to the lysate. The tube was then inverted two to five times to mix the contents and incubated at 37°C for fifteen to sixty min (A).

Two hundred microlitres of protein precipitation solution was then added to the room temperature RNase-treated lysate and then vortexed vigorously at high speed for twenty sec. The sample was incubated on ice for five min after which it was centrifuged at 13000-16000 g for six min. The DNA-containing supernatant was then transferred to a clean 1.5ml tube containing 600µl of isopropanol at room temperature. The tube was inverted several times until the thread-like strands of DNA formed a visible mass. The sample was again centrifuged at 13000-16000 g for four min (A). The supernatant was gently poured off and the tube drained on a clean absorbent paper. Six hundred microlitres of 70% ethanol (room temperature) was added to the sample and gently mixed by inverting the tube several times. The sample was again centrifuged at 13000-16000 g for four min. Ethanol was carefully aspirated from the sample and the tube was drained on clean absorbent paper. The pellet was left to air dry for ten to fifteen min. One hundred microlitres of Tris-EDTA (TE) Buffer (pH7.5) was added to the air-dried pellet and the sample was incubated overnight at room temperature (A). The purified DNA was stored at -20°C (for details of reagents and buffers used see appendix B).

3.3 PCR

Randomly Amplified Polymorphic DNA (RAPD) patterns were obtained by performing arbitrarily-primed PCRs (AP-PCR) (47). A 1:100 and a 1:50 dilution from the purified DNA of the isolates was made which was tested at 260, 280 and 320nm using a Unicam helios gama spectrophotometer. At 260nm the amount of DNA was measured, at 280nm the protein content was measured and at 320 the amount of salts present were measured for each dilution. The value obtained at 280nm was then divided by the value at 260nm to give a ratio of 1.95 for the 1:100 dilution, indicating pure DNA. A ratio smaller than 1.9 indicates the presence of impurities. Optimum concentrations of each individual reagent used for the AP-PCR, was determined using the Taguchi method of optimization (Fig.1) (48).

Taguchi Method for PCR Optimization

Date:

Target size:

Primers:

Reaction nr.	Water (µl)	Variable 1 (µl) *dNTP's*	Variable 2 (µl) *primers*	Variable 3 (µl) $MgCl_2$
1		A	A	A
2		A	A	B
3		B	B	B
4		B	B	A
5		C	C	B
6		C	C	C
7		C	C	A
8		A	A	C
9		B	B	C

A = Highest concentration

C = Lowest concentration

PCR Cocktail:

Components	1 × reaction	X reactions
Aliquot		

Concentrations (final):

Variable 1: A_____ B_____ C_____

Variable 2: A_____ B_____ C_____

Variable 3: A_____ B_____ C_____

Figure 1: An illustration of how the Taguchi method is used for optimization of PCRs (48).

Primer length and sequence are of critical importance in designing the parameters of a successful amplification: the melting temperature Tm of a NA duplex increases both with its length, and with increasing (G+C) content: a simple formula for calculation of the Tm is:

$$Tm = 4(G + C) + 2(A + T) - 1.5°C \text{ (49).}$$

The primers used had the following melting temperatures:

M13 (5'–TTATGTAAAACGACGGCCAGT–3'): Tm = 55.9°C

H5 (5'–AGTCGTCCCC–3'): Tm = 35.1°C

A series of reactions consisting of several different combinations of each reagent, were set up. After determining the optimum combination of concentrations necessary for each reagent, a PCR containing the first of the isolated organisms was set up under the following conditions:

- Pre-denaturation at 94°C for 4 min. and 40 cycles of:
- 1 min. at 94°C
- 1 min. at 35°C
- 2 min. at 72°C
- 5 min. at 72°C (48)

One extra reaction was made with every PCR. The cocktail was made up adding all the reagents necessary for the reactions, except the sterile water, DNA and the *Thermus aquaticus* (*Taq*) DNA polymerase. After the cocktail was made up, the correct amount of each reaction was aliquoted into the marked PCR tubes. Then the correct volume of sterile water was added. Lastly the DNA and finally the *Taq* DNA polymerase of each reaction was added. For each set of PCRs a negative control was included containing sterile water instead of DNA.

Amplification products were analyzed by electrophoresis in a 1.4% agarose gel and were detected by ethidium bromide staining under UV light. Product bands were visible but very vague. A set of reactions was therefore set up with different dilutions of DNA to find an optimum concentration of DNA to yield clearer bands. The 1:20 dilution gave the best results with 25pmol primer thus two microlitres of the 1:20 dilution of DNA was used but only partial amplification resulted. Due to *P. aeruginosa's* high G+C content of 67.2%, 5% of Dimethylsulfoxide (DMSO) was added (1,49). Adding the DMSO resulted in more specific bands, however still only partially amplified. The Touch down method was performed starting the first 10 cycles with an annealing temperature of 38°C while the remaining 30 cycles was run at 35°C (50). The *Taq* DNA polymerase was added to the reaction mixture, just before the PCR was run to prevent formation of primer-primer dimers. Still only partial amplification resulted. A PCR was done with varying $MgCl_2$ concentrations a concentration of 2mM still yielded the best results. Different primer concentrations were also used but 25pmole yielded the best results. Better results weren't obtained therefore repeat PCRs were performed by adding 1µl of initial PCR product to a new PCR reaction mixture but with no improvement of the results (51). PCRs were also done with an annealing temperature of 32°C. Finally PCR products were precipitated in a final attempt to obtain complete amplification (52). Even with the PCR products prisipitated they were only partially amplified. When all attempts to obtain amplification failed a letter was written to the corresponding author of the article used, to ask for advice but no reply was given (see appendix B for details).

3.4 ELECTROPHORESIS

Agarose gels were made by adding thirty-five millilitres of 1×TAE buffer (in a container two to four times the volume of the solution) to 0.52 grams of agarose powder. The agarose solution was heated in a microwave oven on high power until bubbles appeared. It was then removed and gently swirled to resuspend any settled powder and gel pieces. The solution was again heated on high power until it came to a boil. It was held at boiling point for one min. or until all the particles were dissolved. The solution was then cooled to 50-60°C. While the agarose solution was cooling down ethidium bromide was added to a final concentration of 0.5µg/ml (1.25µl). The agarose was then poured into a horizontal gel-casting tray (3-4mm thick) and a comb was carefully placed in the gel. The gel was left to set at room temperature. After setting, the gel was placed in the electrophoresis chamber. The chamber was flooded with running buffer until the buffer reached thirty-five millimetres above the surface of the gel and the gel comb was carefully removed (53). The wells were gently flushed with running buffer prior to loading the samples. Five microlitres of loading dye was then added to twenty microlitres of each sample and the samples were loaded. The gel was run at 110 volts (V) until the loading dye migrated two thirds through the gel. The gel was then removed and viewed under UV light. Isolates with patterns that differed by 1 or more discrete bands were considered different (for details of buffers used see appendix B) (47).

3.5 SUSCEPTIBILITY TESTING

3.5.1 MUELLER-HINTON AGAR PLATES:

A petri dish and MacCartney bottle was marked for every dilution in the dilution series. Three hundred millilitres of distilled water was added to 11.4g of Mueller-Hinton (MH) agar. After mixing well the agar was autoclaved at 121°C for fifteen min. with a pressure of 15 psi. The agar was then left to cool down to approximately 50°C. After reaching 50°C 19ml volumes of agar were aliquoted into the already marked sterile MacCartney bottles and put into a water bath (50°C). One millilitre of each dilution of antibiotic was then added to the corresponding marked bottle. The agar mixture was swerved around to mix and immediately poured into the corresponding petri dish. The agar plates were left to set on a level surface at room temperature. The set agar plates were kept overnight in the incubator (35°C) (54).

3.5.2 THE DILUTION SERIES:

A 1:10 dilution was made of the antibiotic stock solution (see appendix C) to be tested in order to obtain a final concentration of 2560µg/ml. The dilution series with a range of 512-0.125µg/ml was set up according to the National Committee of Clinical Laboratory Standards (NCCLS) (54).

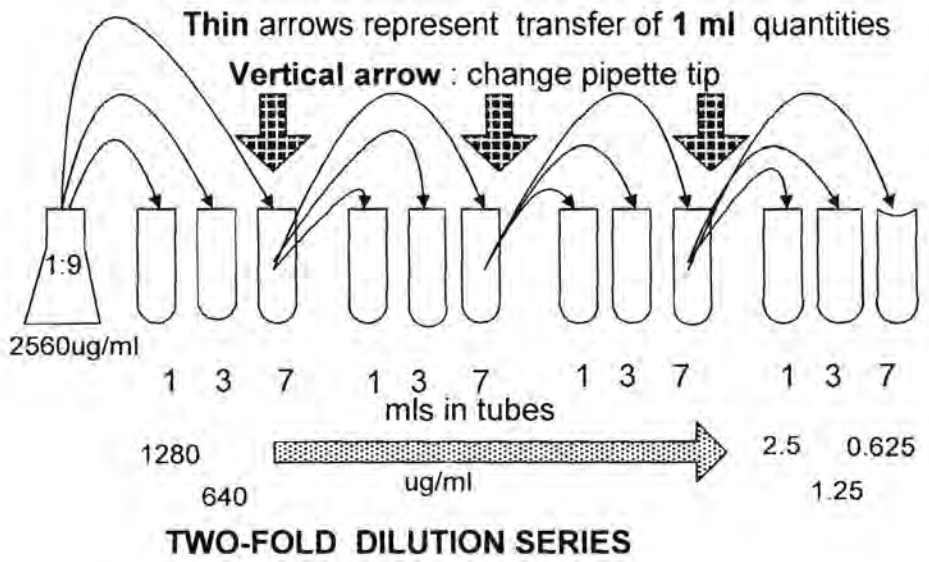

Figure 2: Diagram of agar broth dilution method

3.5.3. INOCULUM STANDARDIZATION:

The top of four to five colonies of the overnight inoculum was touched and inoculated into five millilitres MH broth. The turbidity was then adjusted to that of a 0.5 McFarland standard by adding sterile broth (54).

3.5.4 INOCULATION OF AGAR PLATES:

An inocula-replicating device that can inoculate 36 organisms at once was used for inoculation. Each agar plate was marked for orientation using sterile Indian ink. An aliquot (200µl) of each microbial suspension was placed into the replicator wells. Then the first control plate without antibiotic was inoculated. Thereafter all the plates were inoculated starting with the lowest concentration first. A second control plate was inoculated last. Inoculum spots were left to dry after which the inoculated agar plates were incubated at 35°C for 16 to 20 hours. Negative controls (agar plates without antibiotic) and a positive control using the *P. aeruginosa* ATCC 27853 strain were included (54).

3.6 RESISTANCE TRENDS

From March 1998 to September 2001 a hundred isolates were gathered. Relevant data such as age, sex, ward, date of isolation and source was documented. The data of all the strains (resistant to all the drugs tested) from every year over the study period was compared to determine the resistance trends.

3.7 SYNERGY

The same stock solutions prepared for the MICs were used for the Time-kill curves. Time-kill curves were performed to determine possible synergism according to a method described in the literature (55). In calculating the concentration of antibiotic needed for the killing curves a concentration of sixteen times the MIC value was used due to the very high MIC values of these organisms (see appendix D). Even at these extremely high antibiotic concentrations, growth occurred. Alternative methods for determining synergy was considered such as the double-disc diffusion test (see appendix E) as well as the latest E-test method described by Lewis *et al* (36,56). Both these methods were performed to obtain results, which were hoped to confirm synergy.

3.7.1 DISC DIFFUSION:

Organisms were plated onto blood agar plates from the stock culture and incubated overnight at 35°C. The overnight cultures were inoculated into sterile saline to obtain a 0.5 MacFarland standard. This suspension was then applied onto Mueller-Hinton agar plates, containing 5% sheep blood, using a sterile cotton swab. The discs tested were then placed on these inoculated plates and the plates were incubated overnight at 35°C (36).

3.7.2 E-TEST METHOD:

The organisms were plated onto blood agar plates from the stock culture and incubated overnight at 35°C. Overnight cultures were inoculated into sterile saline to obtain a 0.5 MacFarland standard. M-H agar plates, containing 5% sheep blood, were flooded with this suspension and left in the incubator to dry for approximately 15 min. The first E-test strip (e.g. Amikacin) was then applied to the dry plate, which was then incubated at 35°C for one hour. After one hour the first E-test strip was removed and a second (e.g. Piperacillin) was placed exactly where the first one was. The plate was again incubated at 35°C for one hour. After the hour had passed the first reading was taken and the second strip was removed. The next E-test strip (e.g. Tazobactam) was then placed exactly where the previous one was. The plate was then incubated at 35°C for one hour. After an hour of incubation the second reading was taken and the third E-test strip was removed. The next E-test strip (e.g. Cefepime) was then placed exactly where the previous one was and the plate was incubated overnight at 35°C. The final reading was taken after 24 hours (56).

CHAPTER 4: RESULTS

4.1 PREVALENCE

Patient demographic data and source of specimen of the patients from whom the isolates were gathered is summarized in table 1. The prevalence study performed indicated that *P. aeruginosa* is prevalent in the PAH, particularly in endotrachial aspirates. *P. aeruginosa* is secondly most prevalent in blood cultures, followed by urine samples, pus swabs, and finally catheter tips (Fig. 3). Of the isolates gathered during the study period (March 1998 until September 2001); 62% were isolated from male patients while 38% were isolated from female patients. Nine of these patients were babies while two were children aged sixteen. The rest of the patients these isolates were gathered from were adults ranging in age from twenty to ninety years. Due to ethical considerations the human immunodeficiency virus (HIV) status of these patients could not be obtained. The most common colony morphology of these strains was pleomorphic with a sticky consistency. Of these isolates; 52% produced pyocyanin (blue) while 43% did not produce any type of pigment. The remaining 5% produced another type of pigment, presumably pyomelanin (brown). Strains that appeared to be in duplicate were two different isolates of *P. aeruginosa* with different colony morphology, obtained from the same specimen.

Prevalence of *Pseudomonas aeruginosa* from March 1998 to September 2001

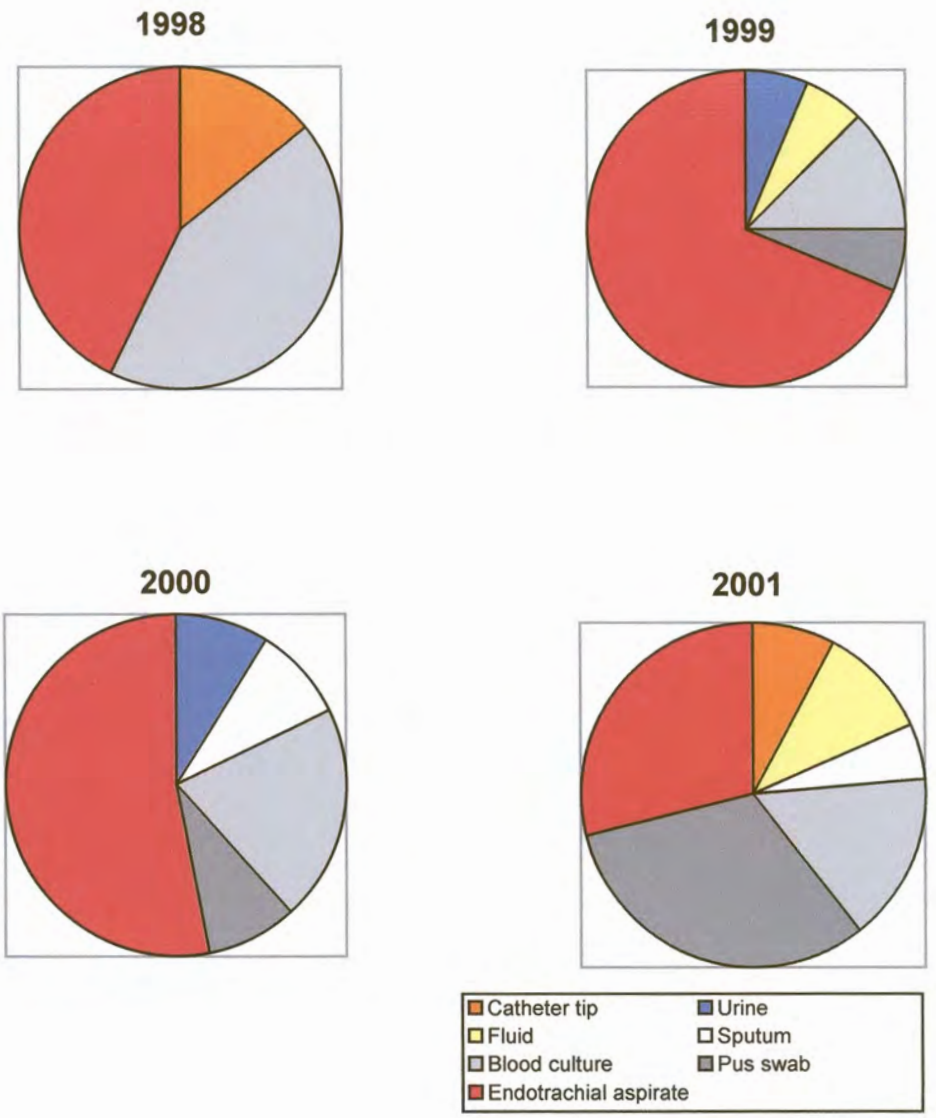

Figure 3: Illustration of the presence of multi-drug-resistant *P. aeruginosa* in the ICUs of the Pretoria Academic Hopital.

TABLE 1: Epidemiology and antibiogram of *P.aeruginosa* isolates tested[a]

STRAIN NO.	DATE ISOLATED	GENDER	WARD	MINIMUM INHIBITORY CONCENTRATIONS (MICs)							
				AK	AZ	CP	CT	TB	IP	MP	P
P1	1998-03-23	M	23 ICU	>256	32	128	32	>256	64	64	>256
P2	1998-12-14	M	23 ICU	>256	64	256	64	>256	64	128	>256
P3	1998-12-15	F	23 ICU	>256	32	128	32	>256	64	128	>256
P4	1998-12-15	F	23 ICU	>256	32	128	32	>256	64	128	>256
P5	1998-12-23	M	23 ICU	>256	64	128	64	>256	64	128	>256
P6	1998-12-24	M	23 ICU	>256	64	128	64	>256	64	128	>256
P7	1998-12-30	M	51A ICU	64	64	64	32	256	32	128	256
P8	1999-01-06	M	51A ICU	128	64	64	32	256	32	128	256
P9	1999-10-06	M	54 ICU	>256	64	128	64	>256	64	128	>256
P10	1999-01-07	M	23 ICU	>256	32	128	64	256	64	64	>256
P11	1999-01-08	M	23 ICU	32	>256	>256	64	>256	32	256	>256
P12	1999-01-08	M	23 ICU	>256	32	128	32	>256	64	64	>256
P13	1999-01-12	M	23 ICU	>256	32	64	32	>256	64	64	>256
P14	1999-08-25	M	*	>256	128	256	64	>256	64	256	>256
P15	1999-10-06	F	54 ICU	>256	64	128	64	256	>256	64	>256
P16	1999-10-12	F	*	>256	128	256	64	>256	>256	128	>256
P17	1999-10-13	M	54 ICU	>256	64	64	16	128	128	32	256
P18	1999-10-13	M	54 ICU	>256	128	>256	64	>256	>256	256	>256
P19	1999-10-13	M	54 ICU	>256	64	128	64	256	>256	64	>256

[a] Abbreviations: AK, Amikacin; AZ, Aztreonam; CP, Cefepime; CT, Ceftazidime; TB, Tazobactam; IP, Imipenem; MP, Meropenem; P, Piperacillin

* ICU wards of private hospitals

TABLE 1: Epidemiology and antibiogram of *P.aeruginosa* isolates tested continued

STRAIN NO.	DATE ISOLATED	GENDER	WARD	MINIMUM INHIBITORY CONCENTRATIONS (MICs)							
				AK	AZ	CP	CT	TB	IP	MP	P
P20	1999-10-13	M	54 ICU	>256	64	128	64	256	>256	64	>256
P21	1999-10-13	F	*	>256	128	>256	64	>256	>256	128	>256
P22	1999-10-25	M	*	>256	>256	64	64	>256	>256	32	>256
P23	1999-11-12	M	23 ICU	>256	64	128	64	256	>256	64	>256
P24	2000-01-14	M	54 ICU	>256	64	128	64	256	256	64	>256
P25	2000-01-14	F	54 ICU	>256	64	128	64	256	>256	64	>256
P26	2000-01-14	F	54 ICU	>256	64	128	64	256	>256	64	>256
P27	2000-01-27	M	Orthopaedic	32	16	16	16	16	64	32	32
P28	2000-01-31	F	11 ICU	>256	32	64	64	256	32	64	>256
P29	2000-02-05	F	11 ICU	>256	64	128	64	>256	64	128	>256
P30	2000-02-14	M	11 ICU	>256	64	64	64	64	>256	128	64
P31	2000-02-21	F	23 ICU	>256	64	128	64	256	>256	64	>256
P32	2000-03-03	M	60	128	64	128	64	256	>256	32	>256
P33	2000-03-09	M	23 ICU	32	8	16	8	8	32	16	16
P34	2000-03-20	M	54 ICU	>256	32	128	64	256	64	64	>256
P35	2000-03-23	M	23 ICU	>256	32	128	64	256	64	64	>256
P36	2000-03-26	M	23 ICU	>256	32	128	64	256	64	128	>256
P37	2000-05-08	M	23A ICU	64	16	32	16	32	64	32	32
P38	2000-05-13	F	23 ICU	>256	32	32	64	>256	64	128	>256
P39	2000-05-16	M	54 ICU	32	8	8	8	16	8	0.5	32

TABLE 1: Epidemiology and antibiogram of *P.aeruginosa* isolates tested continued

STRAIN NO.	DATE ISOLATED	GENDER	WARD	MINIMUM INHIBITORY CONCENTRATIONS (MICs)							
				AK	AZ	CP	CT	TB	IP	MP	P
P40	2000-05-16	M	23A ICU	32	8	16	8	32	32	16	32
P41	2000-05-17	F	11 ICU	>256	32	128	64	>256	64	128	>256
GW-1	2000-05-23	F	54 ICU	128	16	64	64	64	64	64	128
P42	2000-05-23	M	60 ICU	32	16	16	16	16	64	32	32
P43	2000-06-02	M	54 ICU	>256	32	128	64	256	32	64	>256
P44	2000-06-12	M	60 ICU	128	32	128	64	256	64	64	256
P45	2000-06-15	M	16	128	64	64	32	64	64	128	64
P46	2000-06-24	M	23 ICU	128	32	64	64	128	64	32	256
P47	2000-06-26	F	11 ICU	128	32	128	64	256	64	128	>256
P48	2000-06-27	M	60 ICU	128	32	128	64	256	64	128	256
P49	2000-07-07	F	15	>256	32	128	64	256	64	64	>256
P50	2000-07-10	F	54 ICU	64	32	256	64	>256	64	128	>256
P51	2000-08-18	F	54 ICU	32	16	8	16	16	64	32	16
P52	2000-09-15	M	*	>256	32	256	64	256	128	64	>256
P53	2000-09-20	M	54 ICU	64	16	8	16	16	64	32	16
P54	2000-10-24	F	23 ICU	64	32	256	64	>256	64	128	>256
P55	2000-11-04	M	58	32	8	128	8	16	32	16	128
P56	2000-12-15	M	Orthopaedic	>256	32	64	64	>256	32	64	>256
P57	2000-12-22	M	Orthopaedic	64	8	32	8	16	64	16	16
P58	2001-01-11	M	54 ICU	128	16	32	16	32	64	32	64
P59	2001-01-15	M	51 ICU	>256	32	64	64	256	32	64	>256

TABLE 1: Epidemiology and antibiogram of *P.aeruginosa* isolates tested continued

STRAIN NO.	DATE ISOLATED	GENDER	WARD	MINIMUM INHIBITORY CONCENTRATIONS (MICs)							
				AK	AZ	CP	CT	TB	IP	MP	P
P60	2001-01-16	F	23 ICU	>256	32	64	64	256	32	64	>256
P61	2001-01-23	F	23 ICU	>256	32	64	64	256	32	64	>256
P62	2001-01-26	F	KAL 27	32	8	16	16	16	32	32	32
P63	2001-01-26	F	51 ICU	>256	32	64	64	256	32	64	>256
P64	2001-01-27	M	51A ICU	32	128	64	64	>256	128	64	>256
P65	2001-01-27	M	23 ICU	>256	32	128	64	>256	32	64	>256
P66	2001-01-31	F	KAL 27	32	8	16	16	16	64	32	32
P67	2001-01-31	M	KAL 14	>256	32	64	32	64	64	64	64
P68	2001-02-01	F	12 ICU	256	32	64	32	256	16	64	256
P69	2001-02-01	M	KAL 28	32	8	16	16	32	32	32	32
P70	2001-02-13	F	Orthopaedic	>256	32	128	64	256	64	64	>256
P71	2001-02-13	F	Orthopaedic	>256	32	128	64	256	64	64	>256
P72	2001-02-16	M	23 ICU	128	8	32	64	64	8	16	64
P73	2001-02-21	F	15	>256	32	128	64	256	64	64	>256
P74	2001-02-21	M	KAL ICU	>256	32	256	64	>256	128	128	>256
P75	2001-02-23	F	15	>256	32	64	64	>256	32	64	>256
P76	2001-02-23	F	15	>256	64	128	32	>256	64	128	>256
P77	2001-03-06	M	Orthopaedic	>256	32	128	64	256	64	64	>256
P78	2001-03-09	F	23A ICU	256	8	32	8	8	32	32	16
P79	2001-03-12	F	11 ICU	64	8	4	8	16	4	8	16
P80	2001-03-12	M	Orthopaedic	64	16	32	64	128	32	32	256

TABLE 1: Epidemiology and antibiogram of *P.aeruginosa* isolates tested continued

STRAIN NO.	DATE ISOLATED	GENDER	WARD	MINIMUM INHIBITORY CONCENTRATIONS (MICs)							
				AK	AZ	CP	CT	TB	IP	MP	P
P81	2001-03-16	F	11 ICU	>256	8	32	8	64	32	32	64
P82	2001-03-22	M	?	32	64	16	32	64	64	64	64
P83	2001-03-23	F	23A ICU	32	8	16	8	8	32	32	128
P84	2001-03-23	F	23A ICU	32	8	16	8	8	32	32	128
P85	2001-03-27	M	Orthopaedic	>256	32	128	64	256	64	64	>256
P86	2001-04-09	M	Orthopaedic	>256	32	128	32	>256	64	128	>256
P87	2001-04-11	F	*	>256	32	128	64	256	32	64	>256
P88	2001-04-16	F	54 ICU	256	64	64	32	256	32	128	256
P89	2001-04-21	M	KAL 1	128	64	64	32	256	64	128	256
P90	2001-05-05	F	51A ICU	32	4	16	8	8	32	32	256
P91	2001-06-14	M	54 ICU	>256	128	16	16	16	256	32	16
P92	2001-06-14	M	14	16	8	4	8	8	2	2	8
P93	2001-06-18	M	Orthopaedic	>256	32	128	64	>256	64	128	>256
P94	2001-06-18	F	51A ICU	>256	32	64	64	128	64	128	64
P95	2001-06-20	M	Orthopaedic	>256	32	128	64	256	64	64	>256
P96	2001-08-31	M	23 ICU	16	64	64	64	>256	16	128	>256
P97	2001-08-31	M	23 ICU	128	64	64	64	128	64	128	64
P98	2001-09-03	M	*	128	64	64	32	256	32	128	256
P99				>256	256	>256	64	>256	64	>256	>256

4.2 MOLECULAR STUDIES

Unfortunately poor results were obtained using the method described by Hsueh *et al.* (47). The following figures show the results obtained. From these figures it is clear that only partial amplification could be achieved. A detailed explanation about these results and lack thereof is given in chapter five.

Figure 4: A photograph showing five microlitres of purified DNA run on a 1% TBE agarose gel for half an hour at fifty volts (last two lanes).

Figure 5: A picture of a PCR done according to the initial PCR method (47).

Figure 6: Results of a PCR performed using the touch down method (50) run on a 1% TAE agarose gel for forty min at ninety volts.

Figure 7: A photograph of the results of precipitating PCR products (52) of the touch down method run on a 1.4% TAE agarose gel for half an hour at ninety volts.

4.3 SUSCEPTIBILITY TESTING

As all the attempts to enhance amplification failed MICs of all hundred isolates were determined in duplicate using Amikacin (MIC ≥ 32), Aztreonam (MIC ≥ 32), Ciprofloxacin (MIC ≥ 4), Piperacillin (MIC ≥ 128), Tazobactam (MIC ≥ 128/4), Ceftazidime (MIC ≥ 32), Cefepime (MIC ≥ 32), Imipenem (MIC ≥ 16) and Meropenem (MIC ≥ 16) according to the agar dilution method described by the NCCLS (54).

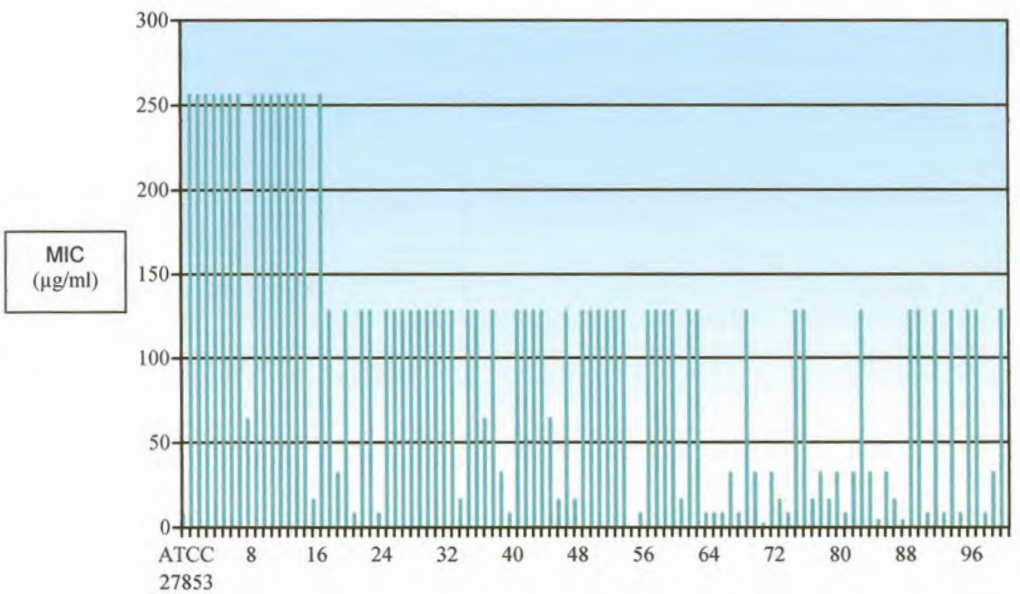

Figure 8: Representation of MIC results of all 100 isolates tested with Amikacin.

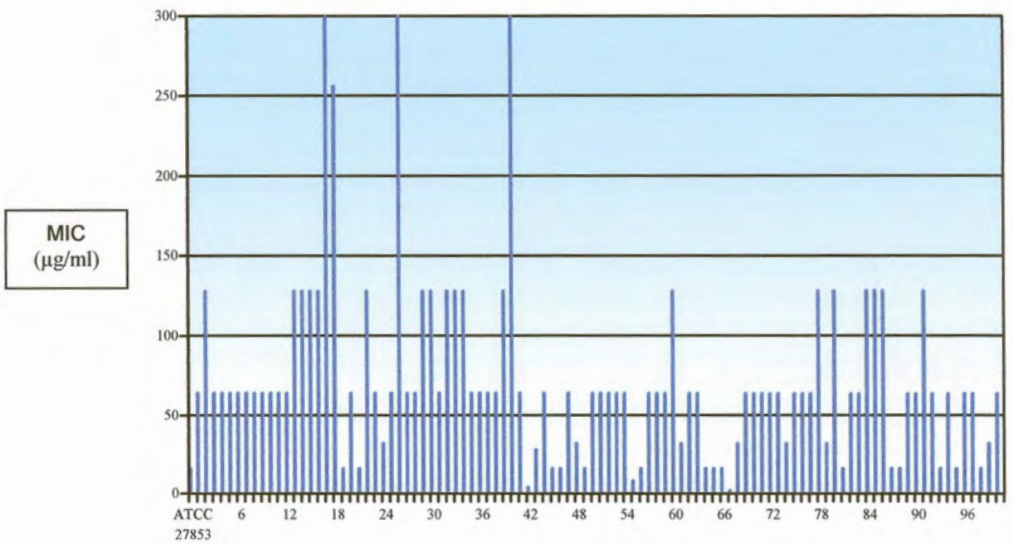

Figure 9: Representation of MIC results of all 100 isolates tested with Aztreonam.

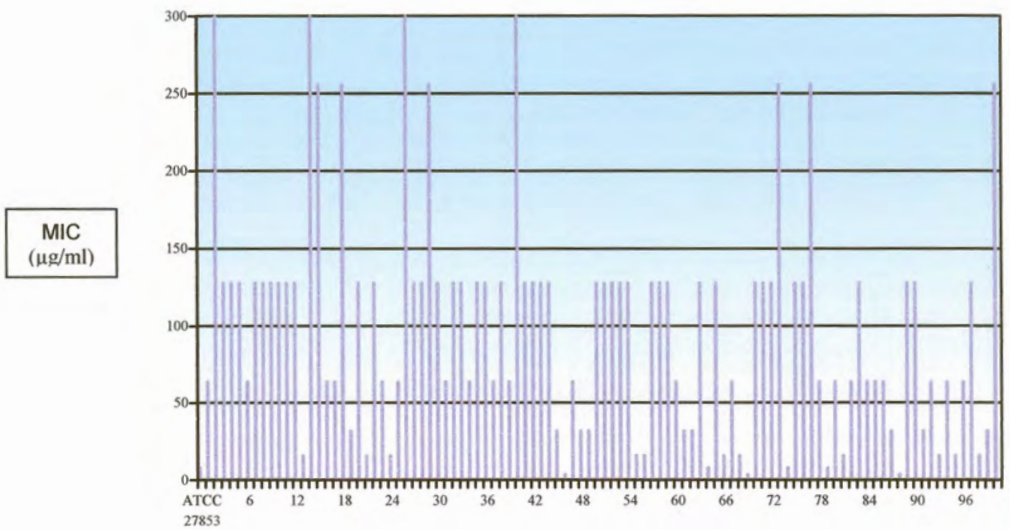

Figure 10: Representation of MIC results of all 100 isolates tested with Cefepime.

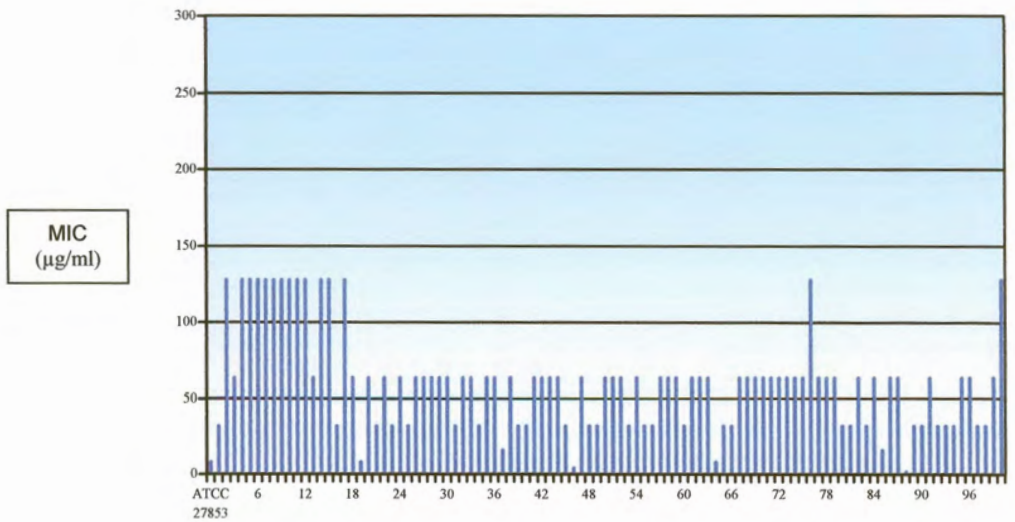

Figure 11: Representation of MIC results of all 100 isolates tested with Imipenem.

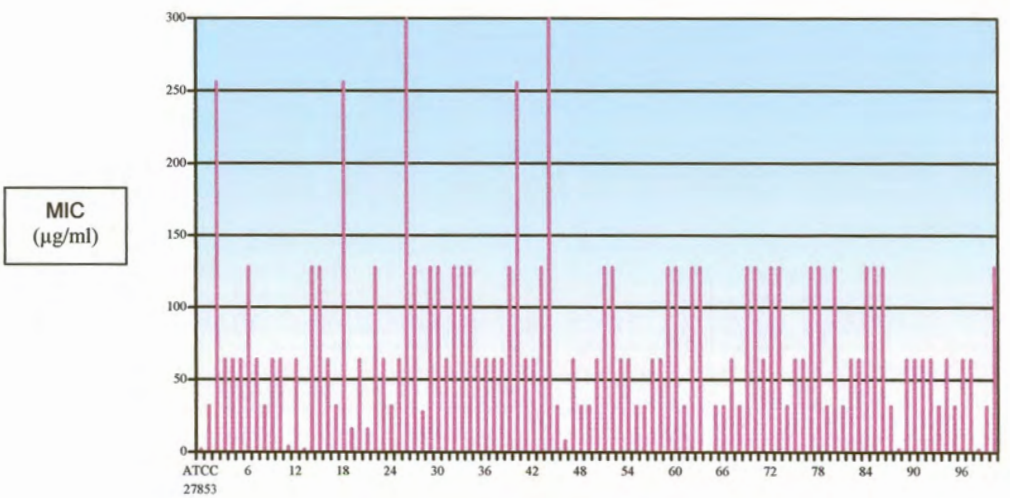

Figure 12: Representation of MIC results of all 100 isolates tested with Meropenem.

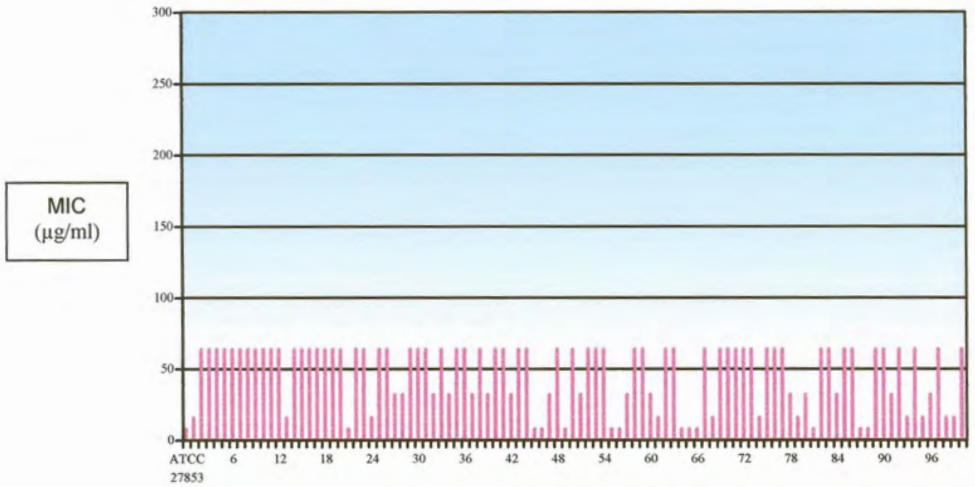

Figure 13: Presentation of MIC results of all 100 isolates tested with Ceftazidime.

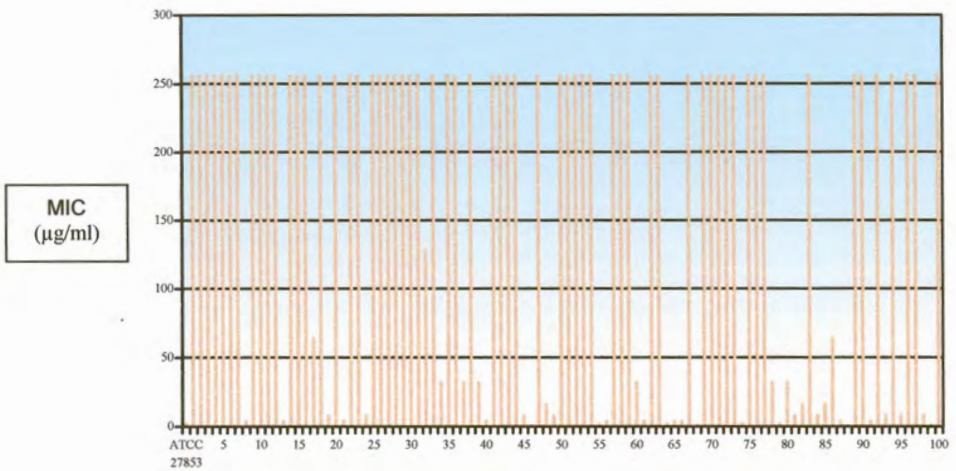

Figure 14: Representation of MIC results of all 100 isolates tested with Ciprofloxacin.

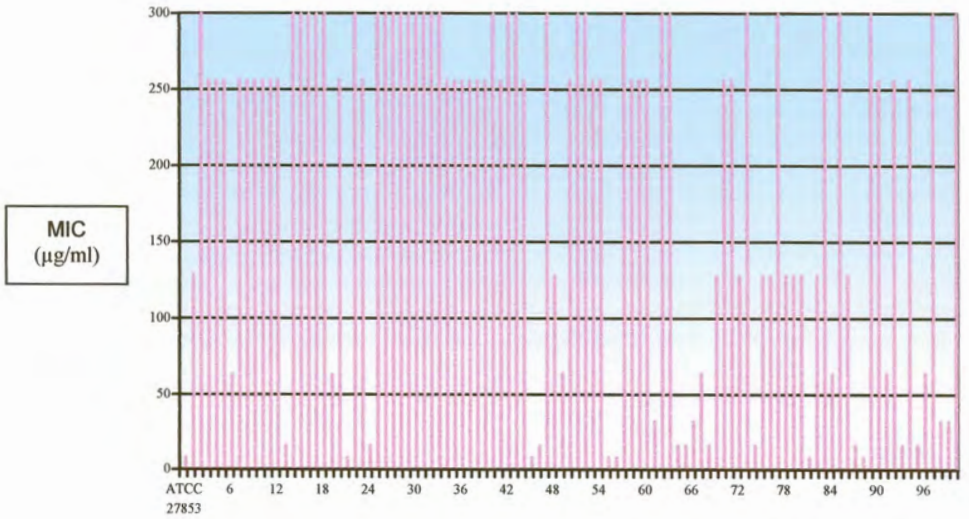

Figure 15: Representation of MIC results of all 100 isolates tested with Tazobactam.

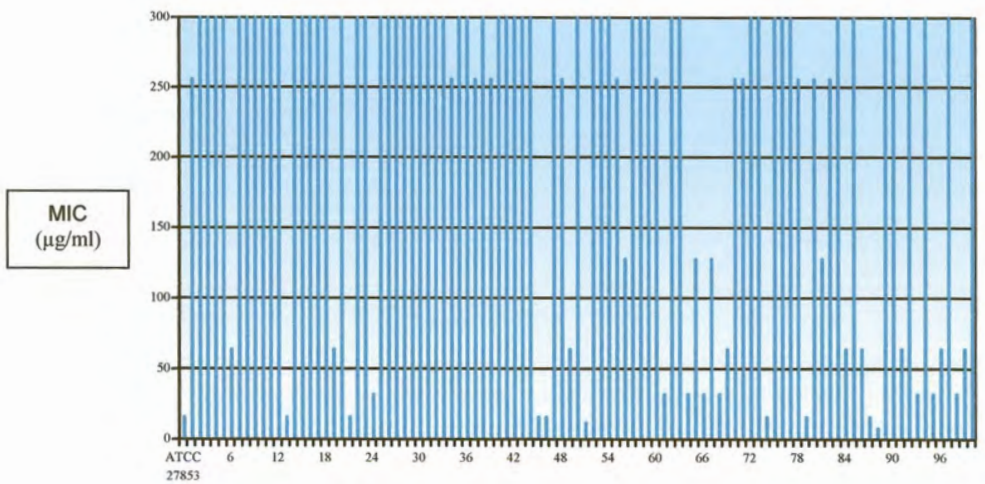

Figure 16: Representation of MIC results of all 100 isolates tested with Piperacillin.

Of the isolates tested 73% were resistant to Amikacin with 15% of them having MIC values of 256µg/ml or more while the remaining 27% were Amikacin sensitive. Nineteen percent of isolates were sensitive to Aztreonam and 81% resistant with 4% of the resistant strains having MICs ≥ 256µg/ml. For Cefepime, 82% of strains were resistant and 18% sensitive with 9% of the resistant organisms having MICs ≥ 256µg/ml. Ninety-eight percent of tested isolates were resistant to Imipenem while 2% were Imipenem sensitive. There weren't any organisms with MIC values ≥ 256µg/ml. Six percent of isolates were meropenem sensitive and 94% were resistant with 5% having MICs ≥ 256µg/ml. For Ceftazidime, 76% were resistant and 24% sensitive with none of the resistant organisms having a MIC of ≥ 256µg/ml. Eight percent of tested strains were sensitive to Ciprofloxacin and 92% were resistant with 62% of them having MICs ≥ 256µg/ml. Twenty-eight percent were Tazobactam sensitive and 72% were resistant with 61% of these resistant organisms having MICs ≥ 256µg/ml. Finally 73% of isolates were resistant to Piperacillin and 27% were sensitive with 70% of the resistant ones having MICs ≥ 256µg/ml. These results indicate that the highest level of resistance was shown towards Piperacillin with 70% of Piperacillin resistant organisms having MICs ≥ 256µg/ml. The lowest level of resistance was found against Ceftazidime with 76% of isolates being resistant and none of them had a MIC value ≥ 256µg/ml. The majority of tested strains however were most resistant to Imipenem (98%) while Tazobactam had the most sensitive strains (28%).

The isolates tested were divided into four different groups according to their specific susceptibility patterns. Group one consisted of isolates sensitive to all drugs tested (3%). Group two's organisms were resistant only to imipenem and meropenem (3%). Group three consisted of isolates resistant to more than three drugs tested (33%), while group four's organisms were resistant to all antibiotics tested (61%). Multi-drug resistant isolates thus made up 94% of the total isolates tested.

4.4 RESISTANCE TRENDS

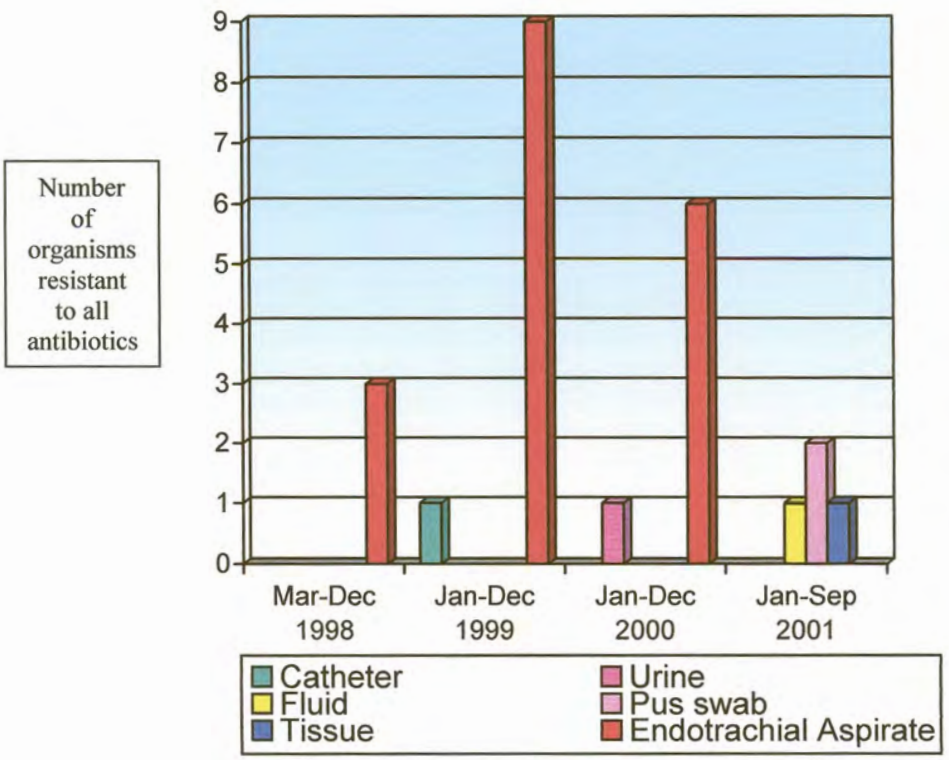

Figure 17: A graph illustrating the resistance trends over the study period.

In comparing the data of every year from 1998 to 2001, a definite decrease in the number of multi-drug resistant *P. aeruginosa*, was observed. A large number of these multi-drug resistant organisms was observed in 1999. This gradually decreased in 2000 and 2001.

4.5 DOUBLE-DISC DIFFUSION

Double-disc diffusion tests were done on twenty-five of the isolates that were resistant to all routinely used antipseudomonal drugs. These tests were performed in duplicate using Ciprofloxacin, Amikacin, Piperacillin, Tazobactam, Ceftazidime, Cefepime, Imipenem, and Meropenem.

Table 2: Disc diffusion results

ISOLATE NUMBER	SYNERGY WITH CIPROFLOXACIN	SYNERGY WITH AMIKACIN	DISC DISTANCE (cm)
P3	None	Piperacillin Tazobactam Ceftazidime Meropenem	1 1 2 1
P4	None	Piperacillin Cefepime Meropenem	1 1.5 1
P5	None	Piperacillin Tazobactam Cefepime Ceftazidime Meropenem	1 1 1.5 2 1
P7	None	Piperacillin Tazobactam Cefepime Ceftazidime Meropenem	1 1 1.5 1.5 1
P8	Cefepime Meropenem	Cefepime Meropenem	1.5 1

Table 2: Disc diffusion results continued

ISOLATE NUMBER	SYNERGY WITH CIPROFLOXACIN	SYNERGY WITH AMIKACIN	DISC DISTANCE (cm)
P9	None	Piperacillin	1
		Tazobactam	1
		Cefepime	1.5
		Ceftazidime	1.5
P10	None	Piperacillin	1
		Tazobactam	1
		Cefepime	1.5
		Ceftazidime	1.5
		Meropenem	1
P11	None	Piperacillin	1
		Tazobactam	1
		Cefepime	1.5
		Ceftazidime	1.5
		Meropenem	1
P15	None	Piperacillin	1
		Tazobactam	1
		Cefepime	1.5
		Ceftazidime	1.5
		Meropenem	1
P25	None	Cefepime	2
P29	None	Cefepime	1
		Meropenem	1.5
P30	None	Cefepime	1.5
		Meropenem	1.5
P32	None	Cefepime	1.5
		Meropenem	1.5

Table 2: Disc diffusion results continued

ISOLATE NUMBER	SYNERGY WITH CIPROFLOXACIN	SYNERGY WITH AMIKACIN	DISC DISTANCE (cm)
P33	None	Cefepime	1.5
P35	None	Piperacillin Tazobactam Cefepime Ceftazidime	1 1 1.5 1.5
P43	None	Cefepime	1.5
P44	None	Piperacillin Tazobactam Cefepime Ceftazidime Meropenem	1 1 1.5 1.5 1
P50	None	Piperacillin Tazobactam Cefepime Ceftazidime	1 1 1.5 1.5
P52	None	Cefepime	1.5
P54	None	Piperacillin Tazobactam Cefepime Ceftazidime Meropenem	1 1 1.5 1.5 1
P72	None	Piperacillin Tazobactam Cefepime Ceftazidime	1 1 1.5 2

☐ Resistant to all antibiotics

The results showed overwhelming evidence of synergy with Amikacin but virtually none with Ciprofloxacin. Only with P8, synergy occurred with Ciprofloxacin in combination with Meropenem or Cefepime. In five of the tested organisms (20%) synergy was found with Amikacin in combination with Piperacillin, Tazobactam, Cefepime, Ceftazidime or Meropenem. However, Cefepime showed the strongest synergism with Amikacin (36% of the tested organisms) followed by Meropenem and Piperacillin (32% of the tested organisms).

4.6 E-TESTS

Antibiotic synergy tests were performed on 50 multi-drug resistant isolates, chosen randomly from susceptibility groups 3 and 4 (table 3). Taking the disc diffusion results into consideration only Amikacin, Piperacillin, Tazobactam, Cefepime, Ceftazidime and Meropenem were tested.

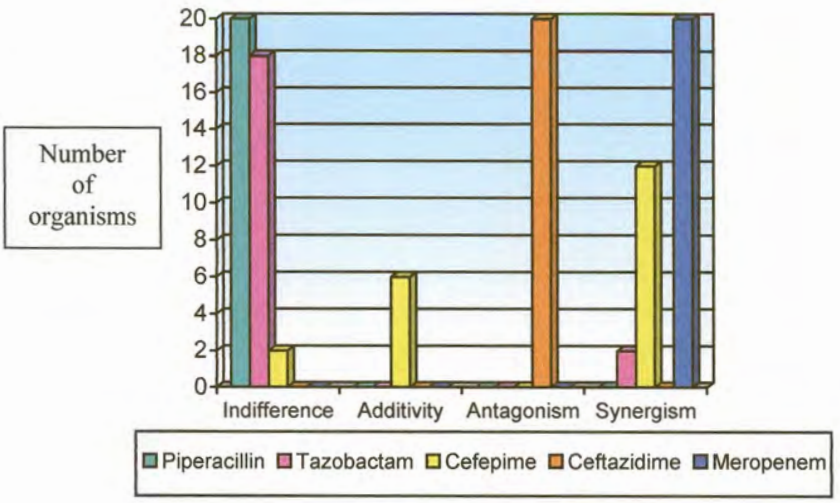

Figure 18: Indicates the E-test results of the 25 organisms that were resistant to all the routinely used antipseudomonal drugs.

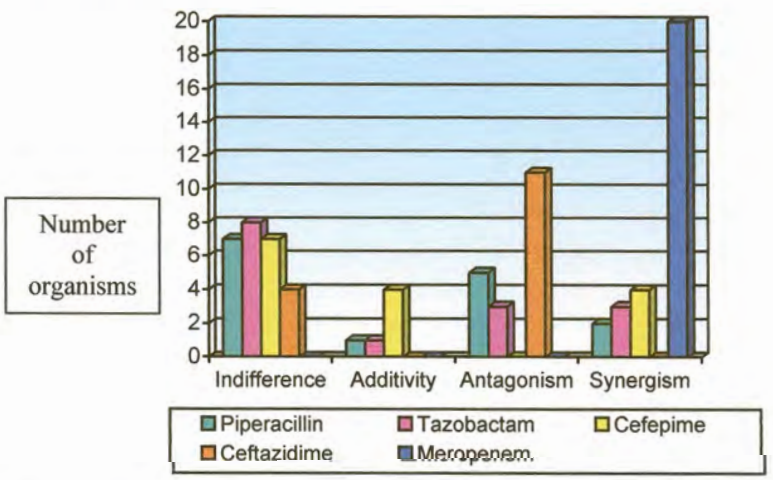

Figure 19: A graph showing the E-test results of the 25 organisms that were sensitive to some of the tested antibiotics.

Table 3: Summary of results obtained from susceptibility and synergy studies.

Antibiotic tested	**% Sensitivity obtained with antibiotic alone. (n = 50)**	**Antibiotic in combination with Amikacin. (n = 50)**		
		% Synergy (*p* value)	% Antagonism (*p* value)	% Additivity (*p* value)
Piperacillin	26	6 (0.01)	16 (0.28)	10 (0.06)
Tazobactam	8	14 (0.37)	10 (0.74)	6 (0.71)
Cefepime	4	60 (< 0.0001)	0 (0.16)	20 (0.02)
Ceftazidime	14	4 (0.09)	74 (<0.0001)	0 (0.007)
Meropenem	0	100 (< 0.0001)	0†	0†

† No calculable difference.

Twenty-six percent of these isolates tested sensitive to piperacillin, 8% were sensitive to piperacillin-tazobactam. Four percent was sensitive to cefepime, 14% tested sensitive to ceftazidime while none (0%) were sensitive to meropenem. Statistically significant antagonism was found with ceftazidime in combination with amikacin ($p < 0.0001$). Meropenem or cefepime combined with amikacin respectively, resulted in statistically significant *in vitro* antibiotic synergy ($p < 0.0001$). Piperacillin in combination with amikacin additionally exhibited synergy with a p value of 0.01.

CHAPTER 5: DISCUSSION

5.1 GENERAL

P aeruginosa is one of the most common nosocomial pathogens in intensive care units (15). This pathogen is one of the most important causes of infection in immunosuppressed patients as well as chronic infectious disease (57). The prevalence of multi-drug resistant *P. aeruginosa* isolates in nosocomial infections has increased over the last decade, and various antimicrobial combinations have been proposed as an alternative for such strains in clinical practice (16). This organism also plays a prominent role as an aetiological agent involved in serious burn wound infections. Because of its ability to colonize wounds easily and rapidly in a compromised host as well as its ability to develop resistance to most commonly used antimicrobial agents, it can be very difficult to control or eliminate *P. aeruginosa* in burn wounds (58). Cystic fibrosis is characterized by chronic endobronchial infection and progressive obstructive lung disease that is the cause of premature death in 90% of patients. *P. aeruginosa* is the pathogen most commonly associated with these endobronchial infections (59). Considering the above it is essential to monitor the prevalence as well as susceptibility patterns of these organisms regularly. The prevalence study results indicated that *P. aeruginosa* occurred mainly in endotrachial aspirates. Thirty-eight percent of organisms were isolated from these specimens. Eighteen percent of organisms were isolated from blood cultures. A definite increase in the number of positive blood cultures was observed in 2000. This was due to an outbreak of *P. aeruginosa* strains producing GES-2 β-lactamases. This outbreak caused an increase in mortality with a mortality rate of 25%. The high prevalence (Fig.3) of *P. aeruginosa* in the PAH clearly shows that it is a reality to be dealt with cautiously.

5.2 MOLECULAR STUDIES

After comparing the PCR conditions and primer composition used in the cloning study to the factors affecting PCRs, some possible factors were found that might explain the lack of amplification that occurred in the cloning study performed. The first factor might be the annealing temperature. The annealing temperature chosen for a PCR depends directly on the nucleotide sequence, length and concentration of the primers (49). It is desirable (but not absolutely necessary) that the two primers have a close melting temperature (Tm) of 5°C (B). Primer M13 had a Tm of 55.9°C while H5 had a Tm of 35.1°C. Thus, an annealing temperature (Ta) of 30°C might have yielded better results. The ideal range for Tm for both primers stretches from 55-72°C (49). The Tm range of primers used in the study was 35.1-55.9°C, which is considerably lower than the recommended range.

The preferable G+C content ranges from 50-60% (49). The G+C content of M13 and H5 was 42.9% and 70% respectively. Compared to these guidelines M13's G+C content is too low while H5's G+C content is too high. M13 doesn't end with a G, C or CG or GC combination, which might increase priming efficiency (B). H5 ends with 3 runs of C's (-CCC-3') at the 3'-end, which might promote mispriming at G or C-rich sequences because of stability of annealing (49). Lastly, the reaction buffer of the Taq polymerase used didn't contain gelatin or Bovine Serum Albumin (BSA), which could have been added (49). Due to time constraints and costs involved the PCRs could not be repeated with these factors in mind.

5.3 SUSCEPTIBILITY PROFILE

Three percent of all the isolates tested were sensitive to all the drugs tested, while three percent were resistant only to imipenem and meropenem. Thirty-six percent of tested isolates were sensitive to some of the drugs tested while an alarming sixty-one percent were resistant to all the drugs tested. As summarized in table 5 the distribution of isolates were as follows: In 1998 43% of isolates originated from non-invasive specimens while 57% were obtained from invasive specimens. Fourteen percent of isolates from invasive specimens were sensitive to some drugs tested and 86% were resistant to all drugs tested. Hundred percent of isolates from non-invasive specimens were resistant to all drugs tested. All the isolates tested in 1998 were from surgical ICUs. For 1999 81% of organisms were isolated from non-invasive specimens and 19% from invasive specimens. Thirty-eight percent of these organisms were isolated from surgical ICUs. Only 6% of the isolates from non-invasive specimens were sensitive to some of the drugs tested. In the year 2000 77% of organisms were isolated from non-invasive specimens and 23% were from invasive specimens. Sixty-one percent of organisms isolated in 2000 were from surgical ICUs. Four percent of isolates from non-invasive specimens were sensitive to all the drugs while 37% were sensitive to some of the drugs. Fifty-nine percent of organisms isolated from non-invasive specimens were resistant to all the drugs tested. Sixty-two percent of isolates from invasive specimens were sensitive to some of the drugs while 38% were resistant to all the drugs tested. For 2001 61% of isolates were gathered from non-invasive specimens and 39% were from invasive specimens. Eighty-five percent of isolates gathered in this year were from surgical ICUs. 8% of isolates from non-invasive specimens were sensitive to all the drugs while 36% were sensitive to some of the drugs tested. The remaining 56% of isolates from non-invasive specimens were resistant to all the drugs tested. Six percent of organisms isolated from invasive specimens were sensitive to all the drugs while 56% were sensitive to some of the drugs tested.

Thirty-eight percent of isolates from invasive specimens were resistant to all the drugs tested. There was a greater number of resistant isolates cultured from invasive specimens in 1998. Resistant colonizers were mainly found in 1999. A steady increase of isolates gathered from invasive specimens collected in surgical ICUs can be observed from 2000 to 2001. These isolates tend to be more sensitive than resistant however. This susceptibility profile definitely agrees with the general fact that this organism is commonly resistant to a variety of antipseudomonal agents.

TABLE 5: Distribution of isolates cultured, with specific focus on invasive and non-invasive specimens.

YEAR	% ISOLATED FROM SURGICAL ICUs	% OF ISOLATES FROM INVASIVE SPECIMENS	SENSITIVTY		
		% OF ISOLATES FROM NON-INVASIVE SPECIMENS	R TO ALL DRUGS TESTED	S TO ALL DRUGS TESTED	S TO SOME DRUGS TESTED
1998	86%	57%	86%	-	14%
		43%	100%	-	-
1999	38%	19%	100%	-	-
		81%	94	-	6%
2000	61%	23%	38%	-	62%
		77%	59	4%	37%
2001	85%	39%	38%	6%	56%
		61%	56%	8%	36%

5.4 RESISTANCE TRENDS

The high number of multi-drug resistant *P. aeruginosa* isolates from 1998 to 2000 mainly came from endotrachial aspirates (Fig.17). This suggests that *P. aeruginosa* is more of a colonizer than a pathogen. Worth noting however is the increase of multi-drug resistant *P. aeruginosa* isolates gathered from blood cultures (2001) (Fig.17). This indicates that this organism is turning into an invasive pathogen rather than a colonizer. The reason for this is could be that some strains of *P. aeruginosa* are more invasive than others, and some form of selection probably occurred in the case of the blood cultures in 2001.

5.5 SYNERGISM PROFILE

Double-disc diffusion synergy tests with Piperacillin, Tazobactam, Ceftazidime, Cefepime, Imipenem and Meropenem showed synergism with Amikacin but virtually none with Ciprofloxacin. This does not correlate with findings in the literature (60). Synergism with Ciprofloxacin in combination with Meropenem or Cefepime was only found in one of the organisms tested (P8) (table 2). This could possibly be due to efflux activity particularly that of the multi-substrate efflux pump MexE-MexF-OprN (43). A possible explanation for the high percentage of synergism with Piperacillin could be that it is one of the smallest antibiotic molecules, which can penetrate and be diffused easily. Molecules such as Ceftazidime and Meropenem are much bigger and do not diffuse that easily. Therefore it had to be placed very close to the Amikacin disc to allow synergism.

Factors found to be taken in to consideration when performing this method were:

- Zone sizes of each individual antibiotic disc
- Distance between discs
- Breakpoints.

By experimentation it was observed that if the organisms is relatively sensitive to one or both drugs the discs could be placed 1.5-2cm from each other. However, if the organisms is highly resistant to one or both drugs the discs should be placed >1.5-1cm from each other. The problem with highly resistant organism is that their zone sizes are too small or non-existent. In such a case the breakpoint for this drug has been exceeded therefore other drugs such as Colistin should be considered for testing (61).

Lewis *et.al.* defined antagonism as an increase of ≥3 dilutions in the resultant MIC whereas Bolström *et.al.* defined an increase of ≥1 dilution as antagonism (56,62). Lewis's interpretation was used for the E-test method of synergy testing (56). Cefepime exhibited remarkable additivity ($p = 0.02$) and synergy ($p < 0.0001$) in resistant organisms, indicating the possible future use of this combination in clinical cases. Concerning ceftazidime in combination with amikacin, exhibition of significant antagonism ($p < 0.0001$), make this combination an unlikely future clinical choice to treat invasive *P. aeruginosa* infections in this hospital. Additionally the mainly antagonistic response seen with ceftazidime could be indicative of the presence of mutational derepression of Amp C type chromosomal ß-lactamases (8, 63), or newly identified integron-borne class A β-lactamases such as GES-2 (64). Meropenem and amikacin in combination exhibited marked synergy and could act as a reserve choice regimen for treatment of these isolates.

CONCLUSION

The prevalence of multi-drug resistant *P. aeruginosa* observed, clearly shows that this organism is a reality to be dealt with cautiously. The susceptibility profile painted a bleak picture with a very high percentage of organisms being resistant to all routinely used antipseudomonal antibiotics. The resistance trends over the study period showed a definite decrease in the number of multi-drug resistant *P. aeruginosa* isolates. This should, not give any re-assurance, however. Clinicians should choose their therapeutic drugs with much more caution keeping these results in mind and consulting on a more regular basis with a clinical microbiologist. Although no formal antibiotic policies for empiric treatment are in place currently, they may become of use in future. The synergism profile strongly indicates that Meropenem or Cefepime in combination with Amikacin is the best combination therapy options available. The findings of this study clearly illustrate the value and necessity of closely monitoring these and other similar organisms and their susceptibility patterns.

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ELECTRONIC MATERIAL

A. Wizard Genomic DNA purification kit; Promega/Technical resources/Protocols/TM050. Available from URL: http://www.promega.com/genomic.

B. Yu R.M.K. Web Tools for Molecular Biologists / PCR Technology / PCR and Multiplex PCR guides & troubleshooting. Available from URL: http://www.geocities.com/richyuhk/

APPENDIX A A-1

1. CULTURE MEDIA

MacConkey agar medium:

MacConkey agar	(Oxoid) Ltd, Basingstoke, Hampshire, England)
Sterile plastic petri dishes (90mm)	(Concorde Plastics, Industria, Johannesburg)

51.5 gram of MacConkey agar was dissolved in one litre of distilled water and then sterilized at 121°C for fifteen min. with a pressure of 15 psi. After cooling to 50°C the agar was poured into sterile petri dishes and left to set. MacConkey agar medium was stored at 4°C.

Stock culture medium:

Stock culture medium	(Difco Laboratories, Detroit, MI)

38 gram of stock culture was added to one litre of distilled water and then placed in a steamer until the agar dissolved. The medium was then poured into sterile MacCartney bottles and sterilized at 121°C for fifteen min. with a pressure of 15 psi. Stock culture was stored at 4°C

Mueller-Hinton Broth (MH):

Mueller-Hinton Broth	(Becton Dickinson and Company, Cockeysville, MD)

Brain Heart Infusion Broth (BHI):

Brain heart infusion broth	(Difco Laboratories, Detroit, MI)

3.7 gram of BHI broth was dissolved in 100ml of distilled water and then sterilized at 121°C for fifteen min. with a pressure of 15 psi.
BHI broth was stored at 4°C.

Sterile saline:

NaCl	(E. Merck, Dramstadt, Germany)

8.5 gram of NaCl was dissolved in one litre of distilled water and then sterilized at 121°C for fifteen min with a pressure of 15 psi.
Sterile saline was stored at 25°C.

2. IDENTIFICATION

API20NE system	(bioMerieux, La Balme les Grottes)
MacConkey agar plates	(Oxoid Ltd, Basingstoke, Hampshire, England)
Stock culture medium	(Difco Laboratories, Detroit, MI)
Sterile saline	(E. Merck, Dramstadt, Germany)

APPENDIX B B-1

BUFFERS

TE BUFFER:

1.211g Tris-HCl (Sigma Chemical co, St. Louis, MD)

0.336g EDTA (E. Merck, Dramstadt, Germany)

1L distilled water

0.5 M EDTA:

186.1g EDTA (E. Merck, Dramstadt, Germany)

800ml distilled water

50 × TAE BUFFER:

242g Tris base (Sigma Chemical co, St. Louis, MD)

57.1g Glacial acetic acid (Saarchem, Johannesburg)

100ml 0.5 M EDTA (E. Merck, Dramstadt, Germany)

1L distilled water

Make a 1:50 dilution (1× TAE)

PHOSPHATE STOCK BUFFERS

BUFFER A:

27.22g KH_2PO_4 (E. Merck, Dramstadt, Germany)

1L deionized H_2O

BUFFER B:

45.64g $K_2HPO_4 \cdot 3H_2O$ (E. Merck, Dramstadt, Germany)

1L deionized H_2O

0.1M PHOSPHATE BUFFERS:

pH 6.0 Buffer:

43.85ml Stock buffer A

6.15ml Stock buffer B

100ml deionized H_2O

pH 7.0 Buffer:

19.5ml Stock buffer A

30.5ml Stock buffer B

100ml deionized H_2O

Saturated $NaHCO_3$:

$NaHCO_3$ (BDH Chemicals Ltd; Poole, England)

100ml deionized H_2O

1. MOLECULAR STUDIES

1.1 DNA ISOLATION:

Mueller-Hinton Broth (Becton Dickinson and Company, Cockeysville, MD)

Micropipettes and sterile tips
Nuclei Lysis Solution
RNase Solution
Protein Precipitation Solution
} (Promega Corporation Madison, WI,)

Isopropanol
70% Ethanol
} (Sigma Chemical Co, St. Louis, MD)

TE Buffer (pH7.5)

1.2 POLYMERASE CHAIN REACTION (PCR):

200µl PCR tubes

Two arbitrary oligonucleotide primers:

M13 (5'–TTATGTAAAACGACGGCCAGT–3') } (Integrated DNA Technologies, Inc. , 1710 Commercial Park Coralville, IA)

H5 (5'–AGTCGTCCCC–3') }

Taq DNA Polymerase

Tris-HCl (pH8.3)

$MgCl_2$

DNTPs,

KCl

} (Promega Corporation Madison WI)

Micropipettes and sterile tips

DNA extract (purified DNA)

1.3 ELECTROPHORESIS:

1×TAE Buffer	35ml	
Agarose	0.52g	(FMC Bioproducts Maple Street, Rockland, ME)
Ethidium bromide	10mg/ml	(Sigma Chemical co, St. Louis, MD)
Loading Buffer 1kb DNA ladder		(Promega Corporation Madison, WI)

APPENDIX C C-1

1. DETERMINATION OF MICs

MacConkey agar plates

Mueller-Hinton (MH) agar plates

BHI broth

0.01M Phosphate buffer of pH 6.0 and 7.0 respectively

Saturated solution of $NaHCO_3$*

Antibiotics tested:

Amikacin	(Bristol-Myers Squibb (Pty) Ltd, Princeton, NJ)
Cefepime	
Aztreonam	
Piperacillin	(Wyeth World Wide, Colledgeville, PA)
Tazocin (Pip/Taz)	
Imipenem	(Merck and Co. Inc., Whitehouse Station, NJ)
Ciprofloxacin	(AB Biodisc, Salna, Sweden)
Meropenem	(R and D Lund, Lund, Sweden)
Ceftazidime	(AB Biodisc, Salna, Sweden)

* Na_2HCO_3 should be used instead, but could not be obtained.

1.1 ANTIBIOTIC STOCK SOLUTIONS:

The following formula may be used to determine the amount of powder or diluent needed for a standard solution (55):

$$\text{Volume (ml)} = \frac{\text{Weight (mg)} \times \text{Concentration (µg/ml)}}{\text{Assay potency (µg/mg)}}$$

AMIKACIN Solvent/Diluent: H_2O

$$\text{Volume} = \frac{500\text{mg} \times 917\text{µg/mg}}{25600(\text{µg/ml})}$$

$$= 17.91\text{ml}$$

Therefore, 17.91ml H_2O were added to 0.5g Amikacin to obtain a concentration of 25600(µg/ml).

AZTREONAM Solvent: Saturated $NaHCO_3$ Diluent: H_2O

Received 100% pure aztreonam powder therefore:

$$\text{Volume} = \frac{2000\text{mg} \times 1000(\text{µg/mg})}{25600\ (\text{µg/ml})}$$

$$= 78.13\text{ml}$$

5ml Solvent was used to dissolve the Aztreonam, therefore 73.13ml H_2O was added to 2g Aztreonam to obtain a concentration of 25600(µg/ml).

CEFEPIME Solvent/Diluent: 0.1M Phosphate buffer pH 6

$$\text{Volume} = \frac{500\text{mg} \times 846(\mu\text{g/mg})}{25600\ (\mu\text{g/ml})}$$

$$= 33.05\text{ml}$$

Therefore, 33.05ml diluent were added to 0.5g Cefepime to obtain a concentration of 25600(µg/ml).

CEFTAZIDIME

Received Adatabs™ instead of base powder form.
One adatab = 800µg per 100ml medium

$C_1V_1 = C_2V_2$ 25600/800 = 32 and 3.125 × 32 = 100ml
$800 \times 100 = 25600 \times V_2$
So: $V_2 = 3.125\text{ml}$

Therefore, 100ml of H_2O were added to 32 adatabs of Ceftazidime to obtain a concentration of 25600(µg/ml).

CIPROFLOXACIN

One adatab = 200µg per 100ml medium

$C_1V_1 = C_2V_2$ 25600/200 = 128 and 0.78 × 128 = 99.84ml

$200 \times 100 = 25600 \times V_2$

So: $V_2 = 0.78ml$

Therefore, 99.84ml of H_2O were added to 128 adatabs of Ciprofloxacin to obtain a concentration of 25600(µg/ml).

IMIPENEM Solvent/Diluent: 0.01M Phosphate buffer pH 7

$$\text{Volume} = \frac{1000mg \times 1000(\mu g/mg)}{25600\ (\mu g/ml)}$$

$$= 39.06ml$$

Therefore, 39.06ml diluent were added to 1g Imipenem to obtain a concentration of 25600(µg/ml).

MEROPENEM Solvent/Diluent: 0.01M Phosphate buffer pH 7

$$\text{Volume} = \frac{500mg \times 1000(\mu g/mg)}{25600\ (\mu g/ml)}$$

$$= 19.53ml$$

Therefore, 19.53ml diluent were added to 0.5g Meropenem to obtain a concentration of 25600(µg/ml).

PIPERACILLIN Solvent/Diluent: H_2O

$$\text{Volume} = \frac{2000\text{mg} \times 1000(\mu\text{g/mg})}{25600\ (\mu\text{g/ml})}$$

$$= 78.13\text{ml}$$

Therefore, 78.13ml H_2O were added to 2g Piperacillin to obtain a concentration of 25600(µg/ml).

TAZOBACTAM Solvent/Diluent: H_2O

$$\text{Volume} = \frac{4500\text{mg} \times 1000(\mu\text{g/mg})}{25600\ (\mu\text{g/ml})}$$

$$= 175.78\text{ml}$$

Therefore, 175.78ml H_2O were added to 4.5g Tazobactam to obtain a concentration of 25600(µg/ml).

To achieve a dilution series ranging from 256-0.125µg/ml, the initial dilution series was set up beginning with1280µg/ml to compensate for the 1:20 dilution that occurs when 1ml of each dilution is added to 19ml of Mueller-Hinton agar.

APPENDIX D D-1

1. TIME-KILL CURVES

Sterile saline

BHI broth

Mueller-Hinton agar plates

Antimicrobial stock solution

Antibiotics tested:	Amikacin, Cefepime, Aztreonam	(Bristol-Myers Squibb (Pty) Ltd, Princeton, NJ)
	Piperacillin, Tazobactam	(Wyeth World Wide, Colledgeville, PA)
	Imipenem	(Merck and Co. Inc., Whitehouse Station, NJ)
	Ceftazidime, Ciprofloxacin	(AB Biodisc, Salna, Sweden)
	Meropenem	(R and D Lund, Lund, Sweden)

Antimicrobial concentrations used were determined using the following formula:

Stock concentration is 25600µg/ml = 25600µg in 1000µl
= 25.6µg in 1µl
=16×MIC in Xµl
X =.....µl

But the final volume is 5ml: X =.....µl×5
= Quantity of antibiotic stock solution to be used.

Example: P2 Amikacin

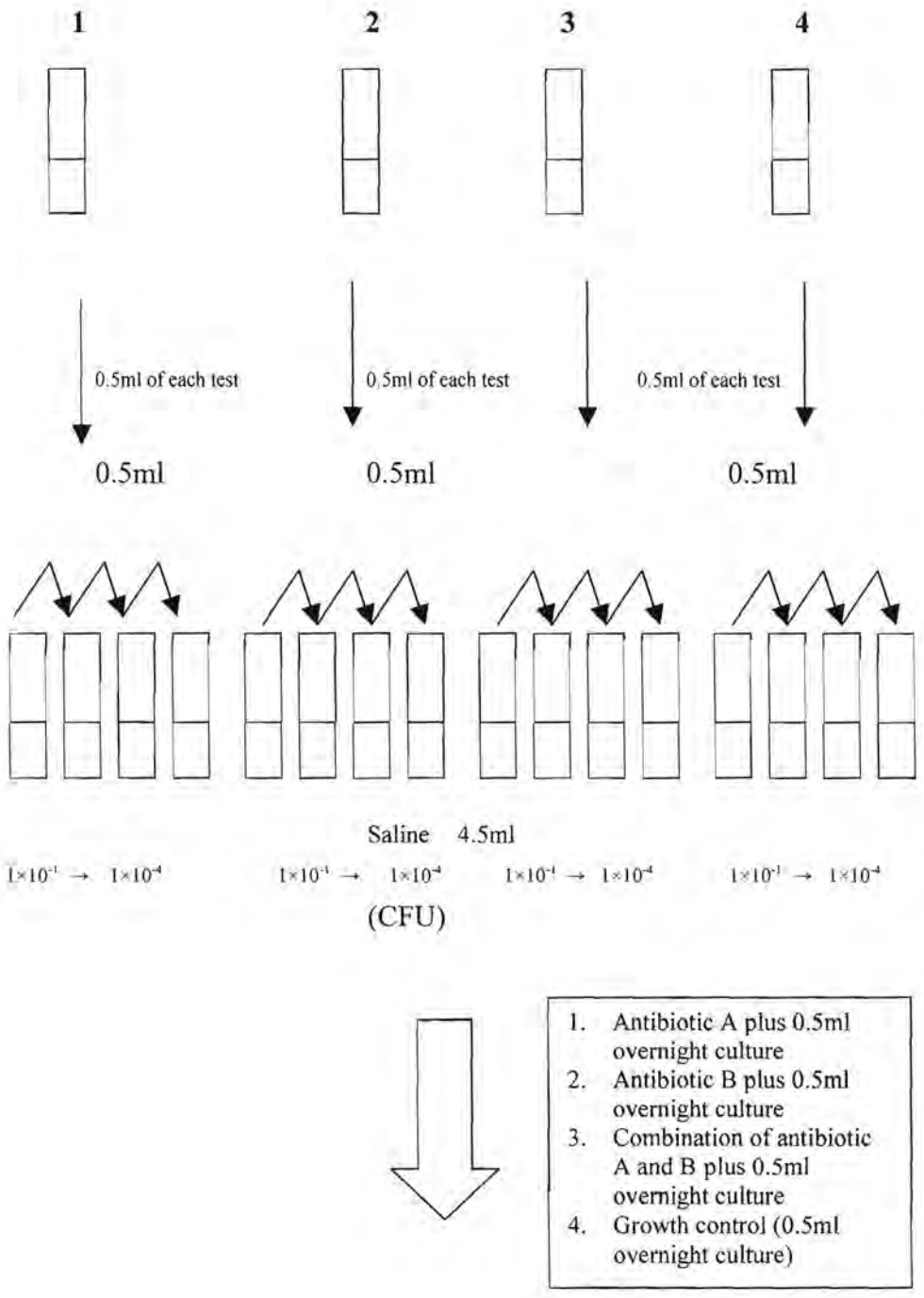
MIC = 256µg/ml

25600 in 1000µl =25.6µg in 1µl
Need 256×16 = 4000µg in Xµl
Therefore X = 156.3µl
But the final volume is 5ml: 156.3×5 = 781µl

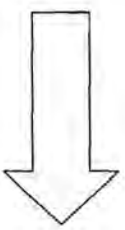
Thus 781µl of Amikacin stock solution was added to 3719µl (4500-781) of sterile BHI broth together with 0.5ml of inoculum (0.5 MacFarland standard).

DILUTION SERIES:

The dilution series was set up according to the following diagram:

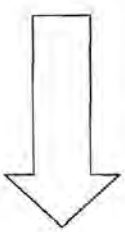


- 0.1ml of each dilution was plated out on Mueller-Hinton agar plates at T_0 within in 5 minutes after inoculum was added.
- The inoculum was spread over the whole surface of the agar plate with a glass rod.
- The agar plates were incubated overnight at 35°C.
- Colonies were counted the next day.
- The rest of the dilution series was immediately incubated for 2 hours.



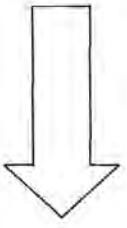
2 hours lapsed

- 0.1ml of each dilution was plated out on Mueller-Hinton agar plates at T_2.
- The inoculum was spread over the whole surface of the agar plate with a glass rod.
- The agar plates were incubated overnight at 35°C.
- Colonies were counted the next day.
- An extra dilution for each test (1×10^{-5}) was added.
- The rest of the dilution series was immediately incubated for 2 hours.



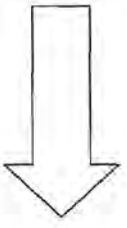
2 hours lapsed

- 0.1ml of each dilution was plated out on Mueller-Hinton agar plates at T_4.
- The inoculum was spread over the whole surface of the agar plate with a glass rod.
- The agar plates were incubated overnight at 35°C.
- Colonies were counted the next day.
- The rest of the dilution series was immediately incubated for 2 hours.



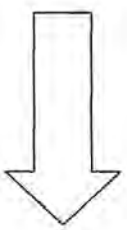
2 hours lapsed

- 0.1ml of each dilution was plated out on Mueller-Hinton agar plates at T_6.
- The inoculum was spread over the whole surface of the agar plate with a glass rod.
- The agar plates were incubated overnight at 35°C.
- Colonies were counted the next day.
- An extra dilution for each test (1×10^{-6}) was added.
- The rest of the dilution series was immediately incubated for 2 hours.



2 hours lapsed

- 0.1ml of each dilution was plated out on Mueller-Hinton agar plates at T_8.
- The inoculum was spread over the whole surface of the agar plate with a glass rod.
- The agar plates were incubated overnight at 35°C.
- Colonies were counted the next day.
- An extra dilution for each test (1×10^{-7}) was added.
- The rest of the dilution series was immediately incubated for 24 hours.



24 hours – T_6

- 0.1ml of each dilution was plated out on Mueller-Hinton agar plates at T_{24}.
- The inoculum was spread over the whole surface of the agar plate with a glass rod.
- The agar plates were incubated overnight at 35°C.
- Colonies were counted the next day.

Figure 20: Flow chart of Time-kill curve method

When inoculating the agar plates it is important to start with the last tube (1×10^{-7}) first, in order to use only one pipette tip. If a colony count of close to zero is expected, inoculate an extra agar plate with 0.1ml undiluted test.

APPENDIX E E-1

1. DISC DIFFUSION

Sterile saline

MacConkey agar plates

Mueller-Hinton agar plates

Antibiotic discs

Antibiotics tested:	Amikacin	(Bristol-Myers Squibb
	Cefepime	(Pty) Ltd, Princeton, NJ)
	Piperacillin	
	Tazobactam	(Wyeth World Wide, Colledgeville, PA)
	Imipenem	(Merck and Co. Inc., Whitehouse Station, NJ)
	Ceftazidime	
	Ciprofloxacin	(AB Biodisc, Salna, Sweden)
	Meropenem	(R and D Lund, Lund, Sweden)

2. E-TEST

Sterile saline

MacConkey agar plates

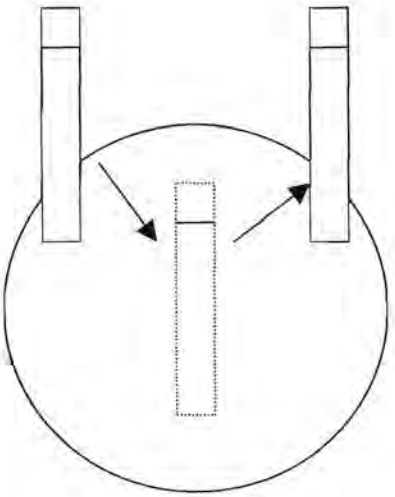
Mueller-Hinton agar plates

E-test strips

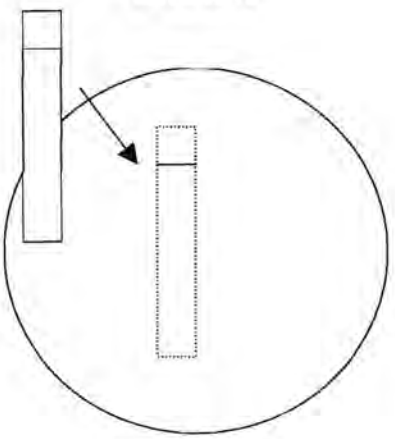
Antibiotics tested:

- Amikacin
- Cefepime
- Ceftazidime
- Piperacillin
- Tazobactam
- Meropenem

(AB Biodisc, Salna, Sweden)



DRUG A



DRUG B

Figure 21: An illustration of the E-test method

Synergy: Decrease of ≥3 dilutions in the resultant MIC.

Additivity: Decrease of ≥2 but <3 in the resultant MIC.

Indifference: Decrease of <2 dilutions in the resultant MIC.

Antagonism: Increase of ≥1 dilution in the resultant MIC.