AN EPIDEMIOLOGICAL STUDY IN THE GREATER DURBAN AREA OF GRAM NEGATIVE BACILLI RESISTANT TO AMINOGLYCOSIDE ANTIBIOTICS

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I declare that this dissertation, except where otherwise stated, represents my own work. It is submitted for the Master's Degree Technology (Medical Technology) to the Cape Technikon. It has not been submitted for any degree or examination to any other Technikon or tertiary institution. Most of the work was carried out at Mangosuthu Technikon, Durban and the University of Durban-Westville. Some of the data on the isolates being investigated was provided by Schering-Plough Research Institute, Kenilworth, USA.

The opinions and conclusions drawn are my own and not neccessarily those of the Cape Technikon.

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SUMMARY

This study was undertaken to investigate resistance to aminoglycoside antibiotics and the transfer of resistance in selected Gram negative bacilli in hospitals in the Greater Durban area in order to determine whether the development of resistance in this region was similar to that found in other countries and whether it was the same in the hospitals in the region. It was intended that the study might expose the existence of nosocomial pathogens of a particular strain or endemic plasmids responsible for aminoglycoside antibiotic resistance.

Strains of *Klebsiella, Enterobacter* and *Serratia* species and *Escherichia coli* resistant to gentamicin, tobramycin, netilmicin or amikacin were obtained.

Resistance of the isolates obtained to the above aminoglycoside antibiotics was confirmed using a disc diffusion technique.

Resistance mechanisms were initially assigned on the basis of resistance to these four aminoglycoside antibiotics. In approximately 50% of the isolates, including donor isolates and their respective transconjugants, resistance mechanisms were confirmed or revised on the basis of a changed resistance profile to a range of 12 aminoglycoside antibiotics in conjunction with DNA/DNA hybridization tests.

Bacterial conjugation studies were performed on selected isolates to investigate the transfer of aminoglycoside resistance from *Klebsiella pneumoniae* isolates to recipient *Escherichia coli.*

Plasmid profiles of all isolates and *Escherichia coli* transconjugants were compared to establish similarities.

Isolates in three of the four genera of bacteria and all isolates collectively, demonstrated the greatest incidence of resistance to tobramycin. Amikacin resistance was, in all groups of isolates, the least frequently encountered.

Collectively, the most frequent mechanisms of resistance were the AAC(3)-V and AAC(6')-1 enzymes

One large hospital showed a high frequency of the AAC(3)-V modifying enzyme while in other hospitals a wider range of enzyme resistance mechanisms were evident.

Plasmid profiles were generally dissimilar within and between different genera and the different hospitals.

Although the part played by plasmids was not demonstrated, the *in vitro* transfer of aminoglycoside resistance between different genera of bacteria was.

Another large hospital showed signs of the possible existence of an endemic plasmid or strain of *Klebsiella pneumoniae* in certain units in the hospital as a number of isolates demonstrated similar or identical plasmid profiles, aminoglycoside resistance patterns and assigned resistance mechanisms.

This study has given rise to a possible further and more intensive investigation of additional isolates from the hospital showing a high frequency of the AAC(3)-V enzyme and the other hospital in which commonality was evident in a number of *Klebsiella pneumoniae* isolates.

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AAC	acetyltransferase
ANT	adenylyltransferase
APH	phosphotransferase
bp	base pair
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
EDTA	ethylene-diamine-tetra-acetic acid
g	gravities
gm	gram
h	hour
M	molar
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
N	normal
R plasmid	resistance plasmid
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
U	unit
μg	microgram
μl	microliter
V	volt

CHAPTER 1

INTRODUCTION

INTRODUCTION

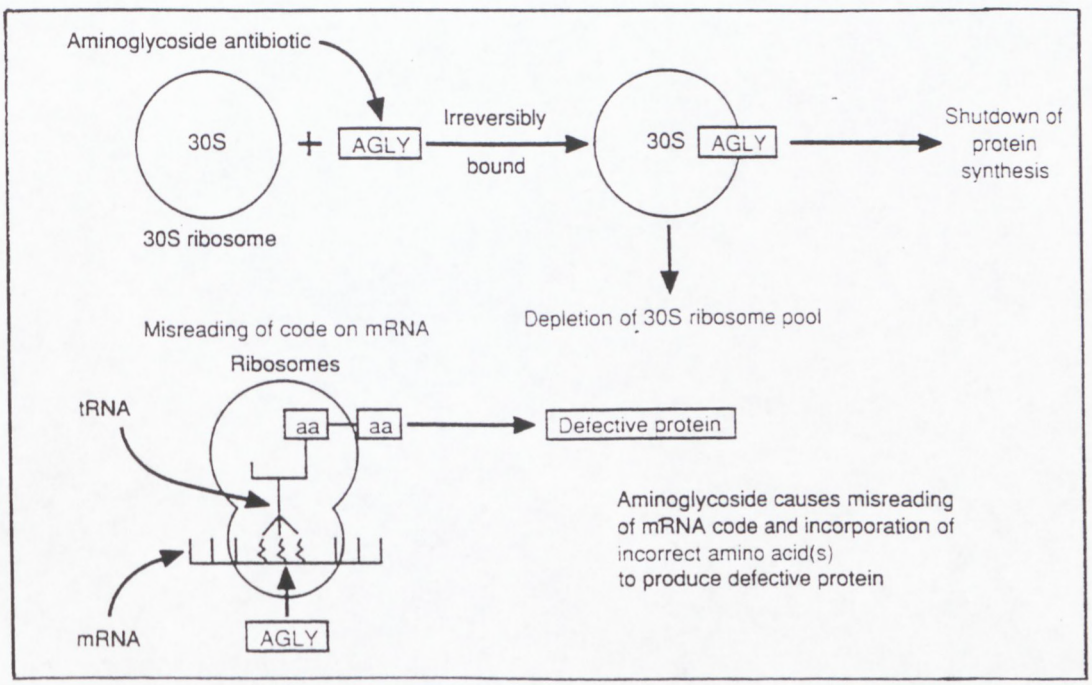
The aminoglycoside antibiotics in the class aminoglycoside-aminocyclitol antibiotics are extremely useful antimicrobial agents, particularly in infection due to members of the Enterobacteriaceae (Felmar *et al*, 1994). However, their widespread usage has contributed to the emergence of resistant strains of bacteria although Felmar *et al* (1994) feel that aminoglycoside resistance is of lesser concern than resistance to other antibiotics such as the β-lactams.

In the Enterobacteriaceae the aminoglycoside antibiotics pass through the bacterial cell walls by way of porin channels which are lined with porin protein and are designed to admit cationic molecules to the periplasmic space. The aminoglycosides are then translocated across the cell membrane by an energy-dependent proton-motive force and bind to the ribosomes just below the membrane (Neu, 1991).

Aminoglycosides are complex sugars which differ in molecular nucleus, which may be streptidine or 2-deoxystreptamine, and in the aminohexoses linked to the nucleus. Free NH_4 and OH groups by which aminoglycosides bind to specific ribosomal proteins are essential to their activity. Streptomycin has a central streptidine ring while other aminoglycosides including gentamicin, tobramycin, netilmicin and amikacin have a 2-deoxystreptamine nucleus. Aminoglycoside antibiotics kill bacteria by binding to the S12 protein of the 30S ribosome causing the shutdown of protein synthesis and misreading of mRNA code which induces the formation of nonfunctional protein complexes (Greenwood, 1989; Neu, 1991; Felmar *et al*, 1994). This is illustrated in Figure 1.

Streptomycin was introduced in 1944 followed shortly after by neomycin (Davies, 1986). Both aminoglycoside antibiotics had toxic side effects although streptomycin was used effectively to control tuberculosis for which it still is used. Neomycin was limited mainly to topical application. In the 1950's and 1960's kanamycin which was less toxic than neomycin was used mainly in Gram negative infection (Davies, 1986). In 1963 gentamicin was discovered and, in 1968, tobramycin (Davies, 1986). Both had a broader spectrum of activity than that of kanamycin. Increased usage of gentamicin and tobramycin led to the emergence of increased numbers of resistant bacterial strains. A number of semisynthetic derivatives including amikacin (a kanamycin derivative) and netilmicin were developed to overcome the problem of resistance (Greenwood, 1989). Later, dibekacin (also a kanamycin derivative) and isepamicin (derived from gentamicin)

Figure 1 : Inhibition of protein biosynthesis by aminoglycoside antibiotics



(Neu, 1991)

became available. Isepamicin has a greater resistance to inactivating enzymes and it was found that, in worldwide studies, isepamicin retained its activity against isolates resistant to gentamicin, tobramycin, netilmicin and amikacin (Minguez *et al*, 1990; Miller *et al*, 1995 [1]; Miller *et al* 1997).

As the newer aminoglycosides became more frequently used so too did the emergence of aminoglycoside resistant clinical strains of bacteria. Jacoby *et al* (1990) found that the usage of amikacin as the principal aminoglycoside over a period of time resulted in increased resistance to the drug. However, Friedland *et al* (1992) concluded that usage as a first-line aminoglycoside usually does not lead to increased resistance to amikacin but increased usage, especially in overcrowded conditions, may result in a significant increase in resistance of harmful bacterial flora resident in the hospital environment. Mayer (1986) also suggested that the most resistant organisms were usually found in units where patients were immunocompromised, had undergone surgery or were hospitalized for long periods of time. Jacoby *et al* (1990) found that amikacin resistance appeared first in *Pseudomonas aeruginosa* followed by other enteric organisms in debilitated and compromised patients who had spent some time in intensive care units and had been treated with multiple antibiotics including amikacin. These were high risk patients for nosocomial infections (hospital acquired).

Nosocomial infection is a worldwide problem with possible serious consequences in hospitalized patients. The repeated isolation of identical organisms from a number of patients probably represents patient to patient transmission from a common source or by a common mechanism (Pfaller, 1991).

In many instances the bacteria causing these infections are resistant to a number of drugs including the aminoglycosides. The Gram negative bacilli of the genera *Escherichia, Klebsiella, Enterobacter, Serratia* and *Citrobacter* are often referred to as **coliform bacilli** mainly because of their common properties and particularly their ability to ferment lactose. The coliform bacilli, together with the genus *Proteus*, are responsible for approximately 45% of nosocomial infections in the United States. The mean nosocomial rate in 1984 was 3.3% of patients discharged from hospitals participating in the National Nosocomial Infections Survey (Guentzel, 1991).

A number of studies have indicated that the usage of particular aminoglycoside antibiotics contributes to resistance rates in bacteria (Jackson, 1979; McGowan, 1983) and that usage is important in the selection of strains with different enzyme resistance mechanisms (European Study Group on Antibiotic Resistance, 1987; Friedland *et al*, 1992; Kallova *et al*, 1997; Miller *et al*, 1997).

Resistance to the aminoglycoside antibiotics in facultatively aerobic Gram negative bacilli is largely due to plasmid-mediated enzymes which inactivate the antibiotics (Davies, 1986). Plasmids may be transferred between bacteria of the same genus and bacteria of different genera (Mayer, 1988). In Japan in the 1960's a large plasmid or R-factor was found to be carrying antibiotic resistance genes in bacterial strains that had acquired simultaneous resistance to four antibiotics. These bacteria were responsible for an epidemic of bacterial dysentery (Freifelder and Malacinski, 1993). The spread of aminoglycoside resistance genes between bacteria is of significant importance in the treatment of disease. Due to their transferable antibiotic resistance genes, plasmids are regarded as important in nosocomial infections (John and Twitty, 1986).

Although it has not been clearly established, it is thought that resistance enzymes are located in the periplasmic space in bacterial cells and must be closely associated with the cytoplasmic membrane where they are accessible to acetyl coenzyme A and ATP. As is diagramatically represented in Figure 2 it appears that in this area of the bacterial cell it is neccessary to modify only small amounts of the antibiotic which then interfere with active transport mechanisms and block all further transport of the drug into the cell (Greenwood, 1989; Neu, 1991).

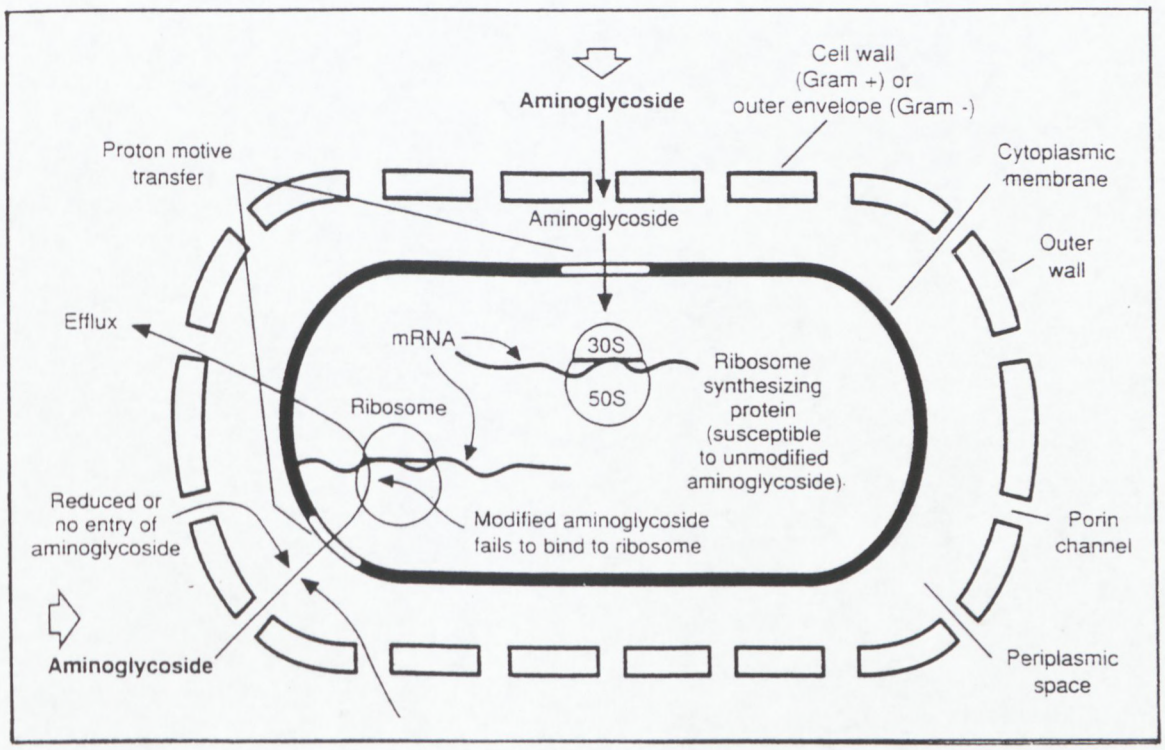
Other factors of lesser importance are a lack of permeability or a low affinity of the drug for bacterial ribosomes (Gilman *et al*, 1992).

A number of different enzymes are responsible for resistance to different groups of aminoglycoside antibiotics. Shaw *et al* (1993) state that there are two theories for the origin of aminoglycoside-modifying enzymes in clinical bacteria. One theory is that the enzymes may have originated from actinomycetes producing aminoglycosides. The presence of the enzymes in these organisms would be for the purpose of self-protection. The second theory is that the resistance enzymes are derived from bacterial genes encoding enzymes for normal cellular metabolism. It is suggested that selective pressure of aminoglycoside usage causes mutations which alter the expression of normal enzymes resulting in their ability to modify aminoglycosides.

Resistance brought about by these enzymes is dependent on the ability of a bacterial strain to produce the enzyme and the chemical structure of a particular aminoglycoside antibiotic.

Known groups of modifying enzymes are the AAC (acetyltransferases), ANT (adenylyltransferases) and APH (phosphotransferases). In describing a particular enzyme the site of modification is given as (3), (3'), (6'), (2") etc. Resistance

Figure 2 : Transfer of the aminoglycoside across the bacterial cell wall

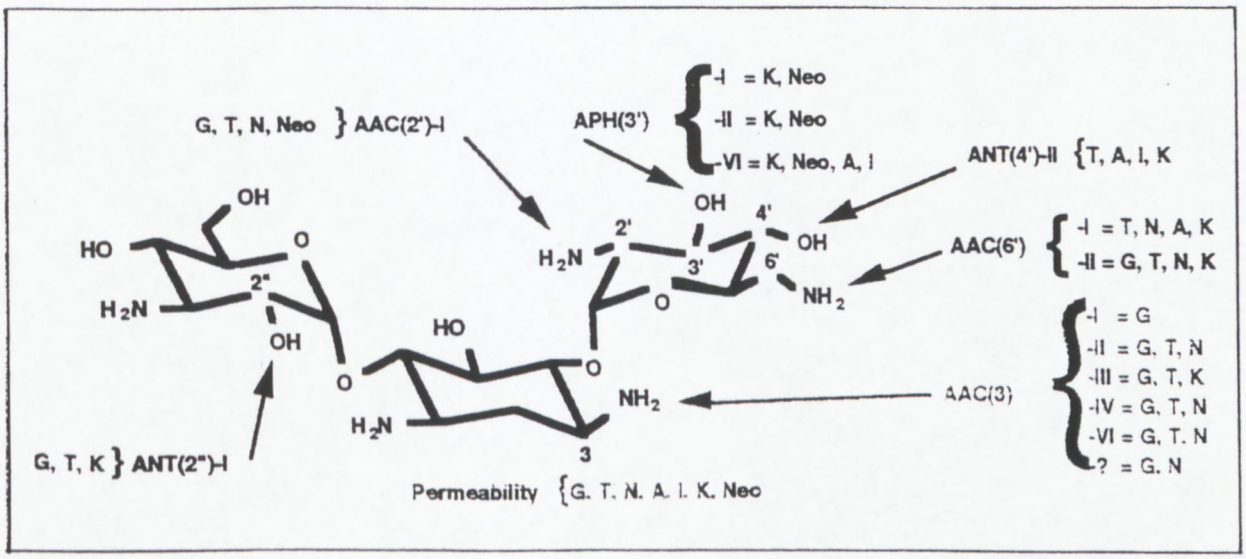


(Neu, 1991)

profiles are given as 1, 11, 111, 1V, V etc. and specific protein designations as *a, b, c* etc. (Shaw *et al*, 1993).

The structure of an aminoglycoside antibiotic which possesses all the hydroxyl and amino groups which may be present in various aminoglycosides is illustrated in Figure 3.

Figure 3 : Structure of aminoglycoside antibiotic possessing all the hydroxyl and amino groups which may be modified by Gram negative bacteria



Key :

G	gentamicin
T	tobramycin
N	netilmicin
A	amikacin
K	kanamycin
Neo	neomycin
I	isepamicin

(Miller *et al*, Schering-Plough Research Institute)

Miller *et al* (Schering-Plough Research Institute) explain the aminoglycoside resistance mechanisms and their mode of action. The explanation which follows is a summary taken from this reference.

Aminoglycoside antibiotics possess many amino and hydroxyl groups which are either not modified or, if modified, do not lead to a loss of activity. Common sites of modification are the 3-, 2'- and 6'- amino and the 2"-, 3'- and 4'- hydroxyl groups. The different substrate and resistance profiles make it possible to determine the aminoglycoside modifying enzyme or enzymes present in a particular isolate. Figure 3 represents an aminoglycoside antibiotic possessing all the possible amino and hydroxyl groups which may be modified by Gram negative bacteria.

The acetyltransferases are the most frequently occurring aminoglycoside resistance enzymes and use acetyl Co-A to acetylate amino groups of specific aminoglycosides which include :

- AAC(2')-1 which acetylates the 2'-N-amino group found in gentamicin, tobramycin, netilmicin and dibekacin and not in amikacin, isepamicin and kanamycin. This enzyme is chromosomal in *Providencia stuartii* but rare in other Gram negative bacilli.

- AAC(6')-1 and AAC(6')-11 which acetylate the 6'-N-amino group. Type 1 is common in Gram negative bacilli and modifies amikacin and not gentamicin. Type 11 modifies gentamicin and not amikacin and is almost exclusive in *Pseudomonas*. Both types modify tobramycin, netilmicin, kanamycin and dibekacin which have free primary amino groups but not isepamicin. The Type 1 enzyme is encoded by a number of genes including the *aac(6')-1c* gene which is chromosomal in *Serratia* species and the *aac(6')-1b* gene which is plasmid mediated in Enterobacteriaceae.

- AAC(3) which acetylates the 3-N-amino group found in all aminoglycosides. The reasons why specific aminoglycosides are inactivated by the large number of AAC(3) enzymes is not fully understood. There are six phenotypes, five of which have been confirmed by genetic studies :

  - AAC(3)-1 is an uncommon enzyme which modifies gentamicin and fortimicin.

  - AAC(3)-11 which modifies gentamicin, tobramycin, netilmicin, 2'-N-ethyl-netilmicin and 6'-N-ethyl-netilmicin. This enzyme is also designated AAC(3)-V as the genes encoding AAC(3)-11 and AAC(3)-V enzymes have been found to be identical. The enzyme is the most frequent cause of gentamicin resistance. In this study it is further referred to as the AAC(3)-V enzyme.

- AAC(3)-111 is an unusual enzyme resistance mechanism which modifies gentamicin, tobramycin, kanamycin and 5-epi-sisomicin.

- AAC(3)-1V which modifies gentamicin, tobramycin, netilmicin, apramicin, 2'-N-ethyl-netilmicin and 6'-N-ethyl-netilmicin. The enzyme has been found in *Escherichia coli* but is very rare in other Enterobacteriaceae.

- AAC(3)-V1 which modifies gentamicin, netilmicin and 6'-N-ethyl-netilmicin has been found infrequently.

- AAC(3)- ? which modifies gentamicin, netilmicin, 2'-N-ethyl-netilmicin and 6'-N-ethyl-netilmicin. This phenotype has been seen mainly in *Acinetobacter*.

The adenylyltransferases use ATP to adenylylate hydroxyl groups and include ANT(2")-1 which modifies gentamicin, tobramycin, dibekacin and kanamycin and does not modify or slowly modifies netilmicin, amikacin and isepamicin. Netilmicin, amikacin and isepamicin have hydroxyl groups in the 2"- position but are not modified or the rate is too slow to cause resistance. ANT(2")-1 is commonly found among the Enterobacteriaceae. The less common ANT(4')-11 modifies tobramycin, amikacin, isepamicin and kanamycin but not gentamicin, netilmicin and dibekacin. The aminoglycosides which are modified have hydroxyl groups in the 4"- position.

The phosphotransferases use ATP to phosphorylate 3'-hydroxyl groups and include APH(3')-1, APH(3')-11, APH(3')-111, APH(3')-1V, APH(3')-V, APH(3')-V1 and APH(3')-V11. APH(3')-1 is the most frequently encountered enzyme in the Enterobacteriaceae and APH(3')-11 and APH(3')-V1 are rarely found in the Enterobacteriaceae. APH(3')-V11 has been found in *Campylobacter*. The remaining enzymes are not found in Gram negative bacilli. The APH(3')-1 enzyme modifies kanamycin and neomycin and not amikacin, isepamicin, gentamicin, tobramycin, netilmicin and dibekacin. Amikacin and isepamicin have hydroxyl groups but are not modified sufficiently to produce resistance. Gentamicin, tobramycin, netilmicin and dibekacin do not have hydroxyl groups in the 3'-position.

Some of the aminoglycosides considered above are experimental. The netilmicin derivatives Sch 21561 (2'-N-ethyl-netilmicin) and Sch 21562 (6'-N-ethyl-netilmicin) are the same as netilmicin but have secondary amino groups at the 2'- and 6'-positions. They enable the distinction between AAC(2'), AAC(6') and AAC(3) enzymes. Sch 22591 (5-epi-sisomicin) is used to distinguish between ANT(2")-1

and AAC(3)-111 enzymes. Its conformation makes it a poor substrate forANT(2")-1 but it is inactivated by AAC(3)-111 enzyme. It is also a poor substrate for AAC(2'), AAC(3)-1 and AAC(3)-V1 but is modified by AAC(3)-V, AAC(3)-1V and AAC(6')-1 and AAC(6')-11 enzymes. Astm (fortimicin) and Apra (apramycin) are, due to their structures, only inactivated by a few enzymes. Resistance to both is due to either permeability changes or, rarely, a combination of the two modifying enzymes AAC(3)-1 and AAC(3)-1V. Fortimicin is only modified by AAC(3)-1 and apramycin by AAC(3)-1V.

Permeability resistance is not frequently occurring in the Enterobacteriaceae and is found mainly in *Pseudomonas*. It is not specific and results in essentially equal changes to the activity of all aminoglycosides. It occurs as a result of alterations to the membrane or transport proteins or the lack of ribosomal binding of the aminoglycoside.

Mechanisms may be proposed considering the relative resistance of isolates to the range of aminoglycoside antibiotics in clinical use and the experimental aminoglycosides in combination with DNA/DNA hybridization techniques. The relative susceptibilities rather than absolute susceptibilities to the various aminoglycoside antibiotics are considered.

An additional advantage of the use of uncommon aminoglycoside antibiotics is that, for the purpose of typing isolates, selective pressure as a result of usage of the antibiotics has usually not occurred (Pfaller, 1991).

A number of investigators (Miller *et al*, 1980; Van der Klundert *et al*, 1984; Shimizu *et al*, 1985; Dornbusch *et al*, 1990; Jacoby *et al*, 1990; Shaw *et al*, 1991; Ho *et al*, 1993; Shaw *et al*, 1993; Miller *et al*, 1995 [2]; Kallova *et al*, 1997; Miller *et al*, 1997) have used these methods to distinguish the various aminoglycoside resistance enzymes.

A reasonable estimation may be made of aminoglycoside resistance mechanisms based on the pattern of resistance but in isolates containing a number of enzymes acting on similar substrates some enzymes may not be recognised (Shimizu *et al*, 1985; Ho *et al*, 1993). Shaw *et al* (1991) recommend the use of DNA probes in addition to the resistance profile when determining resistance mechanisms to overcome partial duplication of resistance profiles. These limitations are discussed further in the chapter dealing with the methods used in this study.

In studies in eight regions of the world, including South Africa, aminoglycoside resistance mechanisms were determined in 11,079 isolates by correlation of resistance phenotypes with up to 12 aminoglycoside antibiotics and DNA/DNA

DNA/DNA hybridization (Miller *et al*, 1995 [2]). Of these isolates 3,452 were in the *Citrobacter-Enterobacter-Klebsiella* group (C-E-K). 399 isolates in this group were from South Africa. 985 of the isolates in the worldwide studies were *Serratia* species and included 31 isolates from South Africa. *Escherichia coli* isolates were included in the *Escherichia-Morganella-Proteus-Salmonella-Shigella* group (E-M-P-S-S) which numbered 2080. No distinction was made between the species in this group. In the C-E-K group 12 different single enzyme mechanisms occurred and 49 combinations including 21 double, 21 triple and seven with four enzymes. However, 14 mechanisms were found in 94.6% of the isolates. The most frequent enzyme occurring in this group was AAC(6')-1 (modifying tobramycin, netilmicin and amikacin) which was most often found in combination with other enzymes and particularly AAC(3)-V (modifying gentamicin, tobramycin and netilmicin) followed by AAC(3)-1 (modifying gentamicin) and ANT(2")-1 (modifying gentamicin and tobramycin). The most frequent single enzyme mechanism found was AAC(3)-V followed by AAC(6')-1 and ANT(2")-1. The AAC(6')-1 enzyme occurred in 29.6% of the isolates from South Africa as a single mechanism and was found most frequently in combination with ANT(2")-1 (24.1%). AAC(3)-V also occurred as a single mechanism. In *Serratia* species the AAC(6')-1 enzyme was frequently found in combination with other plasmid-mediated enzymes but in South Africa was found alone in 41.9% of isolates. In the E-M-P-S-S group the AAC(6')-1 enzyme was much less common than the AAC(3)-V enzyme but was the most common enzyme found in combinations although it was much less common than in the C-E-K group. ANT(2")-1 enzyme, AAC(3)-1V enzyme (modifying gentamicin, tobramycin and netilmicin) and permeability resistance (all aminoglycosides) were more common.

Studies have shown that the AAC(6')-1 enzyme alone or in combination with other enzymes such as AAC(3)-V or ANT(2") enzymes occurred most frequently in isolates demonstrating a gentamicin/tobramycin/netilmicin/amikacin resistance phenotype in different Gram negative species (Shaw *et al*, 1991).

In making a comparison with a European study by Kresken in 1995, Miller *et al* (1997) concluded that there appeared to be no correlation between the aminoglycoside antibiotic used and resistance rates, although increased usage of aminoglycoside antibiotics probably results in increased aminoglycoside resistance while aminoglycoside resistance mechanisms were related to the particular aminoglycoside antibiotics used. In this comparison the AAC(6')-1 enzyme (modifying tobramycin, netilmicin, amikacin, dibekacin and kanamycin), alone and in combination, was far more frequently encountered in hospitals using amikacin than in those which did not.

Aminoglycoside resistance surveys before 1983 usually demonstrated single aminoglycoside enzyme resistance mechanisms in isolates (Shimizu *et al*, 1985) but more recent surveys demonstrated that most resistant strains carried combinations of mechanisms (Shaw *et al*, 1993).

As mentioned earlier changes in antibiotic resistance frequently come about as a result of interactions between bacteria involving a transfer of DNA in different environments. Transfer of genetic material between cells may occur by transformation in which cells take up free DNA molecules from the medium within which they are suspended, conjugation in which DNA is directly transferred from one cell to another or transduction in which transfer between bacterial cells is mediated by bacteriophages.

The most common and easily demonstrated mechanism is conjugation and early studies showed that the transfer of plasmids was common between members of the Enterobacteriaceae (Rubens *et al*, 1981; Shaberg *et al*, 1981; Tantulavich *et al*, 1981; Farrar, 1983; Datta *et al*, 1984; Griffin *et al*, 1985; Mayer *et al*, 1986).

DNA transferred from one cell to another is usually extrachromosomal plasmid DNA but not all plasmids are conjugative. Conjugative plasmids code for functions that promote their transfer from the donor to the recipient bacteria but non-conjugative plasmids do not. Transfer of the smaller non-conjugative plasmids may be mediated by conjugative plasmids in a process called mobilization (Ellwell and Falkow, 1986; Dale, 1989; Holmes *et al*, 1991; Freifelder and Malacinski, 1993).

Conjugation is a natural process in which DNA may be transmitted from one bacterial cell to another joined by means of a male tubular extension called a pilus which forms a conjugation bridge (Shlaes *et al*, 1986; Freifelder and Malacinski, 1993).

In vitro conjugation may be achieved by mixing two strains of bacteria and incubating the mixture. During this time conjugation occurs. The bacteria are then cultured on a medium which does not allow the parent strain to grow but allows the transconjugant (recipient) to grow. Plasmid DNA that is transferred in this manner may encode pathogenic characteristics and resistance. Plasmids encoding resistance are known as R-plasmids or R-factors (Ellwell and Falkow, 1986; Zeelie, 1990).

R-plasmids, like fertility plasmids (F-plasmids), usually carry genetic information required for conjugation and are said to be self-transmissible (Freifelder and Malacinski, 1993).

Plasmids are usually covalently closed circular, double-stranded DNA molecules ranging from 0.7 to over 250 X 10^6 daltons. They are self-replicating extrachromosomal elements of DNA and the mechanism of replication is similar to that of the bacterial chromosome. In the process the donor cell retains a copy of the plasmid (Ellwell and Falkow, 1986; Lewin, 1987). The presence of plasmids in cells in high numbers is reflected in a correspondingly enhanced level of expression of the genes they carry. Small, non-conjugative plasmids usually have a high copy number, rely on the host for some of the functions neccessary for replication and are randomly distributed between daughter cells at division. Larger, conjugative plasmids usually have a low copy number and replication is linked to chromosomal replication (Dale, 1989; Holmes and Jobling, 1991).

Resistance to antibiotics may increase in a hospital as a result of a number of events. A new plasmid may spread with a recently introduced strain of bacteria, an endemic plasmid may become associated with a new species of bacteria or an existing species of bacteria may become associated with a new plasmid. Alternatively, transpositional events may occur in existing plasmid reservoirs (Shaberg *et al*, 1981; Mayer, 1986; Hawkey, 1987; Mayer, 1988; Pfaller, 1991).

In 1975 in a hospital in the United States a plasmid which encoded resistance to gentamicin, chloramphenicol, sulphonamides and cephalosporins in a *Klebsiella pneumoniae* strain spread to more than 2000 other biotypes and species of Enterobacteriaceae. By 1980 the incidence of this plasmid, pBWH1, had decreased. However, in 1981, there was a resurgence of this plasmid with an identical plasmid fragment profile in a strain of *Enterobacter cloacae* in a unit in the same hospital (Mayer, 1986).

In contrast to the above study in which the plasmid was endemic, an aminoglycoside resistance plasmid with identical plasmid profile was found in nine cities in the United States and Venezuela between 1975 and 1985. This plasmid, pLST1000, varied in its occurrence in the different hospitals (O'Brien *et al*, 1985).

Diverse R plasmids may carry the same determinants for antibiotic resistance. This may be due to the presence of transposable elements (transposons) which are units known to carry genes mediating antibiotic resistance. Many of the genes for aminoglycoside resistance are carried on transposons (Mayer, 1988; Neu, 1991).

Transposons may be found in the bacterial chromosome or may be extra-chromosomal. They are segments of DNA that can move from one site in a DNA molecule to other target sites in the same or a different DNA molecule. The process is called transposition (Mayer, 1988; Holmes and Jobling, 1991; Freifelder and Malacinski, 1993).

Transposons are not self-replicating and must integrate into other replicons to be maintained stably (Holmes and Jobling, 1991). Each transposon encodes the functions neccessary for its transposition including a transposase enzyme that interacts with specific sequences at the end of the transposon (Holmes and Jobling, 1991).

Gram negative bacteria collected during the pre-antibiotic era contained plasmids which usually lacked in resistance determinants. Many of the R plasmids from current clinical isolates belong to the same incompatibility groups but they also determine resistance to antibiotics. This suggests that the R plasmids evolved from the older plasmids by acquisition of resistance determinants (Holmes and Jobling, 1991).

When introduced into a new bacterial host in a plasmid the transposon can jump into the chromosome or indigenous plasmids of the new host and they are sometimes referred to as "jumping genes" ((Elwell and Falkow, 1986; Mayer, 1988; Zeelie, 1990; Holmes and Jobling, 1991). As a result the stability of the mobilising plasmid is not essential for the persistence of the genetic elements located on the transposon (Holmes and Jobling, 1991).

Transposons associated with resistance range in size from 5 kb to 15 kb and are capable of inserting at different sites in the bacterial genome. They may contain a specific region, the integron, into which different resistance genes may insert resulting in multiple antibiotic resistant strains of bacteria. Recchia and Hall (1995) describe gene cassettes, each usually containing a single antibiotic-resistance gene. They are normally integrated at a specific site in an integron but may be excised from an integron and moved by site-specific recombination or, less frequently, integrated at a non-specific site. A number of these discrete genetic units may be present at a site simultaneously. Gene cassettes are regarded as mobile elements although they do not have the genes encoding proteins responsible for movement. Both excision and integration are dependent on integron-encoded integrase. Gene cassettes may also exist free as covalently closed circular molecules.

Information may be easily gained on indigenous plasmids in most hospital micro-organisms and their acquisition and dissemination of antibiotic resistance in hospitals by the extraction and detection of plasmid DNA from bacterial isolates using agarose gel electrophoresis. Plasmid profiles may be compared in assessing the relatedness of clinical isolates in the epidemiology of a bacterial species (Mayer, 1988; Pfaller, 1991; John and Twitty, 1986; Hawkey, 1987; Einstein, 1990). A number of protocols have been described, including miniprep protocols, which all follow the same basic steps and are relatively easy to perform (Birnboim

and Doly, 1979; Ellwell and Falkow, 1986; Hawkey, 1987; Holmes and Jobling, 1991; Pfaller, 1991).

Cellular DNA may be separated from other cell components after which plasmid DNA is separated from chromosomal DNA. Methods are normally dependent on the difference in the physical state of the plasmid and chromosomal DNA. Rapid methods depend on the relative resistance of the supercoiled plasmid to denaturation which is followed by rapid renaturation. Chromosomal DNA is denatured and is randomly broken into linear fragments in the extraction procedure. The conformation of a plasmid affects its migration in gel electrophoresis. The intact plasmid is usually in a CCC (covalently closed circular) form and migrates differently from a linear molecule of the same molecular weight. Plasmid DNA forms separate sharper bands at positions dependent on their size. Although plasmids are usually found in a tightly supercoiled form they may also occur in a nicked open circular form or a linear form both of which migrate more slowly. The smaller the plasmid the faster it will migrate in the agarose gel. They are found well ahead of the chromosomal DNA which usually appears as an easily seen diffuse band while larger plasmids, present as intact circular molecules, run slower than the chromosomal DNA. The approximate molecular mass of CCC plasmid DNA can be estimated by relative migration in agarose gel using plasmid DNA of known molecular mass as a standard. CCC plasmid DNA ranging from 0.6 to 95 x 10^6 daltons (One base pair = 660 daltons) may be resolved by electrophoresis in 0.7% agarose gel (Ellwell and Falkow, 1986; Mayer, 1988; Dale, 1989; Holmes and Jobling, 1991; Pfaller, 1991).

Different terms are used when giving plasmid sizes : megadaltons and kilobases. 1 megadalton = 1.51 kilobases and the average sized gene is approximately 1 kb. This gene would encode a protein of 35,000 daltons (Mayer, 1988). The term base pair (bp) is also used in this study and, according to the above calculation, 1 bp is equivalent to 662 daltons.

When a single plasmid or a number of plasmids are present in isolates purified plasmid DNA may be treated with specific restriction endonucleases which make double-stranded breaks at specific recognition sequences within the genome. After separation by electrophoresis the fragments generated may be compared in different isolates to determine whether the plasmids are related or identical (Thompson *et al*, 1974; Shaberg, Tomkins and Falkow, 1981; Ellwell and Falkow, 1986; Hawkey, 1987; Mayer, 1988; Pfaller, 1991).

Epidemiological typing methods have, in the past, included antibiograms, biotyping, serotyping, bacteriocin typing and phage typing and, more recently, plasmid

analysis which was regarded as the best means of distinguishing between strains (Pfaller, 1991) before the introduction of more advanced techniques in molecular biology.

The objectives of this study were to demonstrate the part played by plasmids in the transfer of resistance between bacterial cells. It was intended to achieve this by determining whether similar or identical plasmid profiles were present in isolates resistant to aminoglycoside antibiotics and also by investigating their transfer in *in vitro* conjugation studies. The purpose in attempting to establish commonality of plasmid profiles in these isolates was to determine whether common indigenous plasmids were responsible for aminoglycoside resistance in the individual hospitals or in the region as a whole. This knowledge was intended to provide information in the epidemiology and localization of plasmids bearing resistance genes, thereby assisting in antibiotic selection by clinicians.

The Gram negative bacilli *Klebsiella* species, *Enterobacter* species, *Escherichia coli* and *Serratia* species were included in this study as they frequently acquire resistance as a result of plasmid-mediated enzymes although *Serratia* species also contain chromosomal genes for aminoglycoside resistance.

Further aims were to determine the prevalence of resistance to aminoglycoside antibiotics to the above mentioned species in the individual hospitals and the region as a whole. The isolates selected for testing in this study were those resistant to one or more of the aminoglycoside antibiotics gentamicin, tobramycin, netilmicin and amikacin as they were the most commonly used aminoglycoside antibiotics in the hospitals in the region.

As the study progressed it became apparent that it would be neccessary to establish the mechanisms of resistance in the isolates to better assess common characteristics as, with some exceptions, little commonality was evident in plasmid profiles of the isolates. Mechanisms were initially assigned for all isolates on the basis of resistance to gentamicin, tobramycin, netilmicin and amikacin. In 49 of the isolates in the study, the initially assigned mechanisms were either confirmed or revised on the basis of resistance phenotype demonstrated with a range of 12 aminoglycoside antibiotics (including the above four) in conjunction with DNA/DNA hybridization probes.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Samples and Processing

Consecutive isolates resistant to one or more of the aminoglycoside antibiotics, gentamicin, tobramycin, netilmicin and amikacin, were collected from the laboratories of King Edward V111 Hospital, Addington Hospital, RK Khan Hospital and Wentworth Hospital between November 1994 and August 1995.

A total of 117 isolates were collected. Of these isolates 17 (Nos. 49, 62, 71, 75, 76, 77, 78, 81, 87, 91, 92, 94, 99, 104, 105, 111 and 114) were identified as organisms not included in this study, were not recovered or were not resistant to the aminoglycoside antibiotics.

Isolates were collected on a pre-arranged, regular basis. In three of the four hospitals the support from the technical staff was constant. In one of the larger hospitals it was neccessary to continually re-establish the requirements for the study as staff held senior positions on a rotational basis and satisfactory communication was difficult to maintain. In this one hospital continuity may have been affected in relation to the collection of consecutive isolates.

The organisms selected for this study included *Klebsiella* species, *Enterobacter* species, *Escherichia coli* and *Serratia* species. The dates referred to with regard to the collection of specimens are the dates that the isolates were obtained from the laboratories and are not the dates on which the specimens were received in the particular laboratory. Where possible it has been indicated which wards the isolates were obtained from. When neccessary, it was attempted retrospectively to obtain the identity of the patients from whom the isolates were obtained.

Following collection from the hospital laboratories the identity of each isolate was confirmed using the API 20 E identification system for Enterobacteriaceae (Bio Merieux SA) and susceptibility was tested to gentamicin, tobramycin, netilmicin and amikacin. The use of a commercially available identification system was an advantage as the method was standardized and easy to perform Conjugation studies were carried out whenever sufficient numbers of isolates could be processed at the same time. The isolates were preserved using the Microbank system (Davies Diagnostics). Plasmid extraction and electrophoresis of plasmid DNA was performed at later stages in batches. After all the isolates had been collected susceptibility tests to a range of 12 aminoglycoside antibiotics supplied by Schering-Plough were carried out on 38 randomly selected isolates, five donor isolates and their respective transconjugants and a control *Escherichia coli* (strain J53). Finally, these isolates were prepared for DNA/DNA hybridization studies and mailed to Schering-Plough Research Institute.

2.2 Methods

2.2.1 Determination of antibiotic resistance

Resistance of the isolates was confirmed by using the Kirby-Bauer disc diffusion method. Each organism was tested for susceptibility to gentamicin, amikacin, tobramycin and netilmicin. Antibiotic disc content and inhibition zones used to determine susceptibility are shown in Table 1.

Table 1 : Values considered when determining resistance

Antibiotic	Gentamicin	Amikacin	Tobramycin	Netilmicin
Disc content in μg	10	30	10	30
Zone diameter in mm				
Resistant	<12	<14	<12	<13
Intermediate	13-14	15-16	13-14	14-16
Susceptible	>15	>17	>15	>17
Approximate MIC correlates in μg/ml				
Resistant	>8	>32	>8	>32
Susceptible	<4	<16	<4	<8

(Acar J.F.; 1986, 28-32)

The same Kirby-Bauer disc diffusion technique was employed at a later stage in the study when selected isolates were tested against a wider range of aminoglycoside antibiotics in determining resistance mechanisms. The zone sizes obtained are shown in Appendix C, Table C1 (pp 98-99).

At all times when antibiotic susceptibility tests were carried out a standardized procedure was employed. The medium used was Mueller-Hinton agar inoculated with a standardized inoculum of the isolates and incubated at 37°C, aerobically for 16 hours. The variables were the use of different batches of Mueller-Hinton agar and repeat testing of selected isolates was performed up to one year after initial collection. All isolates were stored at -70°C.

2.2.2 Bacterial conjugation

A recipient strain of *Escherichia coli* (J53), resistant to nalidixic acid and susceptible to gentamicin and tobramycin, was used. Single colonies of donor and recipient organisms growing on LB (Luria-Bertaini) agar containing 20μ/ml gentamicin or tobramycin (depending on the resistance of the isolate) and 64μ/ml nalidixic acid respectively were separately inoculated into 2ml of LB broth containing the respective antibiotics.

After 3 hours incubation at 37°C 1 ml each of donor and recipient cultures were transferred to 2ml of LB broth in a Bijou bottle and vortexed.

2ml of this mating mixture was transferred to a sterile Petri dish (to increase aeration). Both sets of liquid mating mixtures (Bijou bottles and Petri dishes) were incubated at 37°C for 2 hours and room temperature for 22 hours. The reason for the additional incubation at room temperature was that in previous experiments more transconjugants had been obtained than when incubating at 37°C for 2 hours or for 24 hours.

Selection plates were prepared using MacConkey agar containing nalidixic acid (64μg/ml) and either gentamicin (20μg/ml) or tobramycin (20μg/ml).

Donor isolates were inoculated on control LB agar containing 64μg/ml nalidixic acid and LB agar containing 20μg/ml of either gentamicin or tobramycin.

Recipient *Escherichia coli* was inoculated on control LB agar containing 64μg/ml nalidixic acid, LB agar containing 20μg/ml gentamicin and LB agar containing 20μg/ml tobramycin.

The selection plates were flooded with each liquid mating mixture using a sterile pasteur pipette. Excess liquid was removed. Donor and recipient controls and selection plates were incubated overnight at 37°C.

Possible transconjugants were identified from selection plates using API tests (bioMerieux sa) and, using Mueller-Hinton agar and the Kirby-Bauer disc diffusion method, susceptibility to gentamicin, tobramycin, amikacin and netilmicin was tested.

Transconjugants were then preserved using the Microbank system (Davies Diagnostics).

2.2.3 Determination of resistance mechanisms

Resistance mechanisms were determined in a number of phases :

(a) Initially the enzyme mechanisms were assigned on the basis of resistance to gentamicin, tobramycin, netilmicin and amikacin of all 100 isolates and six transconjugants.

Enzymes known to be commonly found in the bacterial species in this study, based on the discussion in chapter one, were considered in assigning mechanisms. These enzymes included AAC(6')-1, AAC(3)-V, ANT(2")-1 and ANT(4')-11. Because of its lack of activity against the above-mentioned aminoglycoside antibiotics the APH(3')-1 enzyme could not be considered.

The activity of these enzymes against the four aminoglycosides is shown in Table 2.

The enzymes which were not commonly found or were not known to occur in the Enterobacteriaceae were not considered. These enzymes included AAC(2')-1, AAC(6')-11, AAC(3)-1, AAC(3)-111, AAC(3)-1V, AAC(3)-V1, APH(3')-11, APH(3')-111, APH(3')-1V, APH(3')-V, APH(3')-V1 and APH(3')-V11.

Table 2 : Determination of aminoglycoside resistance mechanisms in *Klebsiella, Enterobacter, Escherichia coli* and *Serratia* on the basis of susceptibility to gentamicin, tobramycin, netilmicin and amikacin.

Enzyme	Gentamicin	Tobramycin	Netilmicin	Amikacin
AAC(6')-1	-	+	+	+
AAC(3)-V	+	+	+	-
ANT(2")-1	+	+	-	-
ANT(4')-11	-	+	-	+
APH(3')-1	-	-	-	-

KEY : + Modification of the aminoglycoside

(b) 49 of the above isolates, including 37 randomly selected isolates, the six donor isolates and their respective transconjugants, were tested for changes in resistance to 12 aminoglycoside antibiotics including the previously used gentamicin, tobramycin, netilmicin and amikacin together with kanamycin, neomycin, apramycin, fortimicin, 6'-N-ethyl-netilmicin, 2'-N-ethyl-netilmicin, isepamicin and 5-epi-sisomicin.

(c) Hybridization studies were performed on the same group of 49 isolates to detect the genes present.

(d) The initially assigned resistance mechanisms of the respective isolates in (a) above were confirmed by considering the relative susceptibility of each isolate to the 12 antibiotics [(b) above] in conjunction with the detection of genes encoding the enzymes in the DNA/DNA hybridization techniques [(c) above] as described by Shaw *et al* (1989).

As may be seen in Table 2, assigning mechanisms on the basis of resistance to the four aminoglycoside antibiotics gentamicin, tobramycin, netilmicin and amikacin is possible when the most frequently occurring single enzyme mechanisms are present, an exception being the presence of APH(3')-1 enzyme (modifying kanamycin and neomycin). In combinations of enzyme resistance mechanisms the presence of the less commonly ocurring ANT(4')-11 enzyme (modifying netilmicin and amikacin) would be masked by the AAC(6)-1 enzyme (modifying tobramycin, netilmicin and amikacin) as would be the ANT(2")-1 enzyme (modifying gentamicin and tobramycin) by the AAC(3)-V enzyme (modifying gentamicin, tobramycin and netilmicin). The combination of AAC(6')-1 enzyme with AAC(3)-V enzyme (modifying gentamicin, tobramycin, netilmicin and amikacin) may be distinguished from single enzyme mechanisms but not from other multiple mechanisms demonstrating the same phenotype as a result of overlapping resistance profiles. For the same reason it would not be possible to accurately propose most other combinations of mechanisms.

Table 3 summarizes "A Step-by-Step Procedure for the Identification of Ag R Mechanisms in GNB" (Miller et al, Schering-Plough Research Institute) while specifically considering the bacterial species in this study and the enzyme mechanisms likely to be encountered.

When tested with the full range of aminoglycoside antibiotics (Table 3) the presence of single enzyme mechanisms may be confirmed, particularly with regard to the relative effects on 2'-N-ethyl-netilmicin and 6'-N-ethyl-netilmicin. In addition the presence of APH(3')-1 (modifying kanamycin and neomycin) and permeability

resistance (generalized resistance) may be detected. Combinations of the enzymes AAC(3)-V with AAC(6')-1 may be distinguished from AAC(6')-1 with ANT(2")-1 on the basis of relative changes to 2'-N-ethyl-netilmicin and 6'-N-ethyl-netilmicin.

Table 3 : Steps in the procedure for the determination of aminoglycoside resistance mechanisms in *Klebsiella*, *Enterobacter*, *Escherichia coli* and *Serratia* on the basis of relative resistance to clinically used aminoglycosides and experimental aminoglycosides

	Change in resistance	**Mechanism**
1	Apramycin changed	**Permeability** or AAC(3)-1V
2	Apramycin unchanged but fortimicin changed	AAC(3)-1 or AAC(3)-1 in combination with another enzyme
3	Both apramycin and fortimicin unchanged with changes to 2'-netilmicin and/or 6'-netilmicin :	
3.1	equal changes to 2'-netilmicin and 6'-netilmicin	**AAC(3)-V** or AAC(3) ?
3.2	change to 6'-netilmicin but not 2'-netilmicin	AAC(2')-1 or AAC(3)-V1
3.3	change to 2'-netilmicin but not 6'-netilmicin	**AAC(6')-1** or AAC(6')-11 or **AAC(6')-1 with ANT(2")-1** or AAC(6')-1 with AAC(3)-1
3.4	changes to both 2'- and 6'-netilmicin but a greater change in 2'-netilmicin	**AAC(6')-1 with AAC(3)-V**
4	Apramycin, fortimicin, 2'-netilmicin and 6'-netilmicin unchanged but gentamicin and tobramycin changed (kanamycin also changed)	**ANT(2")-1** or AAC(3)-111
5	Apramycin, fortimicin, 2'-netilmicin, 6'-netilmicin and gentamicin unchanged but tobramycin changed (isepamicin and kanamycin also changed)	only **ANT(4')-11**
6	Apramycin, fortimicin, 2'-netilmicin, 6'-netilmicin, gentamicin and tobramycin unchanged but amikacin changed	only APH(3)-V1
7	Changes only to kanamycin and neomycin	**APH(3')-1**

NOTE : The more frequently occurring aminoglycoside resistance mechanisms in the bacterial species in this study are in bold print.

2.2.4 **Isolation of plasmid DNA**

Initially, a rapid miniprep technique using a minicolumn and DNA binding resin was used to recover plasmid DNA (Reed *et al*).

This proved unsuccessful but it was more than likely not a reflection on the quality of the product but rather the fact that facilities were lacking in the laboratory which was not a routine laboratory and one in which molecular biology tests had not previously been performed.

As a result a routine molecular biology laboratory was used (University of Durban-Westville) for plasmid recovery, cleaveage with restriction endonucleases and gel photography.

A simple but established modification of the method of Birnboim and Doly (1979) was used for plasmid recovery. This was in preference to the time-consuming cesium chloride/ethidium bromide centrifugation method (Short Protocols in Molecular Biology, 1992).

Modification of Birnboim and Doly (1979)

Solution A	:	25 mM Tris. HCl 50 mM glucose 10 mM EDTA 100 µg/ml Rnase A pH 8.0
Solution B (Freshly Prepared)	:	0.2 N NaOH 1% SDS
Solution C	:	3 M sodium acetate, pH 4.8 Phenol (equilibrated with 0.1 M Tris HCl, pH 6.8) Choloroform (chloroform : isoamyl alcohol, 24:1)
TE	:	10mM Tris.HCL 1 mM EDTA pH 8.0

The isolates were inoculated into 5ml of Luria-Bertaini (LB) broth containing the appropriate antiobiotic and grown to saturation at 37°C in an orbital shaker. Bacteria from 1.5ml of culture were harvested in Eppendorf tubes and centrifuged for 1 min at 15 800 g. Bacterial pellets were suspended by vortexing in 100 µl of solution A. 200 µl of solution B was added and the contents of the tubes were mixed gently by inversion and incubated at room temperature for 5 min. This step caused lysis of the bacterial cells with SDS, alkaline (NaOH) denaturation of plasmid and chromosomal DNA and degradation of RNA by RNase A. Denatured chromosomal DNA and proteins were precipitated upon addition of 150 µl of cold solution C and a further 5 min incubation on ice. The precipitated material and cell debris was pelleted by centrifugation at 15800g for 10 min in a microcentrifuge. The plasmid-containing supernatant was extracted with an equal volume of phenol/chloroform by repeated inversion of the Eppendorf tubes and centrifugation at 15800 g for 3 min. to separate the aqueous and organic phases. The upper aqueous phase was subsequently collected.

Following protein removal, the aqueous phase was extracted with an equal volume of chloroform to remove all traces of phenol. Plasmid DNA was precipitated by the addition of 2 volumes of cold 100% ethanol and incubation for 1 h at -20°C or 15 min at -70°C. Precipitated plasmid DNA was pelleted at 15 800 g for 15 min, washed with 70% ethanol and air-dried. Plasmid DNA pellets were resuspended in 20 µl of TE buffer and stored at -20°C.

2.2.5 **Electrophoresis of plasmid DNA**

To a large extent electrophoresis was carried out towards the end of the study in order to group similar species of isolates so that they appeared on common gels. In spite of this it was sometimes neccessary to regroup isolates and repeat electrophoresis when similar profiles appeared on different gels.

The following buffers were used :

Gel loading buffer (6X)	:	40% sucrose 0,25% bromophenol blue
TAE buffer (50X)	:	40 mM Tris base 20 mM glacial acetic acid 2 mM EDTA

Horizontal agarose gel electrophoresis was used to analyse uncleaved plasmid DNA preparations and preparations cleaved with the restriction endonucleases *Eco*RI and *Hin*dIII. Uncleaved DNA preparations in 1 X gel loading buffer were loaded in 0.7% agarose gels and electrophoresed at a constant voltage of 40 V for 18 h in 1 X TAE buffer.

The plasmid preparation from each isolate was digested with 10 U of *Eco*RI and *Hin*dIII for 1 h at 37°C. A typical restriction reaction using a single enzyme comprised the following in an Eppendorf tube:

plasmid DNA	x	µl (200-400ng)
deionised H_2O	17-X	µl
restriction buffer (10X)	2	µl
restriction endonuclease	1	µl (10 U)
	20	µl total volume

DNA digests together with a DNA marker (phage λ DNA cleaved with *Eco*RI and *Hin*dIII) were loaded in 1% agarose gels and electrophoresed at 40 V for 18 h in 1 X TAE buffer. Following electrophoresis, gels were stained with 1 µl/ml ethidium bromide for 15 min. The fluorescence of nucleic acid-containing bands was visualised with a UV transilluminator (UVP, Inc.). Gels were photographed, using Ilford FP4 Plus film, with a Minolta camera fitted with a UV and a Vivitar No. 25 (red) filter.

2.2.6 **DNA/DNA hybridization**

In personal communication with Dr G. Miller (Schering-Plough Research Institute) it was advised that because of the expense and the time taken in preparation and shipping the Institute had deposited their hybridization probes for aminoglycoside resistance with ATCC (American Type Culture Collection). By arrangement the probes could be purchased from ATCC.

It was further suggested that it was neccessary to determine phenotypes by using a range of aminoglycoside antibiotics (some experimental) in addition to gene probes in order to more accurately determine resistance mechanisms as false negatives were frequently obtained with the 19 probes available.

The Institute offered to provide sufficient aminoglycoside antibiotic discs and filter papers for hybridization tests for 50 isolates. In addition the Institute offered to perform the DNA/DNA hybridization on these 50 isolates from the study.

In view of the fact that the laboratory in which most of the study was being undertaken lacked any facilities for radio-active analyses the offer was accepted.

A control strain of *Escherichia coli* (J53), five donor isolates and their respective transconjugants and 38 randomly selected isolates were processed.

Hybridization filters were prepared by placing 10μl of 48 hour cultures of each strain on each of 25 gene screen filter papers.

The filter papers were dried at room temperature and then placed upright on Whatman 3 MM paper saturated with 1 N NaoH for 5 min.

A second sheet of Whatman 3 MM paper was saturated with 1.0 M Tris pH 7.0 and the filter papers were transferred and placed upright on this sheet for 5 min.

Then all the filter papers were placed in a container with 500 ml of 1.0 M Tris pH 7.0 for 3 min.

After drying at room temperature the filters were placed in a plastic bag and mailed to Schering-Plough for hybridization.

In the laboratories of Schering-Plough Research Institute hybridization was carried out by pre-hybridizing for 4 hours at 42°C in the hybridization solution consisting of 50% formamide, 1% SDS, 1M NaCl, 10% dextran sulphate and 100μg/ml sonicated salmon sperm DNA.

After the excess solution was drained off 1 X 10^6 dpm/ml (5ml) of nick translated probe was added to each filter in the prehybridization mix. The following probes were used : *ant(2")-1a, aac(3)-1a, aac(3)-1b, aac(3)-Va, aac(2')-1a, aph(3')-1, aph(3')-11, aph(3')-V1, aac(6')-1b, aac(6')-1c, aac(6')-1f, aac(6')-1l, aac(6')-1m, aac(6')-1n, aac(6')-11b, ant(4')-1, aph(2" + 6'), aph(3')-111.*

The filters were then hybridized overnight at 42°C after which they were rinsed twice in 2 X SSC, 0,1% prewarmed to 55°C, washed twice in 2 X SSC, 0.1% SDS for 30 minutes at 55°C and washed once in 0.1 X SSC, 0.1% SDS for 30 minutes at 55°C.

After drying at room temperature the filters were exposed for 24 - 48 hours to X-ray film. The presence of a specific aminoglycoside resistance gene was observed as a darkened spot on the X-ray film.

2.3 **Media**

LB (Luria-Bertaini) Broth (per liter) :

10.0g	Bacto-tryptone
5.0g	Bacto-yeast extract
5.0g	NaCl

Adjust pH to 7.5 with NaOH and autoclave. Where indicated add the desired antibiotic at the required concentration after allowing to cool to 50°C.

LB Agar

Add 15g agar to 1 liter of LB broth. Adjust to pH 7.5 with NaOH. After autoclaving allow to cool to 55°C before adding the desired antibiotic. Pour 30-35ml of medium into Petri dishes. If neccessary, flame the surface of the medium with a Bunsen burner to eliminate air bubbles. Once the agar has solidified store at room temperature for 1 week or at 4°C for up to 1 month.

(Promega Protocols and Applications Guide. 1991)

Mueller-Hinton Agar (per liter)

5.0g	meat infusion
17.5g	casein hydrolysate
1.5g	starch
14.0g	agar

Dissolve 38g per liter distilled water. Allow to stand for 15 minutes. Boil to dissolve completely. Autoclave at 121°C for 15 minutes. Cool to 45°C and pour into Petri dishes. pH: 7.4 $\pm$ 0.2.

(Biolab Catalogue. 1997)

CHAPTER 3

RESULTS

RESULTS

3.1 Nature of specimens from which aminoglycoside resistant isolates were obtained

Resistant isolates were obtained mainly from swabs, blood cultures and catheters as is reflected in Table 4.

Table 4: Nature of specimens from which aminoglycoside resistant isolates were obtained.

Specimen	Total/Per Cent
Swab	23
Blood Culture	19
Tracheal tube	10
Urine/urinary catheter	9
Sputum	6
Aspirate	6
Venous catheter	5
CSF	2
Stool	1
Not established	19
Total	100

3.2 Reproducibility of antibiotic susceptibility testing

The sizes of zones of inhibition were not recorded during initial susceptibility testing of isolates (Appendix A, pp 80-89) or donor isolates and their respective transconjugants (Table 15, page 65) although the resistance profiles were reported as susceptible, intermediately resistant or resistant, based on zone diameters (Table 1, page 19).

In the 38 isolates, five donor isolates and their respective transconjugants which were subjected to repeat testing with 12 aminoglycoside antibiotics including gentamicin, tobramycin, netilmicin and amikacin, zone diameters were recorded. 36 of the isolates, the five donor isolates and their respective transconjugants produced the same resistance profiles in terms of reporting the results as susceptible, intermediately resistant or resistant. In isolate 41 changes occurred in tobramycin (resistant changed to intermediate resistance) and netilmicin (resistant changed to susceptible). In isolate 53 resistance to netilmicin was changed to intermediate resistance.

3.3 Frequency and hospital distribution of isolates resistant to aminoglycoside antibiotics

Of the 100 isolates obtained between November 1994 and August 1995 54% were identified as *Klebsiella pneumoniae*, 23% as *Enterobacter cloacae* and 12% as *Escherichia coli*. The remaining 11% were identified as *Serratia marcescens*, *Enterobacter aerogenes*, *Klebsiella aerogenes* and *Serratia liquefaciens* (Table 5). 42% were obtained from King Edward Hospital, 44% from Addington Hospital and the remaining 14% from RK Khan Hospital and Wentworth Hospital (Table 5).

Table 5 : Relative percentage and distribution by hospital of aminoglycoside resistant isolates.

	Addington	RK Khan	Wentworth	King Edward	TOTAL / %
Klebsiella pneumoniae	17	3	3	31	54
Klebsiella oxytoca	2				2
Enterobacter cloacae	14	4	3	2	23
Enterobacter aerogenes	1			1	2
Serratia marcescens	5			1	6
Serratia liquefaciens				1	1
Escherichia coli	5	1		6	12
TOTAL / %	44	8	6	42	100

3.4 **Resistance of isolates to aminoglycoside antibiotics**

Collectively all isolates predominantly demonstrated resistance to tobramycin (96%). Amikacin was the antibiotic to which all isolates were least frequently resistant (Table 6).

Table 6 : Relative resistance of all isolates to aminoglycoside antibiotics

	Gentamicin	Tobramycin	Netilmicin	Amikacin
Resistant	77	96	73	16
Intermediate	2	4	16	13
Susceptible	21	nil	11	71
Total/percentage	100	100	100	100

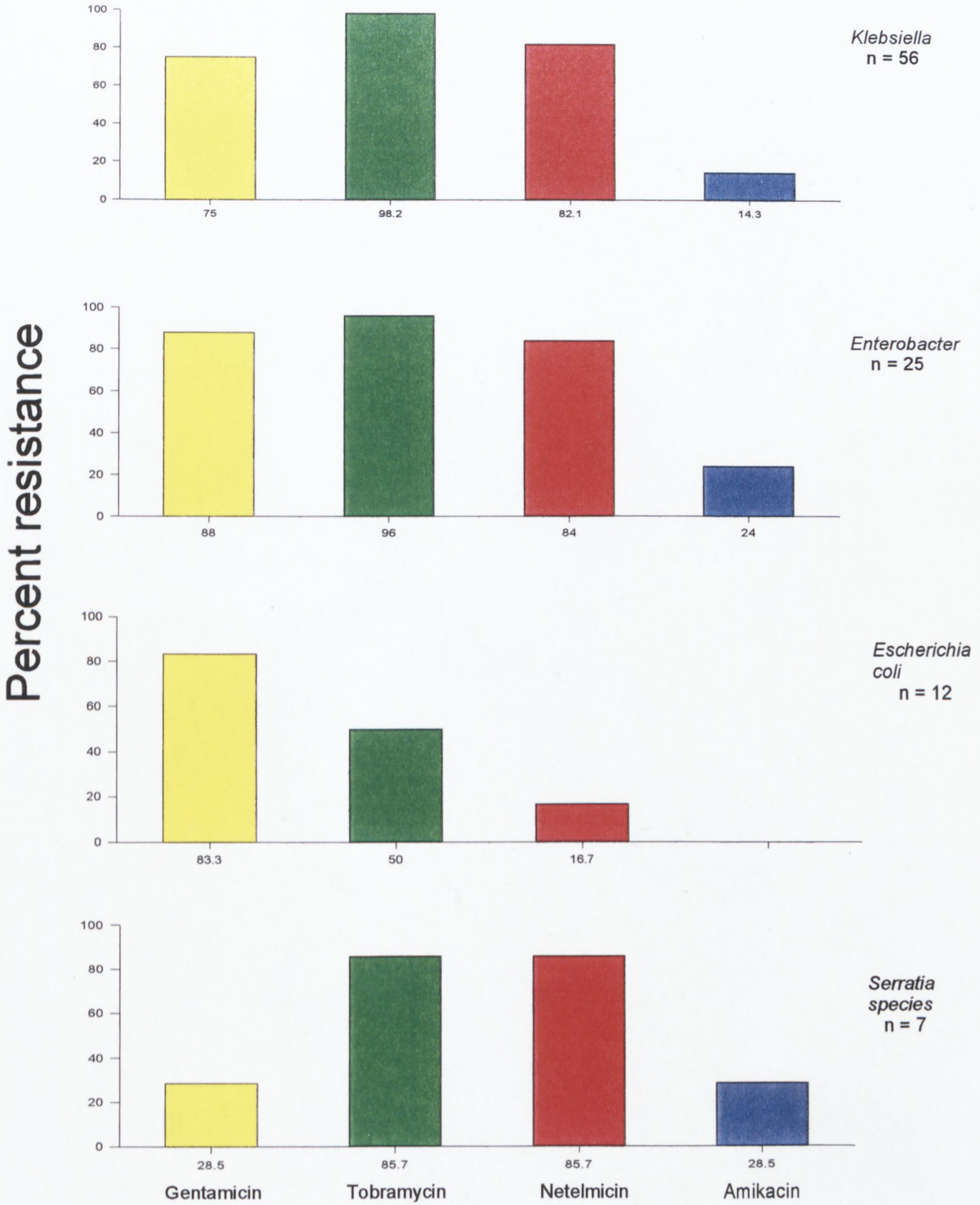
Statistics of aminoglycoside antibiotic resistance in this study were based on absolute resistance. It was noted, however, that a significant percentage of isolates demonstrated intermediate resistance to all aminoglycosides and particularly to netilmicin and amikacin (Table 7).

Table 7 : Intermediate resistance to aminoglycoside antibiotics

	Gentamicin	Tobramycin	Netilmicin	Amikacin
Klebsiella species	nil	1 (1.8%)	5 (8.9%)	6 (10.7%)
Enterobacter species	1 (4%)	1 (4%)	2 (8%)	4 (16%)
Escherichia coli	1 (8.5%)	1 (8.3%)	8 (66.7%)	3 (25%)
Serratia species	nil	1 (14.3%)	1 (14.3%)	nil

The resistance demonstrated by *Klebsiella* species was mainly to tobramycin (98.2%), to a lesser extent netilmicin and gentamicin and least to amikacin (Figure 4).

Figure 4: Resistance of isolates to aminoglycoside antibiotics



A greater percentage of *Enterobacter cloacae* isolates were resistant to both gentamicin (88%) and amikacin (24%) than *Klebsiella pneumoniae* and *Escherichia coli* (Figure 4).

Escherichia coli isolates demonstrated a high frequency of resistance to tobramycin and gentamicin but comparative resistance to netilmicin was reduced and none were resistant to amikacin (Figure 4).

Serratia species demonstrated a high frequency of resistance to tobramycin and netilmicin. Resistance was reduced to gentamicin but relative to the other species of bacteria the percentage of amikacin resistant strains was higher (Figure 4).

All *Klebsiella pneumoniae* isolates (n = 31) received from King Edward Hospital were resistant to gentamicin and susceptible to amikacin (Table 8).

Of the *Klebsiella* species (n = 19) received from Addington Hospital seven were resistant to gentamicin (Table 8). Six of these isolates demonstrated intermediate resistance to amikacin (Appendix B : Table B1, p 92).

Table 8 : Aminoglycoside resistance to isolates in hospitals surveyed

	Hospital	Gentamicin	Tobramycin	Netilmicin	Amikacin	n
Klebsiella species	King Edward	31 (100%)	30 (96.8%)	25 (80.6%)	nil	n = 31
	Addington	7 (36.8%)	19 (100%)	15 (78.9%)	5 (26.3%)	n = 19
	Wentworth	2 (66.7%)	3 (100%)	3 (100%)	2 (66.7%)	n = 3
	RK Khan	2 (66.7%)	3 (100%)	2 (66.7%)	nil	n = 3
Enterobacter species	King Edward	3 (100%)	3 (100%)	2 (66.7%)	1 (33.3%)	n = 3
	Addington	14 (93.3%)	14 (93.3%)	13 (86.7%)	2 (13.3%)	n = 15
	Wentworth	1 (33.3%)	3 (100%)	3 (100%)	3 (100%)	n = 3
	RK Khan	4 (100%)	4 (100%)	2 (50%)	nil	n = 4
Escherichia coli	King Edward	6 (100%)	6 (100%)	1 (16.7%)	nil	n = 6
	Addington	3 (60%)	5 (100%)	1 (20%)	nil	n = 5
	RK Khan	1 (100%)	nil	nil	nil	n = 1
Serratia species	King Edward	nil	2 (100%)	2 (100%)	1 (50%)	n = 2
	Addington	2 (40%)	4 (80%)	4 (80%)	1 (20%)	n = 5

3.5 Resistance mechanisms to the aminoglycoside antibiotics

Resistance mechanisms of the isolates in this study were assigned in three phases :-

- On the basis of phenotype demonstrated when tested with gentamicin, tobramycin, netilmicin and amikacin. This was applied to all isolates in the study.

- On the basis of phenotype demonstrated when tested with gentamycin, tobramycin, netilmicin, amikacin, kanamycin, neomycin, apramycin, fortimicin, 6'-N-ethyl-netilmicin, 2'-N-ethyl-netilmicin, isepamicin and 5-episisomicin. This means of assigning a mechanism was applied to approximately 50% of the isolates in the study.

- DNA/DNA hybridization techniques were employed to confirm or modify the mechanisms assigned in the latter group.

Correlation of the mechanisms assigned above was also carried out in stages :-

- Those mechanisms assigned on the basis of resistance to the initial four aminoglycosides were compared with mechanisms assigned according to relative resistance to the 12 aminoglycosides in the group above.

- Mechanisms assigned according to the 12 aminoglycoside antibiotics were compared with the genotypes detected in the DNA/DNA hybridization studies.

- Initial mechanisms assigned according to the resistance phenotypes demonstrated with gentamicin, tobramycin, netilmicin and amikacin were compared with the mechanisms assigned on the basis of relative resistance to the 12 aminoglycoside antibiotics in conjunction with DNA/DNA hybridization studies.

Final mechanisms were then assigned to all the isolates in the study.

3.5.1 Resistance mechanisms initially assigned on the basis of resistance to gentamicin, tobramycin, netilmicin and amikacin

The mechanisms assigned initially on all 95 isolates and five donor isolates and their respective transconjugants are reflected in Appendix B (pp 90-96).

3.5.2 Resistance mechanisms assigned by relative changes in resistance to gentamicin, tobramycin, netilmicin, amikacin, kanamycin, neomycin, apramycin, fortimicin, 6'-N-ethyl-netilmicin, 2'-N-ethyl-netilmicin, isepamicin and 5-epi-sisomicin in 38 isolates, five donor isolates and their respective transconjugants

The mechanisms assigned on the basis of relative changes in resistance to the 12 aminoglycoside antibiotics are shown in Appendix C (pp 97-99).

3.5.3 Resistance mechanisms assigned by relative changes in resistance to gentamicin, tobramycin, netilmicin, amikacin, kanamycin, neomycin, apramycin, fortimicin, 6'-N-ethyl-netilmicin, 2'-N-ethyl-netilmicin, isepamicin and 5-epi-sisomicin in conjunction with DNA/DNA hybridization tests in 38 isolates, five donor isolates and their respective transconjugants

Of the 38 randomly selected isolates, five donor isolates and their respective transconjugants 30 were assigned a single enzyme resistance mechanism, 11 were assigned two enzyme mechanisms, three were assigned one enzyme mechanism in combination with permeability resistance and four were assigned three enzyme mechanisms (Appendix D, pp 100-104).

The AAC(3)-V enzyme (modifying gentamicin, tobramycin and netilmicin) was assigned as a single aminoglycoside resistance mechanism in 28 isolates according to phenotype. All demonstrated the presence of the *aac(3)-Va* gene in hybridization tests.

The AAC(6')-1 enzyme (modifying tobramycin, netilmicin and amikacin) was assigned as a single aminoglycoside resistance mechanism in two isolates. One isolate demonstrated the presence of the *aac(6')-1b* gene and the other, identified as a *Serratia marcescens*, the *aac(6')-1c* gene.

Four isolates for which the AAC(3)-V plus AAC(6')-1 aminoglycoside enzyme resistance mechanisms were assigned demonstrated the presence only of the *aac(6')-1b* gene.

The *aac(6')-1a* gene was not detected in any of the isolates while the *aac(6')-1b* gene was present in 89% (n = 9) of isolates in combination with the AAC(3)-V enzyme according to phenotype. In all of these isolates the *aac(3)* gene was not demonstrated.

In *Escherichia coli isolates* negative probe results were produced in six of eight isolates (75%). In two of these six isolates the AAC(3)-V enzyme resistance mechanism together with permeability resistance had been assigned according to phenotype. In the remaining four the AAC(3)-V (modifying gentamicin, tobramycin and netilmicin) plus APH(3')-1 (modifying kanamycin and neomycin) enzymes had been assigned (Appendix D : Table D3, p 103).

In one *Klebsiella* and three *Enterobacter* isolates the triple combination of the AAC(3)-V, AAC(6')-1 and APH(3')-1 enzyme resistance mechanisms were assigned according to phenotype. In hybridization tests one of these isolates was positive only for the *aac(6')-1b* gene while another was positive for the *aac(3)-Va*, *aac(6')-1b* and *aac(6')-11b* genes. The remaining two isolates were both positive for the *aac(6')-1b* and *aac(6')-11b* genes (Appendix D : Tables D1 and D2, pp 101-103).

Both *Serratia marcescens* isolates on which DNA/DNA hybridization tests were performed demonstrated the chromosomal *aac(6')-1c* gene and one of these isolates also demonstrated permeability resistance according to phenotype (Appendix D : Table D4, p 104).

3.5.4 Correlation between initial resistance mechanisms assigned on the basis of resistance to gentamicin, tobramycin, netilmicin and amikacin and mechanisms proposed on the relative changes in resistance to gentamicin, tobramycin, netilmicin, amikacin, kanamycin, neomycin, apramycin, fortimicin, 6'-N ethyl-netilmicin, 2'-N-ethyl-netilmicin, isepamicin and 5-epi-sisomicin on 38 isolates, five donor isolates and their respective transconjugants

The results, tabulated in Appendix D (pp 100-104), are summarized in Table 9.

In 35 of the 38 isolates, five donor isolates and the five respective transconjugants (total = 48) the single or double enzyme mechanism initially assigned on the basis of resistance to gentamicin, tobramycin, netilmicin and amikacin agreed with those assigned on the basis of relative changes in resistance to the same four aminoglycoside antibiotics plus the additional eight aminoglycoside antibiotics.

In 46 of the 48 isolates at least one common enzyme mechanism was assigned in both instances.

Table 9 : Correlation between assigned mechanisms based on resistance to gentamicin, tobramycin, netilmicin and amikacin and mechanisms based on resistance to the full range of 12 aminoglycoside antibiotics

		Mechanisms assigned on the basis of resistance to gentamicin, tobramycin, netilmicin and amikacin				
		AAC(3)-V	AAC(6')-1	AAC(6')-1 and AAC(3)-V	ANT(2")-1	Total
Mechanisms assigned on the basis of changed resistance to: gentamicin tobramycin netelmicin amikacin kanamycin neomycin apramycin fortimicin 6'-N-ethyl-netilmicin 2'-N-ethyl-netilmicin isepamicin 5-epi-sisomicin	Concurred	27	2	4	0	33
	Changed to AAC(3)-V	-	0	0	1	1
	Concurred but APH(3')-1 in addition	7	0	2	0	9
	Concurred but APH(3')-1 and AAC(6')-1 in addition	2	0	0	0	2
	Concurred but permeability resistance in addition	0	1	0	0	1
	Changed to AAC(3)-V plus permeability resistance	-	0	1	1	2
	Total	36	3	7	2	48

The APH(3')-1 enzyme (modifying kanamycin and neomycin) which always occurred in combination with either AAC(3)-V (modifying gentamicin, tobramycin and netilmicin) or AAC(3)-V plus AAC(6')-1 (modifying tobramycin, netilmicin and amikacin) was additionally assigned in nine of the test isolates only on the basis of changes in resistance to the full range of 12 aminoglycoside antibiotics.

In one isolate the ANT(2")-1 enzyme (modifying gentamicin and tobramycin) which was assigned for two of the test isolates on the basis of resistance to the initially used four aminoglycoside antibiotics was changed to AAC(3)-V enzyme when tested with the full range of aminoglycoside antibiotics. The other initially assigned ANT(2")-1 enzyme resistance mechanism was changed to AAC(3)-V plus permeability.

When tested with the full range of aminoglycoside antibiotics the AAC(6')-1 and APH(3')-1 enzymes were assigned in addition to AAC(3)-V in two of the test isolates.

Permeability resistance (generalized) was assigned in addition to the initially assigned AAC(6')-1 enzyme mechanism in one test isolate after testing with the full range of aminoglycoside antibiotics.

One test isolate for which the AAC(6')-1 plus the AAC(3)-V enzymes were initially assigned on the basis of resistance to gentamicin, tobramycin, netilmicin and amikacin was changed to AAC(3)-V plus permeability resistance when tested with the full range of aminoglycoside antibiotics.

3.5.5 Correlation between resistance mechanisms assigned according to the relative changes in resistance to gentamicin, tobramycin, netilmicin, amikacin, kanamycin, neomycin, apramycin, fortimicin, 6'-N-ethyl-netilmicin, 2'-N-ethyl-netilmicin, isepamicin and 5-epi-sisomicin and resistance mechanisms determined in DNA/DNA hybridization tests on 38 isolates, five donor isolates and their respective transconjugants

The results, tabulated in Appendix D (pp 100-104), are summarized in Table 10.

In all single enzyme resistance mechanisms (n = 30) assigned according to phenotype it was confirmed that the *aac(3)-Va* gene encoded AAC(3)-V enzyme while the *aac(6')-1b* and/or *aac(6')-11b* encoded the AAC(6')-1 enzyme while in one *Serratia marcescens* isolate the *aac(6')-1c* gene (chromosomal) was present with an AAC(6')-1 phenotype.

In assigned combinations of AAC(3)-V plus AAC(6')-1 (n = 4) only the *aac(6')-1b* gene was demonstrated in hybridization tests.

Where a combination of APH(3')-1 plus AAC(3)-V enzyme resistance mechanisms were assigned according to phenotype (n = 7) the hybridization tests were negative.

Where a multiple enzyme resistance mechanism such as AAC(3)-V plus AAC(6')-1 plus APH(3')-1 the *aph(3')-1* gene was not demonstrated and in three of the four isolates for which this combination was assigned nor was the *aac(3)-Va* gene. The *aac(6')-1b* or *aac(6')-11b* gene was demonstrated in all four isolates.

Table 10 : Correlation between assigned mechanisms based on resistance to gentamicin, tobramycin, netilmicin. amikacin, kanamycin, neomycin, apramycin, fortimicin, 6'-N-ethyl-netilmicin, 2'-N-ethyl-netilmicin, isepamicin and 5-epi-sisomicin and mechanisms based on gene probe results

		Genotype demonstrated with gene probe						
		aac(3)-Va	*aac(6')-1b*	Negative	*aac(3)-Va* + *aac(6')-1b* + *aac(6')-11b*	*aac(6')-1b* + *aac(6')-11b*	*aac(6')-1c*	Total
Mechanisms assigned on the basis of changed resistance to gentamicin tobramycin netilmicin amikacin kanamycin neomycin apramycin fortimicin 6'-N-ethyl-netilmicin 2'-N-ethyl-netilmicin isepamicin 5-epi-sisomicin	AAC(3)-V	28						28
	AAC(6')-1		1				1	2
	AAC(3)-V + AAC(6')-1		4					4
	AAC(3)-V + APH(3')-1			7				7
	AAC(3)-V + AAC(6')-1 + APH(3')-1		1		1	2		4
	AAC(3)-V + permeability resistance			2				2
	AAC(6')-1 + permeability resistance						1	1
	Total	28	6	9	1	2	2	48

The assigned combination of the AAC(3)-V enzyme resistance mechanism with permeability resistance yeilded negative gene probe results in both *Escherichia coli* isolates. However, the assigned combination of the AAC(6')-1 enzyme with permeability resistance in the remaining *Serratia marcescens* isolate yeilded a positive gene probe result for the *aac(6')-1c* gene.

From the above it appeared that the APH(3')-1 enzyme could only be assigned according to phenotype. When the AAC(3)-V enzyme resistance mechanism occurred in combination with one or both of the AAC(6')-1 and APH(3')-1 enzymes or with permeability resistance the *aac(3)-Va* gene was not usually detected in hybridization tests.

In contrast to this observation the genes encoding the AAC(6')-1 enzyme, assigned according to phenotype, were detected in all combinations of resistance mechanisms.

3.5.6 Correlation between initial resistance mechanisms assigned on the basis of resistance to gentamicin, tobramycin, netilmicin and amikacin and resistance mechanisms assigned on the basis of the relative changes in resistance to gentamicin, tobramycin, netilmicin, amikacin, kanamycin, neomycin, apramycin, fortimicin, 6'-netilmicin, 2'-netilmicin, isepamicin and 5-epi-sisomicin in conjunction with DNA/DNA hybridization tests in 38 isolates, five donor isolates and their respective transconjugants

In the 30 isolates ultimately assigned a single mechanism considering changed resistance to the full range of 12 aminoglycoside antibiotics in conjunction with DNA/DNA hybridization tests the same enzyme was initially assigned in 29 of these isolates on the basis of susceptibility to gentamicin, tobramycin, netilmicin and amikacin (Appendix D, pp 100-104).

In ten of the 14 isolates which were ultimately assigned two resistance mechanisms (enzymes and/or permeability resistance) one of the two was initially assigned on the basis of susceptibility to gentamicin, tobramycin, netelmicin and amikacin. In four isolates both enzymes were proposed initially.

In each of the four isolates ultimately assigned three resistance mechanisms at least one of the enzymes implicated was assigned initially considering resistance to gentamicin, tobramycin, netilmicin and amikacin.

Final mechanisms assigned generally agreed with the initially assigned mechanisms. However, permeability resistance (generalized resistance to aminoglycoside antibiotics) and all APH(3')-1 assigned mechanisms (modifying only kanamycin and neomycin) which were found in combination with other enzymes could not be distinguished on the basis of resistance to gentamicin, tobramycin, netilmicin and amikacin (Table 9).

Given the above it was assumed that the assigned mechanisms for each of the remaining 57 isolates would at least correlate with one of the actual enzyme mechanisms present.

3.5.7 **Final assigned resistance mechanisms for all isolates**

With reference to Appendix E (pp 105-110), the most frequent mechanisms of resistance in all isolates are reflected in Table 11. The AAC(3)-V enzyme resistance mechanism was the most frequent followed by AAC(6')-1, both occurring singly. Permeability resistance occurred in three isolates in combination with the AAC(3)-V or AAC(6')-1 enzyme. Combinations of enzyme resistance mechanisms occurred in 18% of the isolates and in all genera. This included four isolates demonstrating the presence of three enzyme resistance mechanisms. The most frequent combinations of enzyme resistance mechanisms (Table 11) in which the AAC(3)-V and AAC(6')-1 enzymes were present occurred in isolates resistant to gentamicin, tobramycin and netilmicin and with varying resistance to amikacin (isolates 6, 9, 28, 29, 68, 12, 58, 61, 3, 26 and 52 in Appendix A : Tables A1, pp 81-84).

In isolates assigned the AAC(6')-1 enzyme resistance mechanism as a single mechanism (Appendix E, pp 105-110) all were susceptible to gentamicin, two demonstrated intermediate resistance to tobramycin and the remaining 20 were susceptible to tobramycin. Of the 22 isolates two were susceptible to netilmicin and two demonstrated intermediate resistance while the remaining 18 were resistant. Seven of the isolates were susceptible to amikacin while seven demonstrated intermediate resistance and eight were resistant.

An interesting observation in this study was the lack of the occurrence of the ANT(2")-1 enzyme (affecting gentamicin and tobramycin and not netilmicin) in any of the resistant isolates. The mechanisms assigned initially on the basis of resistance to gentamicin, tobramycin, netilmicin and amikacin included only two isolates for which the ANT(2")-1 enzyme was proposed. Both isolates were included in the 38 random isolates, five donor isolates and their respective

transconjugants for which resistance to the wider range of aminoglycoside antibiotics was tested in combination with hybridization tests. When tested with 6'-N-ethyl-netilmicin and 2'-N-ethyl-netilmicin both isolates showed changes in resistance, although these changes were reduced in comparison with 42 of the 43 test isolates (Isolates 41 and 57 in Appendix C : Table C1, pp 98-99). On this basis the AAC(3)-V enzyme was then proposed and confirmed in one isolate by gene probe hybridization tests (Appendix D : Tables D1 and D3, pp 101 and 103). None of the 43 isolates hybridized with the *ant(2")-1a* gene probe used (Appendix D, pp 100-104).

Table 11 : Frequency of resistance mechanisms in all isolates

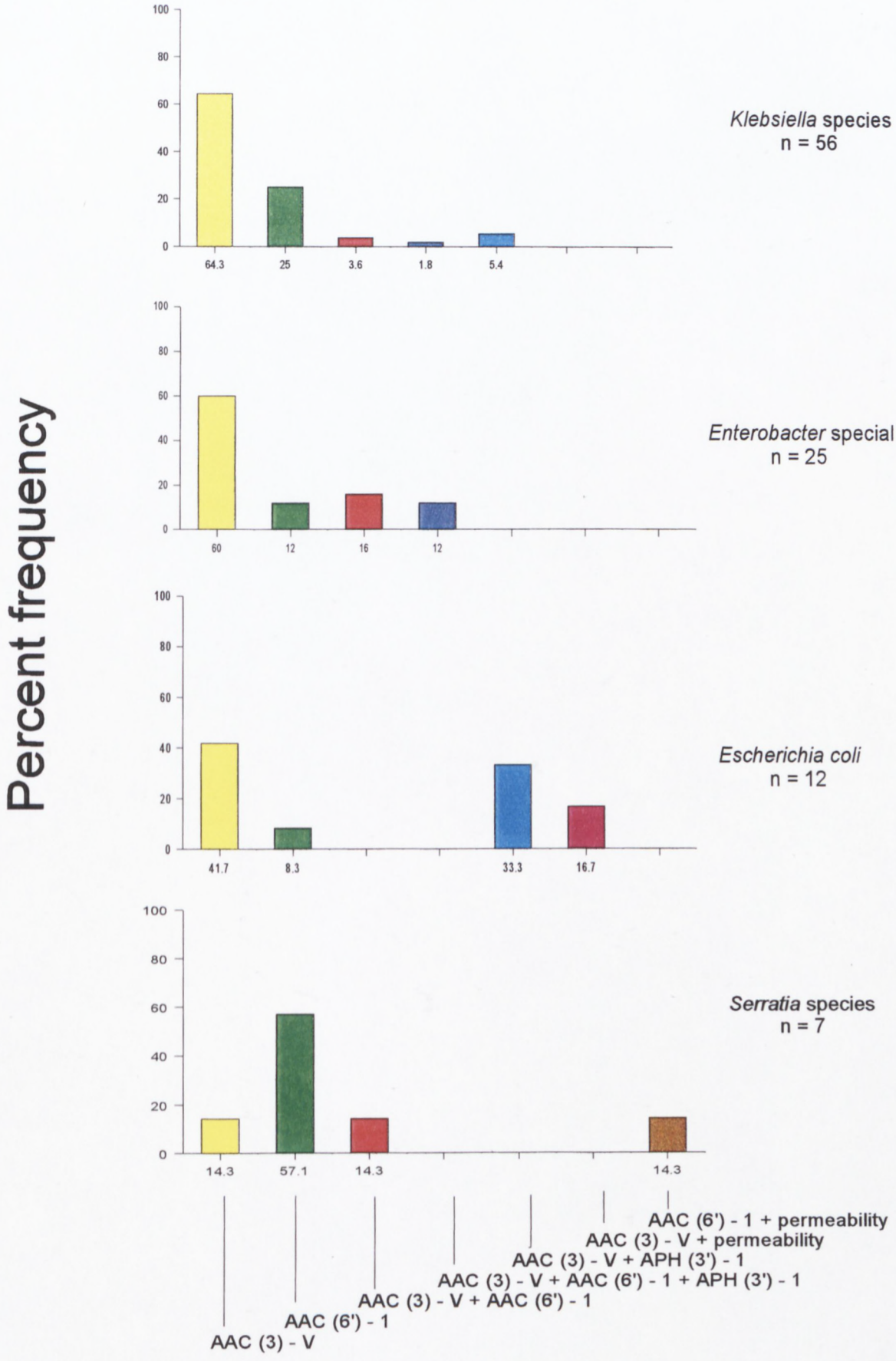
MECHANISM	No./%
AAC(3)-V	57
AAC(6')-1	22
AAC(3)-V + AAC(6')-1	7
AAC(3)-V + APH(3')-1	7
AAC(3)-V + AAC(6')-1 + APH(3')-1	4
AAC(3)-V + permeability	2
AAC(6')-1 + permeability	1
TOTAL	100

Figure 5 reflects the frequency of resistance mechanisms in the species of bacteria in this study.

In *Klebsiella* species, *Enterobacter* species and *Escherichia coli* the most common enzyme resistance mechanism was the AAC(3)-V enzyme (modifying gentamicin, tobramycin and netilmicin) occurring singly. In *Serratia* species the AAC(6')-1 enzyme (modifying tobramycin, netilmicin and amikacin), as a single mechanism, occurred most frequently. Both enzymes occurred in all the hospitals and in all four genera of bacteria as single mechanisms and were also found in various combinations.

The APH(3')-1 enzyme resistance mechanism (modifying kanamycin and neomycin) occurred in combination with AAC(6')-1 and/or AAC(3)-V in *Klebsiella* species, *Enterobacter* species and *Escherichia coli*.

Figure 5: Resistance mechanisms in all isolates



Permeability resistance (generalized resistance to aminoglycoside antibiotics) was noted only in *Escherichia coli* in combination with the AAC(3)-V enzyme and in *Serratia* species in combination with AAC(6')-1 enzyme.

Figure 6 reflects the frequency of resistance mechanisms in *Klebsiella* species in the different hospitals.

All 31 *Klebsiella* species obtained from King Edward Hospital possessed the AAC(3)-V enzyme (modifying gentamicin, tobramycin and netilmicin) as a single mechanism. The other hospitals demonstrated a wider range of resistance mechanisms although the AAC(6')-1 enzyme (modifying tobramycin, netilmicin and amikacin) was predominant at Addington hospital, occurring in 63.1% of *Klebsiella* isolates (n = 19).

Combinations of AAC(3)-V and AAC(6')-1 were found in isolates from Addington Hospital and Wentworth Hospital while APH(3')-1 (modifying kanamycin and neomycin) in combination with with AAC(6')-1 and/or AAC(3)-V occurred in isolates from Addington Hospital and RK Khan Hospital.

Figure 6: Comparative resistance mechanisms of *Klebsiella* species in hospitals surveyed

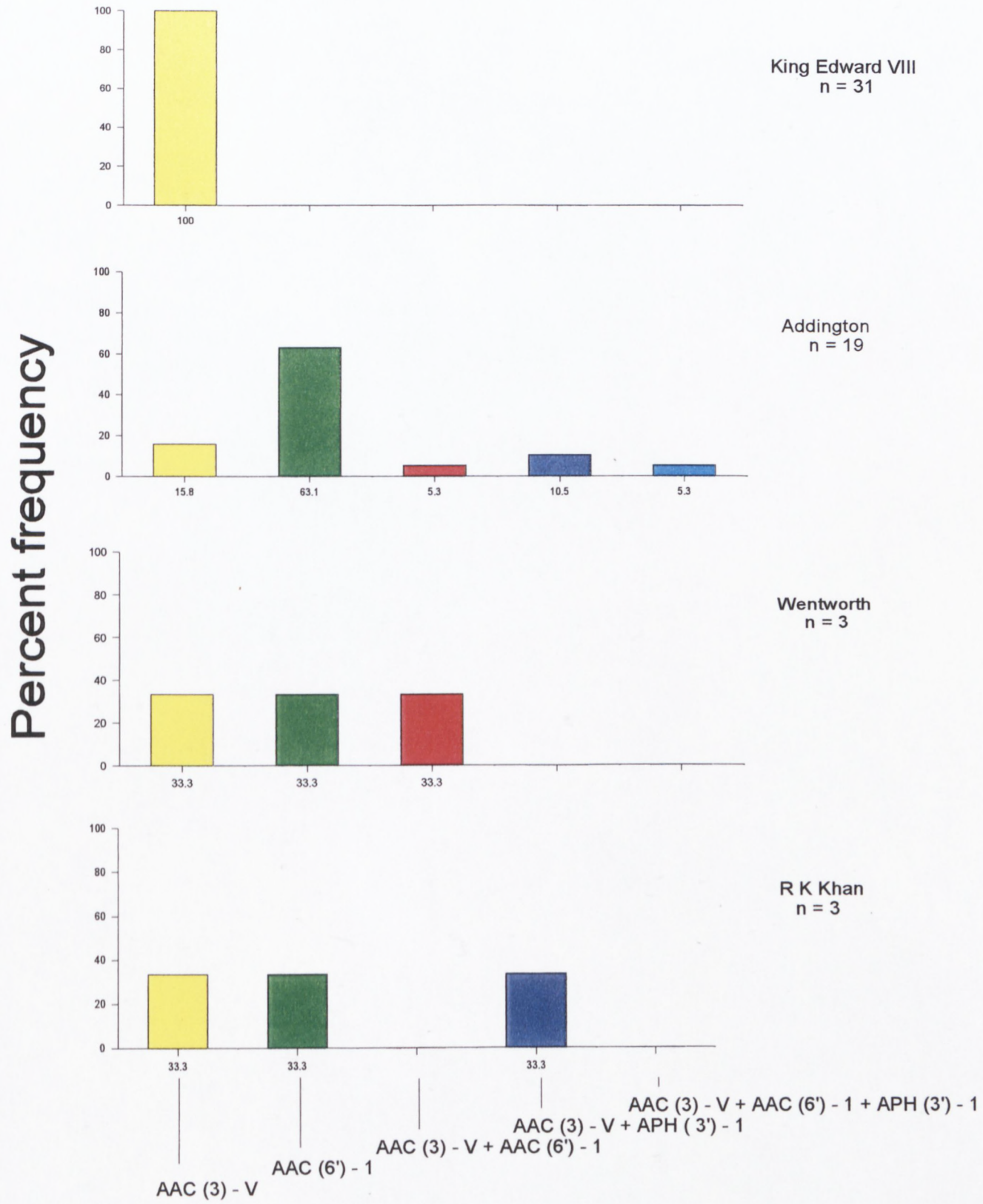


Figure 7 reflects the frequency of resistance mechanisms in *Enterobacter* species in the different hospitals.

AAC(3)-V (modifying gentamicin, tobramycin and netilmicin) was the most commonly occurring single enzyme mechanism in three of four hospitals among *Enterobacter* species (n = 25).

AAC(6')-1 (modifying tobramycin, netilmicin and amikacin), as a single mechanism or in combination with AAC(3)-V, was found in isolates from Addington Hospital and Wentworth Hospital.

APH(3')-1 enzyme (modifying kanamycin and neomycin) in combination with AAC(3)-V and AAC(6')-1 occurred in isolates from both Addington Hospital and King Edward Hospital.

Figure 7: Comparative resistance mechanisms of *Enterobacter* species in hospitals surveyed

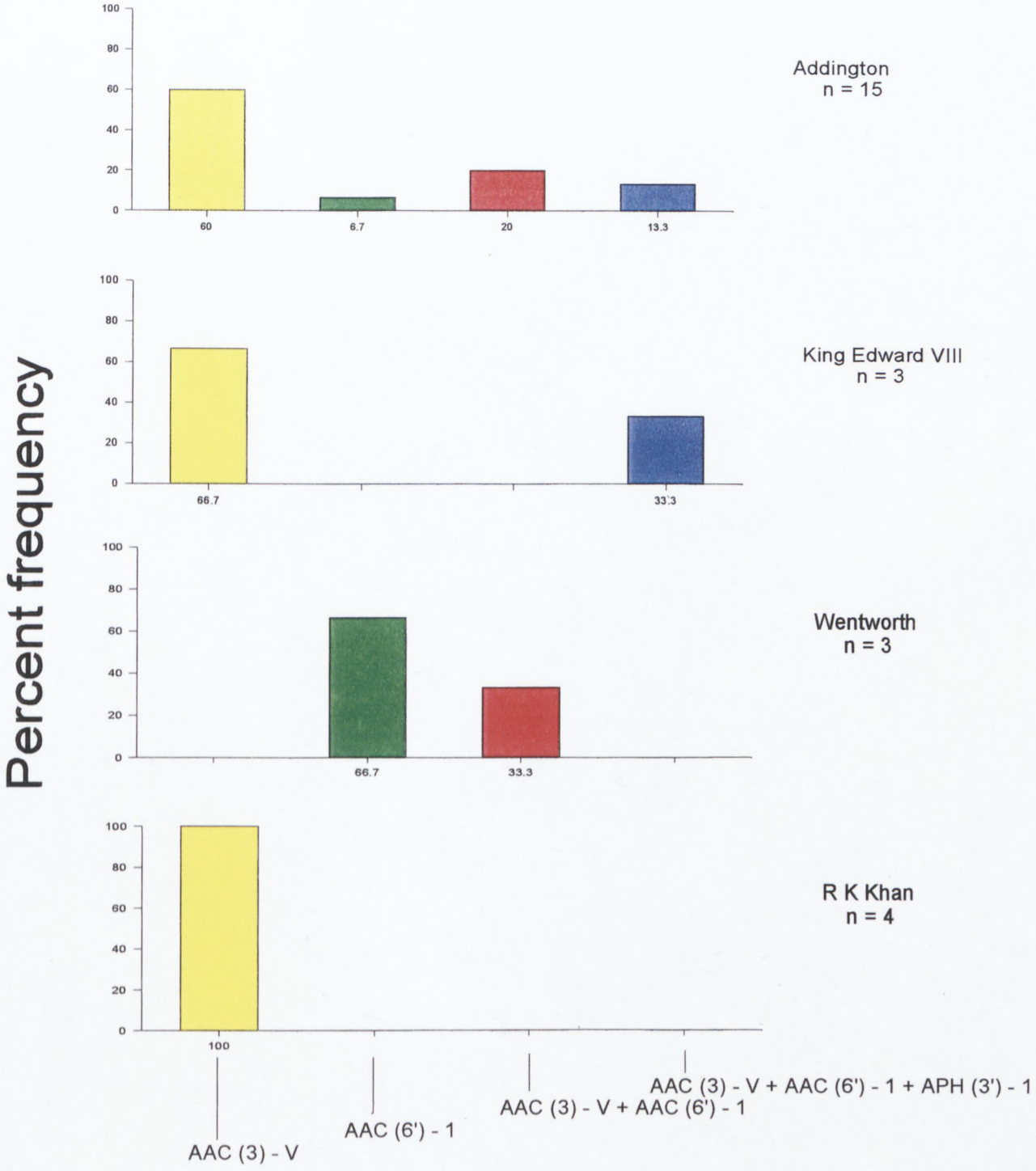


Figure 8 represents the frequency of resistance mechanisms in *Escherichia coli* in the different hospitals.

AAC(3)-V (modifying gentamicin, tobramycin and netilmicin) in combination with APH(3')-1 (modifying kanamycin, neomycin) was the most frequently occurring enzyme resistance mechanism (66.6%) in *Escherichia coli* isolates (n = 6) obtained from King Edward Hospital. This combination was absent in isolates obtained from Addington Hospital where 60% of isolates (n = 5) possessed AAC(3)-V.

Permeability resistance (generalized resistance) in combination with the AAC(3)-V enzyme mechanism occurred in isolates from Addington Hospital and RK Khan Hospital.

Figure 8: Comparative resistance mechanisms of *Escherichia coli* in hospitals surveyed

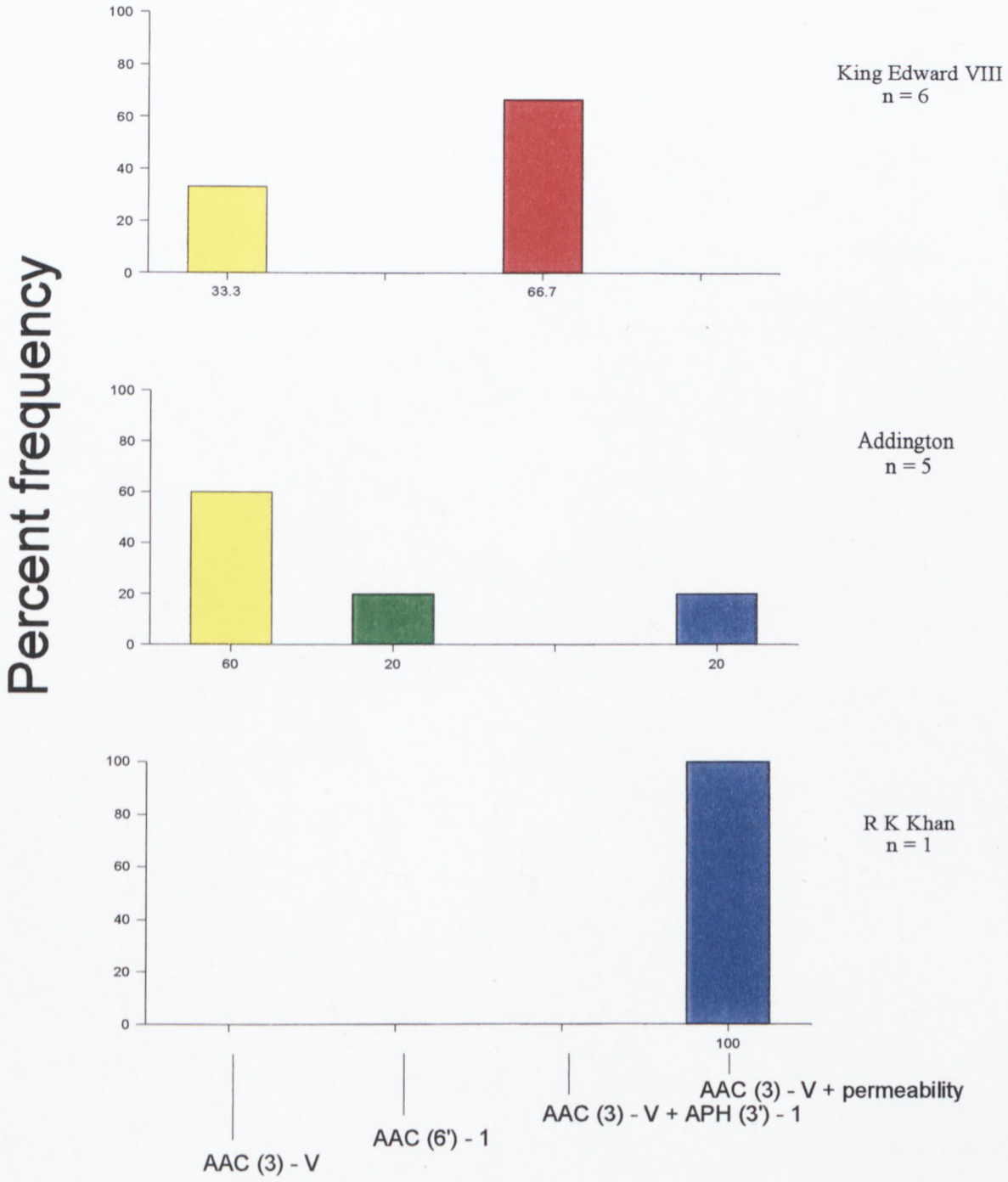
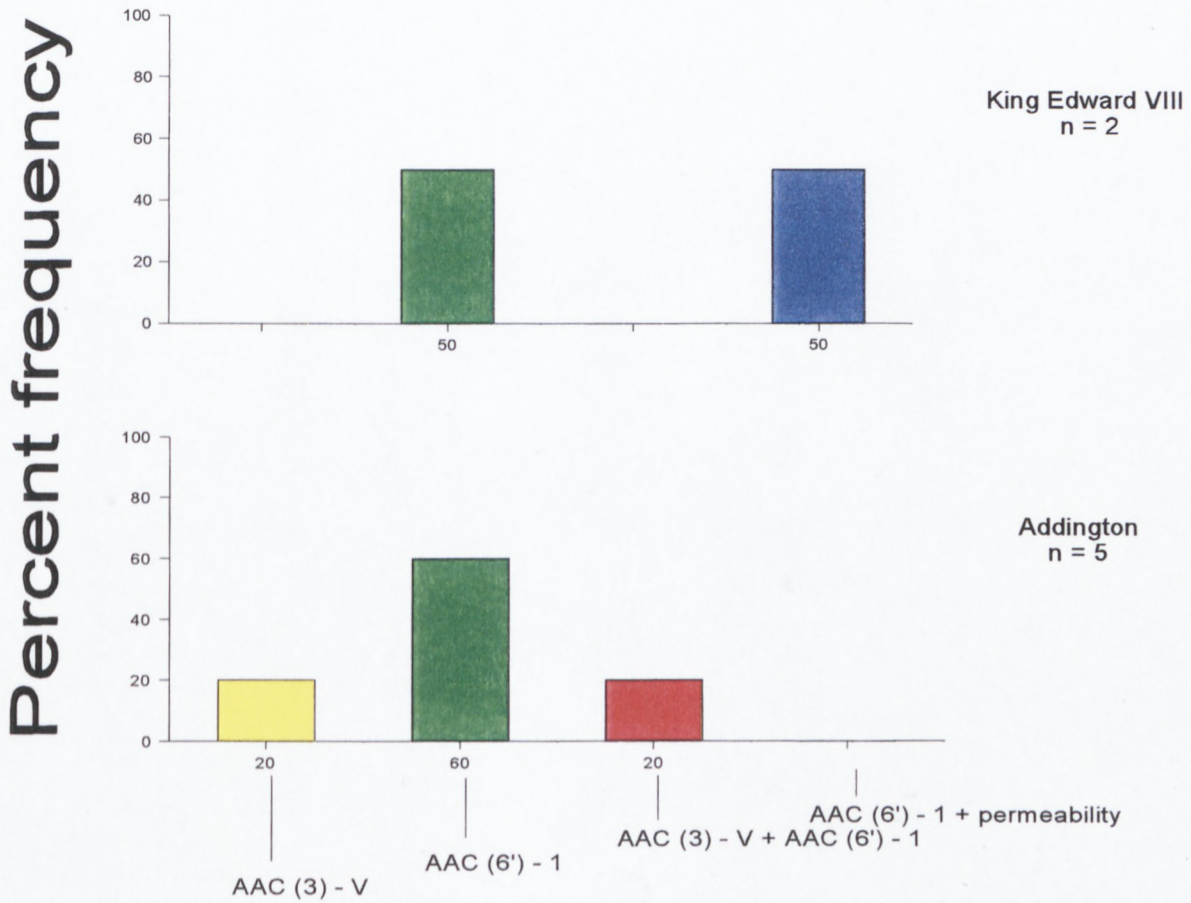


Figure 9 represents the frequency of resistance mechanisms in seven *Serratia* species isolates in the different hospitals.

The AAC(6')-1 enzyme resistance mechanism (modifying tobramycin, netilmicin and amikacin), occurring singly, was the most frequent mechanism in *Serratia* species at King Edward Hospital and Addington Hospital from which all isolates were obtained.

The AAC(6')-1 enzyme also occurred in combination with AAC(3)-V (modifying gentamicin, tobramycin and netilmicin) and in combination with permeability resistance (generalized resistance).

Figure 9: Comparative resistance mechanisms of *Serratia* species in hospitals surveyed

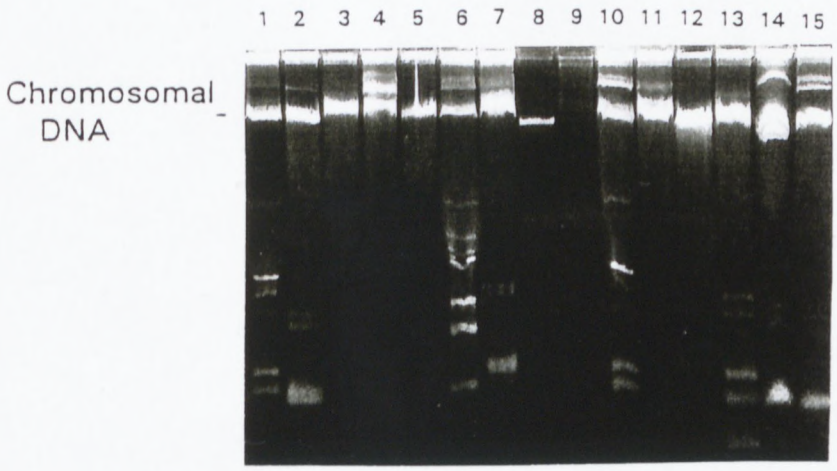


3.6 **Plasmid profile analysis**

In uncleaved plasmid DNA analysis 10 of 15 *Klebsiella pneumoniae* isolates appeared to contain one or more plasmids but none of the profiles were common (Figure 10). Isolates 6, 7, 39, 40, 51, 65, 74 and 79 (lanes 1, 2, 6, 7, 10, 13, 14 and 15) demonstrated plasmid DNA bands of both high molecular weight (between the wells and the chromosomal DNA) and low molecular weight. Isolates 11 and 56 (lanes 4 and 11) appeared to contain plasmids of high molecular weight. Isolates 9, 35 and 61 (lanes 3, 5 and 12) did not contain detectable plasmid DNA while it appeared that DNA might have been lost as a result of technical problems in isolates 41 and 42 (lanes 8 and 9).

Figure 10 : Uncleaved plasmid DNA analysis of *Klebsiella pneumoniae*

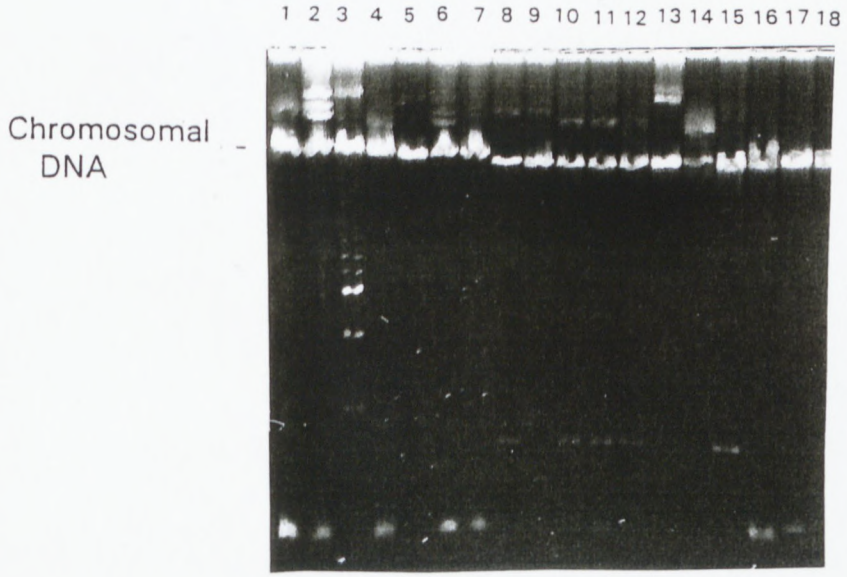
1, Isolate 6 (Addington 3B); 2, Isolate 7 (Addington Surgical); 3, Isolate 9 (Wentworth OPD); 4, Isolate 11 (Wentworth Neuro); 5, Isolate 35 (Addington OPD); 6, Isolate 39 (King Edward); 7, Isolate 40 (King Edward); 8, Isolate 41 (King Edward ICU); 9, Isolate 42 (King Edward Respiratory Unit); 10, Isolate 51 (Addington 5B); 11, Isolate 56 (RK Khan); 12, Isolate 61 (Addington); 13, Isolate 65 (RK Khan); 14, Isolate 74 (King Edward ICU); 15, Isolate 79 (King Edward ICU).



In uncleaved plasmid DNA of *Klebsiella pneumoniae* isolates 16 of the 18 isolates appeared to contain plasmids (Figure 11). Large plasmids were evident in isolates 80,82,83,84,86,89,95,96,97,100 and 102 (lanes 1,2,3,4,6,8,9,10,11,13 and 14). Isolates 80,82,84,86,88,115 and 116 (lanes 1,2,4,6,7,16 and 17) appeared to contain a common plasmid of low molecular weight. Isolates 89,96,97,98 and 100 (lanes 8,10,11,12 and 15) also demonstrated a common plasmid band. Isolates 96 and 97 (lanes 10 and 11) appeared to have identical profiles.

Figure 11 : Uncleaved plasmid DNA analysis of *Klebsiella pneumoniae*

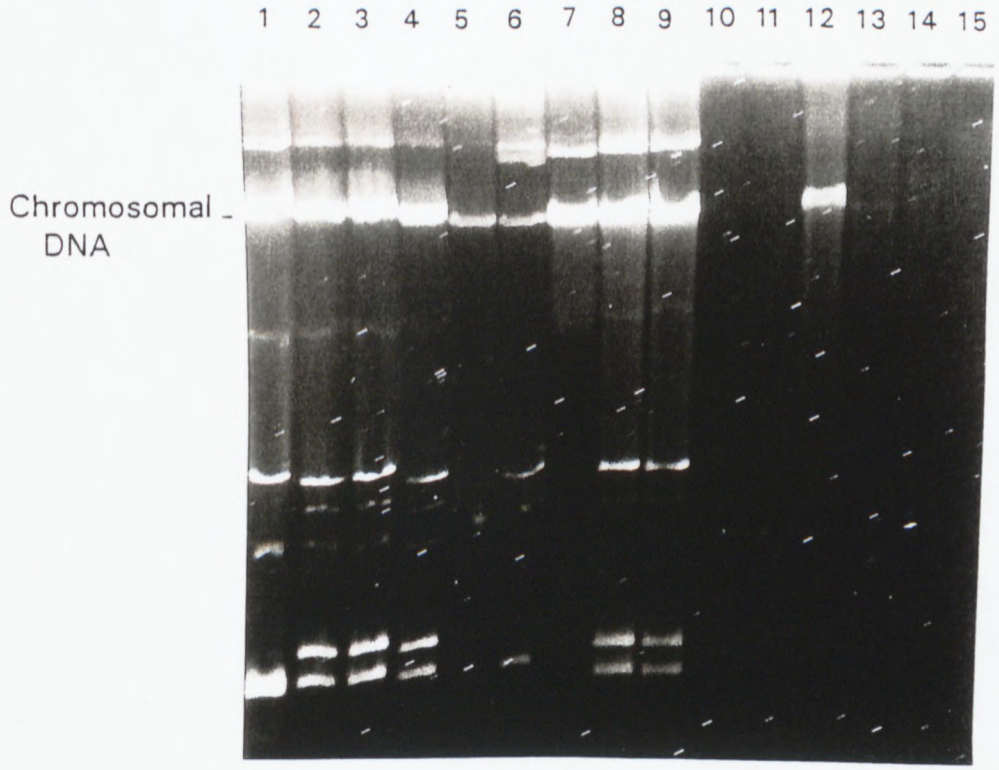
1, Isolate 80 (King Edward ICU); 2, Isolate 82 (King Edward Paediatrics); 3, Isolate 83 (King Edward Paediatrics); 4, Isolate 84 (King Edward ICU); 5, Isolate 85 (King Edward ICU); 6, Isolate 86 (King Edward ICU); 7, Isolate 88 (King Edward ICU); 8, Isolate 89 (King Edward ICU); 9, Isolate 95 (King Edward Paediatrics); 10, Isolate 96 (King Edward Surgical); 11, Isolate 97 (King Edward); 12, Isolate 98 (King Edward Surgical); 13, Isolate 100 (King Edward Surgical); 14, Isolate 102 (King Edward D2); 15, Isolate 112 (King Edward Paediatrics); 16, Isolate 115 (King Edward Paediatric OPD); 17, Isolate 116 (King Edward Paediatric OPD); 18, Isolate 117 (King Edward Paediatric OPD).



In Figure 12 large plasmids were evident in the *Klebsiella pneumoniae* isolates 14, 32, 33, 45, 48, 55, 59 and 60 (lanes 1, 2, 3, 4, 6, 7, 8 and 9). Isolates 32, 33, 45, 59 and 60 (lanes 2,3,4,8 and 9) appeared to have identical profiles. The profile of isolate 14 differed only in that it lacked a single plasmid band of low molecular weight while isolate 48 (lane 6), although similar, lacked two plasmid bands. Plasmid DNA was not detected in the *Serratia marcescens* isolate 43 (lane 12). The absence of DNA in the *Serratia marcescens* isolates 1, 17 and 67 (lanes 10, 11 and 13), the *Serratia liquefaciens* isolate 18 (lane 14) and the *Enterobacter cloacae* isolate 13 (lane 15) may have been due to technical problems.

Figure 12 : Uncleaved plasmid DNA analysis of *Klebsiella pneumoniae*, *Serratia marcescens*, *Serratia liquefaciens* and *Enterobacter cloacae*.

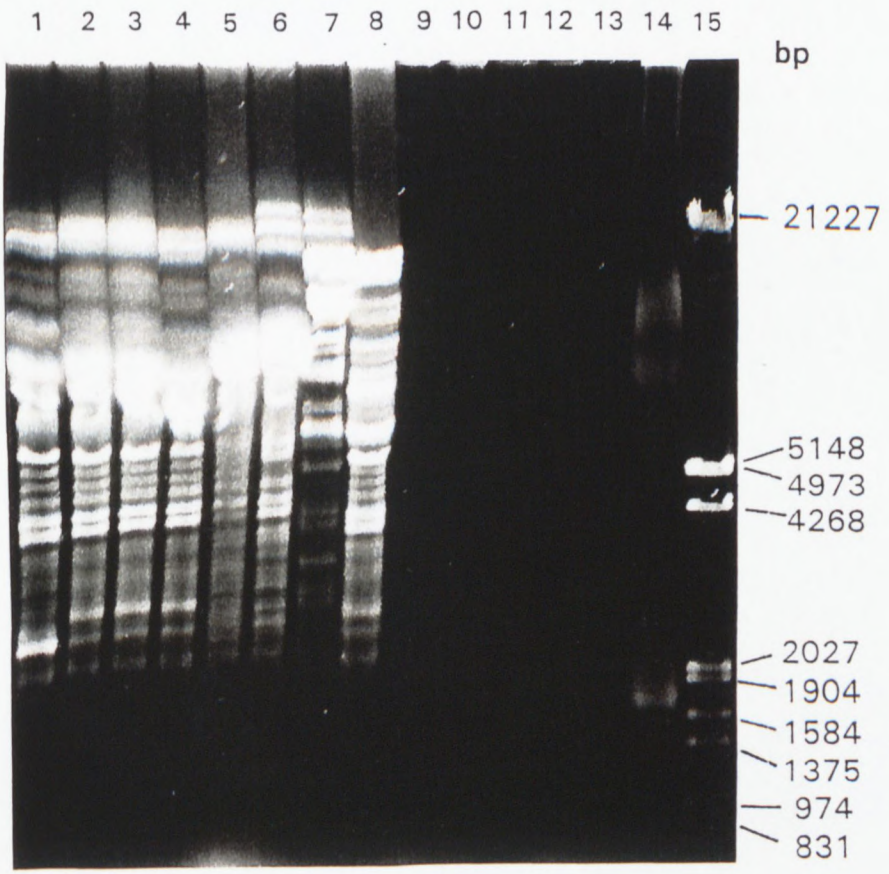
1, Isolate 14 - *K.pneumoniae* (Wentworth Neuro.); 2, Isolate 32 - *K.pneumoniae* (Addington 5B); 3, Isolate 33 - *K.pneumoniae* (Addington 5B); 4, Isolate 45 - *K.pneumoniae* (Addington 3B); 5, Isolate 47 - *K.pneumoniae* (Addington 3B); 6, Isolate 48 - *K.pneumoniae* (Addington HDU); 7, Isolate 55 - *K.pneumoniae* (RK Khan); 8, Isolate 59 - *K.pneumoniae* (Addington 5B); 9, Isolate 60 - *K.pneumoniae* (Addington 5B); 10, Isolate 1 - *S.marcescens* (Addington Burns Unit); 11, Isolate 17 - *S.marcescens* (King Edward ICU); 12, Isolate 43 - *S.marcescens* (Addington 6A); 13, Isolate 67 - *S.marcescens* (Addington 11B); 14, Isolate 18 - *S.liquefaciens* (King Edward ICU); 15, Isolate 13 - *E.cloacae* (Wentworth Thoracic Surgery).



When cleaved with *Eco*R1 restriction endonuclease the *Klebsiella pneumoniae* isolates 14, 32, 33, 45 and 59 (Lanes 1, 2, 3, 4 and 8) were identical in profile (Figure 13). With regard to isolate 60 (lane 9) it appeared that the plasmid DNA was lost either during storage or in the procedure using restriction endonucleases.

Figure 13 : Plasmid DNA analysis of *Klebsiella pneumoniae*, *Serratia marcescens* and *Enterobacter cloacae* cleaved with *Eco*R1 restriction endonuclease and lambda phage molecular weight marker cleaved with *Hind*III and *Eco*R1 restriction endonucleases

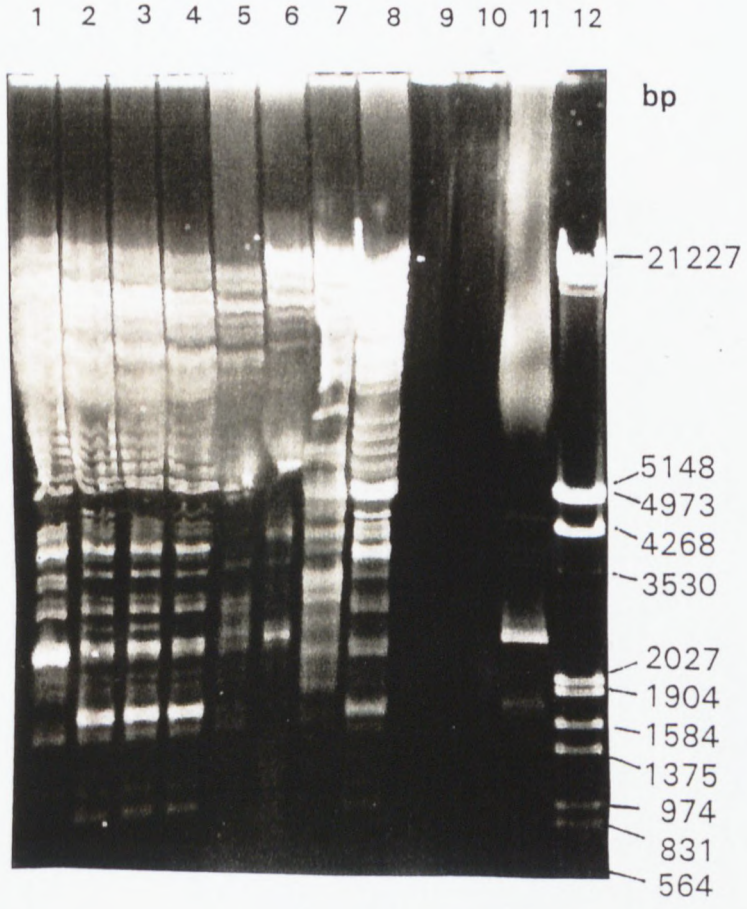
1, Isolate 14 - *K.pneumoniae* (Wentworth Neuro.); 2, Isolate 32 - *K.pneumoniae* (Addington 5B); 3, Isolate 33 - *K.pneumoniae* (Addington 5B); 4, Isolate 45 - *K.pneumoniae* (Addington 3B); 5, Isolate 47 - *K.pneumoniae* (Addington 3B); 6, Isolate 48 - *K.pneumoniae* (Addington HDU); 7, Isolate 55 - *K.pneumoniae* (RK Khan); 8, Isolate 59 - *K.pneumoniae* (Addington 5B); 9, Isolate 60 - *K.pneumoniae* (Addington 5B); 10, Isolate 1 - *S.marcescens* (Addington Burns Unit); 11, Isolate 43 - *S.marcescens* (Addington 6A); 12, Isolate 67 - *S.marcescens* (Addington 11B); 13, Isolate 18 - *S.liquefaciens* (King Edward ICU); 14, Isolate 13 - *E.cloacae* (Wentworth Thoracic Surgery); 15, Lambda phage molecular weight marker; the sizes (base pairs) of the fragments are indicated on the right.



The *Klebsiella pneumoniae* isolates 32,33,45 and 59 were similar in profile when cleaved with *Hind*111 restriction endonuclease (Lanes 2,3,4 and 8 in Figure 14) differing only in that isolates 45 and 59 (lanes 4 and 8) lacked a band of restricted DNA of approximately 3000 bp. Isolate 14 (lane 1) differed in that it lacked a number of bands of DNA fragments. In isolate 60 (lane 9) the same test plasmid DNA was used as that in the gel electrophoresis using the *Eco*R1 endonuclease (Figure 13) which also yeilded an absence of DNA fragments.

Figure 14 : Plasmid DNA analysis of *Klebsiella pneumoniae,* Serratia marcescens, *Serratia liquefaciens* and *Enterobacter cloacae* cleaved with *Hind*III restriction endonuclease and lambda phage molecular weight marker cleaved with *Hind*III and *Eco*R1 restriction endonucleases

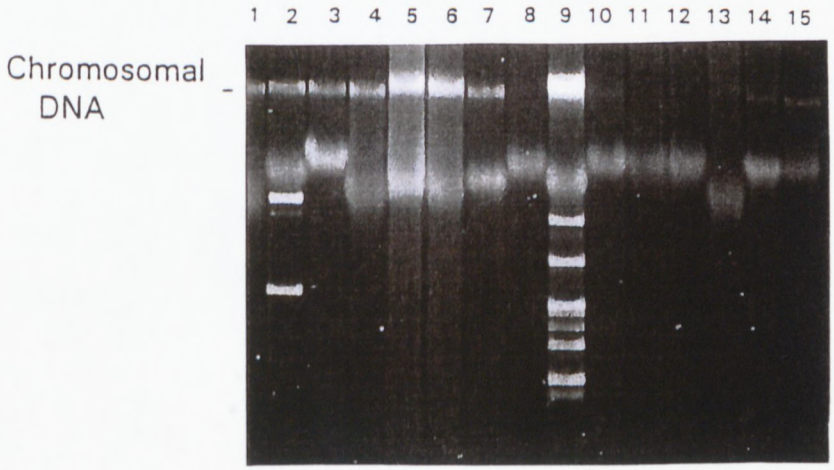
1, Isolate 14 - *K.pneumoniae* (Wentworth Neuro.); 2, Isolate 32 - *K.pneumoniae* (Addington 5B); 3, Isolate 33 - *K.pneumoniae* (Addington 5B); 4, Isolate 45 - *K.pneumoniae* (Addington 3B); 5, Isolate 47 - *K.pneumoniae* (Addington 3B); 6, Isolate 48 - *K.pneumoniae* (Addington HDU); 7, Isolate 55 - *K.pneumoniae* (RK Khan); 8, Isolate 59 - *K.pneumoniae* (Addington 5B); 9, Isolate 60 - *K.pneumoniae* (Addington 5B); 10, Isolate 18 - *S.liquefaciens* (King Edward ICU); 11, Isolate 13 - *E.cloacae* (Wentworth Thoracic Surgery); 12, Lambda phage molecular weight marker; the sizes (base pairs) of the fragments are indicated on the right.



Most of the *Enterobacter cloacae* isolates, with the exception of isolates 5 and 52 (lanes 2 and 9), did not contain detectable plasmid DNA (Figure 15).

Figure 15 : Uncleaved plasmid DNA analysis of *Enterobacter cloacae*

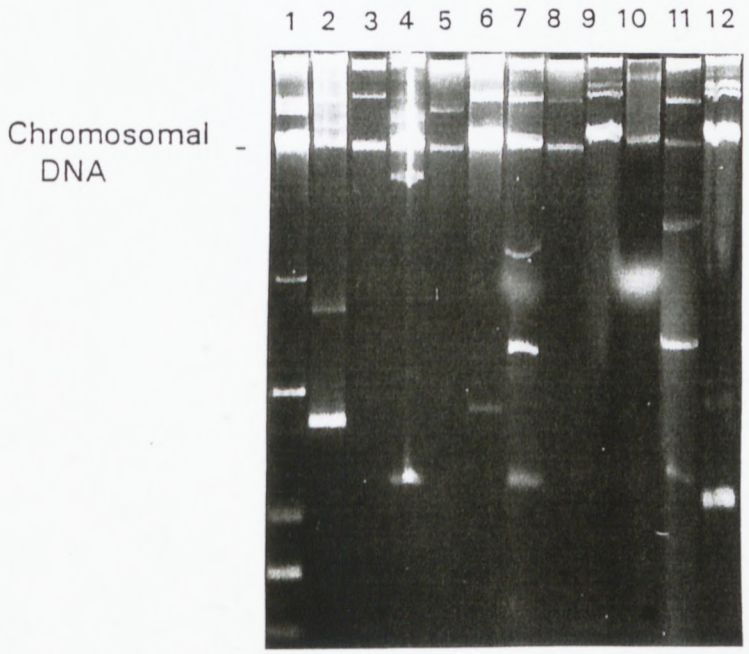
1, Isolate 3 (Addington 6A); 2, Isolate 5 (Addington 3B); 3, Isolate 20 (King Edward ICU); 4, Isolate 26 (Addington 14A); 5, Isolate 28 (Addington 2A); 6, Isolate 29 (Addington 11A); 7, Isolate 36 (Addington 13A); 8, Isolate 38 (Addington), 9, Isolate 52 (King Edward); 10, Isolate 53 (RK Khan); 11, Isolate 54 (RK Khan); 12, Isolate 63 (RK Khan); 13, Isolate 64 (RK Khan); 14, Isolate 66 (Addington); 15, Isolate 69 (Addington 2A).



In Figure 16 all the *Escherichia coli* isolates (lanes 1-9) contained large plasmids and some contained smaller plasmids. However, none of the profiles were common. The three *Enterobacter* species (lanes 10, 11 and 12) appeared to contain plasmid DNA but their profiles were not similar.

Figure 16 : Uncleaved plasmid DNA analysis of *Escherichia coli*, *Enterobacter cloacae* and *Enterobacter aerogenes*

1, Isolate 23 - *E.coli* (Addington OPD); 2, Isolate 30 - *E.coli* (Addington 2B); 3, Isolate 50 - *E.coli* (Addington 12B); 4, Isolate 57 - *E.coli* (RK Khan); 5, Isolate 93 - *E.coli* (King Edward Gynae); 6, Isolate 103 - *E.coli* (King Edward Paediatric); 7, Isolate 108 - *E.coli* (King Edward M1B); 8, Isolate 110 - *E.coli* (King Edward Paediatric); 9, Isolate 113 - *E.coli* (King Edward Paediatric OPD); 10, Isolate 24 - *E.cloacae* (Addington 3A); 11, Isolate 68 - *E.cloacae* (Addington 5C); 12, Isolate 90 - *E.aerogenes* (King Edward ICU).



3.7 **Summary of plasmid profile analysis of isolates**

Plasmid DNA extraction was performed on all isolates. In 75% of the isolates (n = 100) plasmid DNA was detected. In certain of the isolates the presence of plasmid DNA was not evident.

Of the 75 isolates on which electrophoresis was performed DNA was not evident in seven isolates. This may have occurred as a result of the loss of DNA after the extraction procedure and during storage or electrophoresis.

Similarities in plasmid profile were noted in a number of *Klebsiella pneumoniae* isolates while *Enterobacter* species were not similar in profile and the *Escherichia coli* and *Serratia* species either demonstrated no detectable DNA or showed no similarities in plasmid profile.

Klebsiella pneumoniae isolates 32, 33, 45, 59 and 60 were obtained from Addington Hospital. All demonstrated identical plasmid profiles without the use of restriction endonucleases (Figure 12) and when cleaved with *Eco*R1 restriction endonuclease isolates 32, 33, 45 and 59 (Figure 13) were identical in profile. The latter four isolates were similar in profile when cleaved with *Hind*111 restriction endonuclease (Figure 14), differing only in that isolates 45 and 59 appeared to lack a band of plasmid DNA. Plasmid DNA was lost during the processing of isolate 60 after the extraction procedure. It was not possible at a later stage to recover the isolate in order to repeat the process.

The *Klebsiella pneumoniae* isolates 96 and 97 from King Edward V111 Hospital which appeared to have identical plasmid profiles (Figure 11) were found to be from the same patient on retrospective investigation.

3.8 Collation of results of *Klebsiella pneumoniae* isolates demonstrating similar or identical characteristics

Isolates 14, 32, 33, 45, 47, 48, 55, 59 and 60 had many characteristics in common which are reflected in Table 12.

All isolates were susceptible to gentamicin and resistant to tobramycin and were assigned the AAC(6')-1 enzyme resistance mechanism.

Isolates 32 and 33 were identical in uncleaved plasmid DNA profiles and in plasmid DNA profiles when cleaved with both *Eco*R1 and *Hind*III restriction endonucleases. They differed only in antibiotic resistance profile in that isolate 33 was resistant to netilmicin while isolate 32 demonstrated intermediate resistance. Isolate 60 was identical to both isolate 32 and 33 in uncleaved plasmid DNA profile but was susceptible to amikacin. Isolates 45 and 59 differed slightly from isolates 32 and 33 in plasmid DNA profile when cleaved with *Hind*III restriction endonuclease. Both isolates 45 and 59 demonstrated intermediate resistance to amikacin.

It was noted that isolates 32, 33 and 60 were obtained from patients in the same unit at Addington Hospital while isolates 45 and 59 were obtained from patients in another unit. Specimens from which isolates 32 and 33 were obtained were received in the laboratory on the same day but it was not possible to establish patient identities (retrospective investigation). The specimen from which isolate 60 was obtained was received in the laboratory three months later. The specimens from which isolates 45 and 59 were obtained were received three weeks apart and were from different patients (retrospective investigation).

Table 12 : *Klebsiella pneumoniae* isolates demonstrating similar or identical characteristics

Isolate	14	32	33	45	47	48	55	59	60
Hospital	Went	Add	Add	Add	Add	Add	RKK	Add	Add
Unit		5B	5B	3B	3B	HDU		5B	5B
Patient	1	?	?	2	?	?	3	4	?
Date of collection	27/1	20/2	20/2	7/4	7/4	7/4	12/5	17/5	17/5
Antibiotic profile									
Genta	S	S	S	S	S	S	S	S	S
Tobra	R	R	R	R	R	R	R	R	R
Netil	R	I	R	R	R	R	R	R	R
Amik	R	I	I	I	R	R	S	I	S
AAC(6')-1 enzyme mechanism	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Uncleaved plasmid profile (Fig. 12)	1a	1	1	1	2	1b	3	1	1
Plasmid profile *Eco*R1 (Fig. 13)	4	4	4	4	5	4a	6	4	
Plasmid profile *Hind* 111 (Fig. 14)	8	7	7	7a	9	10	11	7a	

KEY Hospital : Add - Addington
Went - Wentworth
RKK - R K Khan

Antibiotic profile : Genta - gentamicin
Tobra - tobramycin
Netil - netilmicin
Amik - amikacin

S - susceptible
R - resistant
I - intermediate

Plasmid profile: Isolates with the same numbers were identical
The suffix a or b indicates strong similarity

3.9 Transfer of resistance genes

3.9.1 Conjugation and antibiotic susceptibility testing

Conjugation studies were performed on all isolates.

The recipient *Escherichia coli* strain, J53, was resistant to nalidixic acid and susceptible to both gentamycin and tobramycin.

Transconjugants were obtained from the mating mixtures incubated in the Bijou bottles and included five *Klebsiella pneumoniae* donors which constituted 5% of all isolates (n = 100) and 8.9% of isolates identified as *Klebsiella* species (n = 56).

Isolate 51 produced what initially appeared to be a transconjugant but, on further investigation, was found to be a contaminant. This contaminant was disregarded for the purpose of statistical data but was processed in the examination of plasmid profiles of the other five transconjugant bacteria.

All donor isolates were able to grow in the presence of gentamicin and tobramycin (Table 13).

Four of the five donors were unable to grow in the presence of nalidixic acid (Table 13).

Table 13 : Growth of Donor Isolates

DONOR ISOLATE *Klebsiella pneumoniae*	GROWTH ON LB AGAR WITH GENTAMICIN (20ug/ml)	GROWTH ON LB AGAR WITH TOBRAMYCIN (20ug/ml)	GROWTH ON LB AGAR WITH NALIDIXIC ACID (64ug/ml)
11	+	+	-
39	+	+	-
40	+	+	-
42	+	+	-
82	+	+	+

All transconjugants growing on LB agar in the presence of nalidixic acid and gentamicin or tobramycin (Table 14) were identified as *Escherichia coli* using API 20E (bioMerieux sa).

All the *Escherichia coli* transconjugants were able to grow in the presence of nalidixic acid and gentamicin (Table 14).

Two of the transconjugants were unable to grow in the presence of tobramycin and nalidixic acid (Table 14).

Table 14 : Growth of Transconjugant *Escherichia coli* (J53)

TRANSCONJUGANT *Escherichia coli*	GROWTH ON LB AGAR WITH NALIDIXIC ACID (64ug/ml) AND GENTAMICIN (20ug/ml)	GROWTH ON LB AGAR WITH NALIDIXIC ACID (64ug/ml) AND TOBRAMYCIN (20ug/ml)
C11	+	+
C39	+	-
C40	+	-
C42	+	-
C82	+	+

Gentamicin resistance was transferred to the five transconjugants. Resistance to tobramycin was transferred to one transconjugant, reduced in three transconjugants in that they demonstrated intermediate resistance and resistance was not transferred in one transconjugant (Table 15).

The recipient *Escherichia coli* from donor isolates 11 and 82 also produced a number of single colonies within the zones of inhibition around the tobramycin and netelmicin antibiotic discs and the tobramycin disc respectively. However, with subsequent testing these colonies demonstrated identical patterns of resistance to those which were initially shown by the majority of bacteria in both transconjugants.

Table 15 : Susceptibility of donor isolates and *Escherichia coli* transconjugants

DONOR ISOLATE *Klebsiella pneumoniae*	DONOR SUSCEPTIBILITY	RECIPIENT *Escherichia coli*	RECIPIENT SUSCEPTIBILITY
	G T N A		G T N A
11	R R R S	C11	R R S S
39	R R R S	C39	R I S S
40	R R R S	C40	R S S S
42	R R R S	C42	R I S S
82	R R R S	C82	R I S S

KEY : **ANTIBIOTICS** **SUSCEPTIBILITY**

G - gentamicin S - susceptible
T - tobramycin R - resistant
N - netilmicin I - intermediate
A - amikacin

3.9.2 Resistance mechanisms of *Klebsiella pneumoniae* donor isolates and *Escherichia coli* transconjugants

Resistance mechanisms were determined by susceptibility tests to a range of aminoglycoside antibiotics (Table 16) in combination with hybridization techniques.

All five isolates and their respective transconjugants demonstrated similar resistance to gentamicin with all zone diameters $\leq$ 7mm.

Table 16 : Zones of inhibition (in mm) of donor isolates and transconjugants

NO.	AP	AS	6N	2N	G	T	A	I	NT	5E	K	NY
11	19	20	<6	<6	<6	<6	18	19	<6	<6	17	19
39	18	23	8	8	<6	8	21	17	13	13	10	16
40	20	21	9	10	<6	8	18	19	12	17	11	19
42	24	23	10	11	<6	10	21	23	12	22	14	20
82	21	22	9	8	<6	8	22	22	10	14	11	19
J53	26	28	28	28	23	20	22	24	26	22	20	22
C11	25	26	14	13	<6	12	22	22	20	20	18	21
C39	25	20	14	15	<6	12	22	22	20	20	18	21
C40	26	28	20	24	7	16	21	23	22	20	20	20
C42	28	28	14	15	7	16	21	23	22	20	20	20
C82	26	26	14	12	7	13	22	21	19	20	20	22

KEY:

AP	Apramycin	A	Amikacin
AS	Fortimicin	I	Isepamicin
6N	6'-N-ethyl-netilmicin	NT	Netilmicin
2N	2'-N-ethyl-netilmicin	5E	5-epi-sisomycin
G	Gentamicin	K	Kanamycin
T	Tobramycin	NY	Neomycin

In all five transconjugants tobramycin zone sizes (in mm) were reduced in comparison to the recipient control, strain J53, but not to the same extent as with gentamicin. This indicated that tobramycin resistance had been transferred to the transconjugants, but to a lesser degree than gentamicin resistance (Table 16).

Similar relative reductions in zone sizes were applicable to 6'-N-ethyl-netilmicin, 2'-N-ethyl-netilmicin and netilmicin in the transconjugant bacteria.

These observations together with the zone sizes shown with apramycin, fortimicin, amikacin, insepamicin, kanamycin and neomycin suggested the presence of the AAC(3)-V modifying enzyme in both donor and recipient bacteria (Table 17). This was confirmed using DNA/DNA hybridization tests (Appendix D : Table D5, p 103).

Table 17 : Resistance mechanisms in donor *Klebsiella pneumoniae* isolates and recipient *Escherichia coli*

DONOR ISOLATES	MECHANISM (GENE PROBE)
11 *Klebsiella pneumoniae*	AAC (3)-V
39 *Klebsiella pneumoniae*	AAC (3)-V
40 *Klebsiella pneumoniae*	AAC (3)-V
42 *Klebsiella pneumoniae*	AAC (3)-V
82 *Klebsiella pneumoniae*	AAC (3)-V

Escherichia coli J53	NONE (GENE PROBE)

TRANSCONJUGANT *Escherichia coli* J53	MECHANISM (GENE PROBE)
C11	AAC(3)-V
C39	AAC(3)-V
C40	AAC(3)-V
C42	AAC(3)-V
C82	AAC(3)-V

3.9.3 **Plasmid profiles of donor isolates and transconjugant *Escherichia coli***

None of the donor isolates demonstrated similar plasmid profiles although four of the five isolates appeared to contain one or more large plasmids between the wells and the chromosomal DNA (isolates 11, 39, 40, 42 and 82 in lanes 4, 6, 7 and 9 in Figure 10 and lane 2 in Figure 11 on pages 53 and 54). Three of the five isolates contained a number of other, smaller plasmids. Due to the presence of a number of plasmids in the donor isolates and only a few detectable large plasmids in some of the transconjugants the use of restriction endonucleases to determine whether similar or identical plasmids had been transferred would have been of limited value.

The transconjugants C11T, C40G and C11G (lanes 1,5 and 10 in Figure 17) appeared to contain single large plasmids although their numbers appeared to be low as was seen by the diminished intensity of the bands in the gel. No detectable plasmids were observed in the remaining transconjugants.

The electrophoresis of transconjugants C42 (lanes 2 and 6) and C82 (lanes 8 and 11) was duplicated as may be seen in Figure 17. Plasmid DNA was absent in all four lanes.

In Figure 17 it appeared that plasmid DNA was absent in the transconjugant C11N (lane 3) but evident in the transconjugants C11T (lane 1) and C11 (lane 10). As mentioned earlier transconjugants C11N and C11T were thought to be variants of C11 but on further testing were found to have the same resistance patterns. Transconjugant C82T (lane 9) which was also originally thought to be a variant of C82 (lanes 8 and 11) was also found to have the same resistance pattern as transconjugant C82. Plasmid DNA was not evident in the duplicated electrophoresis of transconjugant C82 or in C82T.

Plasmid DNA was not present in the control strain (recipient) *Escherichia coli* J53 as is evident in Lane 1 (without restriction endonuclease) and in lane 2 (EcoR1 restriction endonuclease) in Figure 18.

Figure 17 : Analysis of uncleaved plasmid DNA of *Escherichia coli* transconjugants

1, C11T; 2, C42; 3, C11N; 4, C39; 5, C40; 6, C42; 7, C51 (contaminant); 8, C82; 9, C82T; 10, C11; 11, C82.

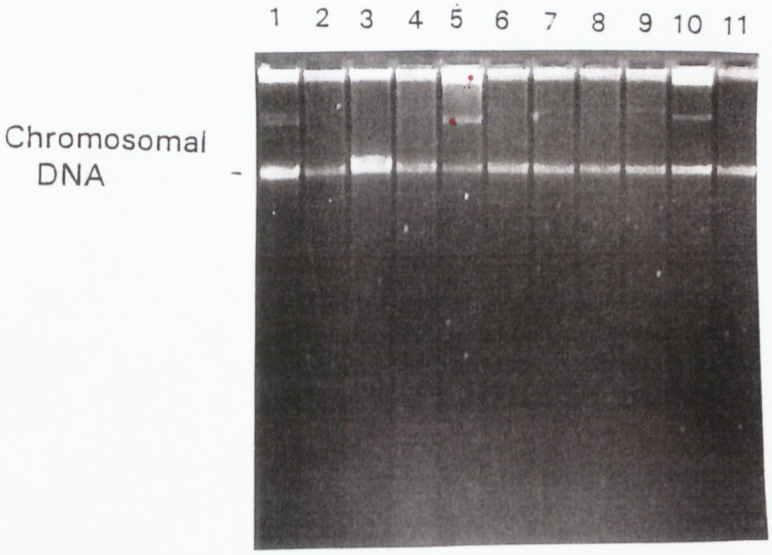
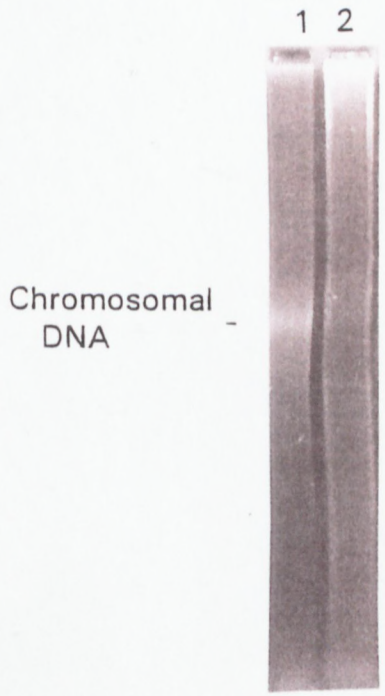


Figure 18 : Plasmid DNA analysis of recipient *Escherichia coli* (J53)

1, J53; 2, J53 with *Eco*R1 restriction endonuclease.



CHAPTER 4

DISCUSSION

DISCUSSION

4.1 Frequency and hospital distribution of isolates resistant to aminoglycoside antibiotics

As was mentioned in Chapter two, problems were encountered in one of the large hospitals with regard to the collection of consecutive isolates. However, the predominance of *Klebsiella pneumoniae* isolates was found in all hospitals in the study.

The 54% frequency of *Klebsiella pneumoniae* in the resistant isolates was similar to a study in Slovakian hospitals (Kallova *et al*, 1997) in which the majority of gentamicin resistant isolates were *Klebsiella* species.

These findings were, however, in contrast to a study in a Hong Kong hospital (Ho *et al*, 1993) in which 73% of the aminoglycoside resistant isolates (n = 179) were *Escherichia coli*. This may have been due to an outbreak of resistant *Escherichia coli* during the time of the study and the authors did not state that the isolates were consecutive.

John and Twitty (1986) observed that *Klebsiella pneumoniae* accounted for the majority of single-genus and multiple-genus outbreaks of nosocomial R-plasmid phenomena.

4.2 Correlation between resistance mechanisms assigned according to phenotype and DNA/DNA hybridization

The failure of certain resistant isolates to hybridize with gene probes may have been due to the possibility that there may be more than one DNA sequence coding for a particular enzyme (Lee *et al*, 1987; Shaw *et al*, 1989; Dornbusch *et al*, 1990).

Generally, in this study, there was correlation between assigned phenotypes and genotypes in the isolates tested although in *Escherichia coli* the genotype was not detected where multiple mechanisms occurred or possible permeability resistance.

This was in contrast to the study of Ho *et al* (1993) in which antibiograms correctly identified modifying enzymes in over 95% of *Escherichia coli* (n = 131)

and the resistance mechanisms in two isolates could not be accurately identified due to the possible presence of permeability resistance.

In a number of other isolates in which multiple mechanisms occurred in the current study only a single gene was detected which concurred with the findings of Ho *et al* (1993) that in mixed production of enzymes the genotype was not reliably reflected by antibiogram.

4.3 Resistance of isolates to aminoglycoside antibiotics and resistance mechanisms

The relative increased resistance of the *Klebsiella* isolates to gentamicin (100%), tobramycin (97%) and netilmicin (81%) and the lower level of resistance to amikacin (0%) together with the higher frequency (100%) of the AAC(3)-V enzyme (modifying gentamicin, tobramycin and netilmicin), at King Edward V111 Hospital could be the result of selective pressure due to the increased usage of the respective antibiotics. It may also have been due to a common *Klebsiella pneumoniae* strain or an endemic plasmid which was not detected in the plasmid analysis. Shimizu *et al* (1985), in studies in the United States, Chile and the Far East on more than 2000 aminoglycoside resistant Gram negative bacteria, found a similar incidence of resistance in countries using either gentamicin or amikacin more frequently. In these countries the AAC(6')-1 enzyme predominated where amikacin was used more frequently and a wider range of enzyme resistance mechanisms occurred where gentamicin was mainly used. The incidence of a 90% frequency of the AAC(3)-V enzyme in one country was likened to an epidemic, either of a single organism or plasmid.

In contrast the increased resistance to amikacin (26%) and the higher frequency (68%) of the AAC(6')-1 enzyme (modifying tobramycin, netilmicin and amikacin) at Addington Hospital could be the result of increased usage of amikacin. These findings support the possibility that an increased usage of a particular aminoglycoside antibiotic results in selection of strains producing modifying enzymes (European Study Group on Antibiotic Resistance, 1987; Friedland *et al*, 1992; Miller *et al*, 1997; Kallova *et al*, 1997)

The occurrence of permeability resistance in this study was limited to *Escherichia coli* isolates. The European Study Group on Antibiotic Resistance (1987) found 51 strains of *Escherichia coli* in which the enzyme resistance mechanisms could not be determined. In these isolates resistance to amikacin was intermediate and

permeability resistance was proposed. Kallova *et al* (1997) found a frequency of 10% permeability resistance in *Escherichia coli*. The occurrence of the AAC(3)-V enzyme in all *Escherichia coli* isolates in this study, either singly or in combination with the APH(3')-1 enzyme or permeability resistance was in agreement with the findings of Ho et al (1993) in which the AAC(3)-V enzyme was demonstrated in 126 of 131 gentamicin resistant *Escherichia coli*; in 86 of these isolates as a single mechanism and in 30 isolates in combination with APH(3')-1.

In this study the 89% incidence of the *aac(6')-1b* gene in combination with other resistance phenotypes was similar to the study of Ho *et al* (1993) in which the *aac(6')-1b* gene always occurred in combination with other resistance genes.

The 18% incidence of combinations of enzyme resistance mechanisms in this study was probably the result of the use of a number of aminoglycosides including gentamicin, tobramycin, netilmicin and amikacin. Miller *et al* (1997) state that an increased complexity of resistance mechanisms is due to an increased usage of aminoglycosides. Kallova *et al* (1997) found an increase in resistance mechanism combinations in more recent studies in comparison to earlier studies and the greatest increase in resistance was observed to netilmicin and amikacin.

The predominant frequency of the AAC(3)-V enzyme followed by AAC(6')-1 in resistant isolates was similar to collated worldwide studies between 1988 and 1993 (Miller *et al*, Schering-Plough Research Institute) in which the most frequent resistance mechanisms encountered in Enterobacteriaceae (n = 5,532) were AAC(3)-V (33%) and AAC(6')-1 (15%). Mechanisms were assigned by the correlation of phenotype with the results of hybridization to gene probes. In both earlier studies (Kettner and Kremery, 1988) and more recent studies in Slovakian hospitals (Kallova *et al*, 1997) found the AAC(3)-V enzyme to be the most frequently occurring enzyme in gentamicin-resistant isolates.

Contributing to the above worldwide studies Klugman (1988-1993), in South Africa, encountered the AAC(6')-1 enzyme (25%), the AAC(3)-V enzyme (23%), the AAC(6')-1 enzyme plus the ANT(2")-1 enzyme (20%) and the AAC(6')-1 enzyme plus the AAC(3)-V enzyme (12%) among Enterobacteriaceae (n = 504). These results differed from the current study in which the incidence of the AAC(3)-V enzyme was higher than that of the AAC(6')-1 enzyme and the ANT(2")-1 enzyme was absent. Klugman's finding of an increased presence of the AAC(6')-1 enzyme was probably related to the use of amikacin as the principal aminoglycoside antibiotic in the unit of the hospital in which the study was undertaken.

In the same study Klugman found the AAC(6')-1 enzyme occurring in excess of 50% of *Serratia* species (n = 31), AAC(6')-1 plus AAC(3)-V in 23% and AAC(3)-V in 12% of the isolates. Although the sample size was smaller (n = 7) in the current study the relative proportions of the incidence of these resistance enzymes in *Serratia* species was similar.

The absence of the ANT(2')-1 enzyme (modifying gentamicin and tobramycin) in all isolates was in contrast to the findings in most other studies. The European Study group on Aminoglycoside Resistance (1987) found the ANT(2") enzyme (28%), the AAC(3)-V enzyme (24%) and the AAC(6')-1 enzyme (15%) the most frequently occurring enzymes in Gram negative bacilli. Miller *et al* (1997) state that the enzyme was predominant in resistant isolates in early surveys in the USA as a single resistance mechanism and found in combination with AAC(6')-1 in Japan. In Europe early surveys differed in that the AAC(3)-V enzyme was more common than the ANT(2")-1 enzyme. Ho *et al* (1993) found the enzyme in 19 of 33 *Klebsiella pneumoniae* isolates in a Hong Kong hospital.

The occurrence of negative probe results for the *aac(3)* gene when the resistance phenotype suggested a combination of mechanisms including the AAC(3)-V enzyme (modifying gentamicin, tobramycin and netilmicin) supports other observations that additional genes exist for the enzyme groups tested (Dornbusch, 1990; Shaw *et al*, 1991). The fact that the presence of the APH(3')-1 enzyme (modifying kanamycin and neomycin) could only be established according to phenotype suggests that there is a possibility that this phenotype may be encoded by other genes.

The frequency of the APH(3')-1 enzyme mechanism (modifying kanamycin and neomycin) is probably not a true reflection of its occurrence either in the hospitals in the region as it may have been present among the 57 isolates which were not tested with the full range of aminoglycoside antibiotics and only isolates resistant to one or more of the aminoglycoside antibiotics gentamicin, tobramycin, netilmicin and amikacin were collected for this study. In the isolates which were tested with the full range of aminoglycoside antibiotics the enzyme was always proposed in combination with other mechanisms. This agreed with the findings in other studies (Shaw *et al*, 1991).

4.4 Plasmid profiles and transfer of resistance genes

The only similarities noted in plasmid profiles of the isolates were the five *Klebsiella*

pneumoniae isolates from Addington Hospital all demonstrating intermediate resistance to amikacin, susceptibility to gentamicin and resistance to tobramycin and netilmicin which suggested the presence of the AAC(6')-1 enzyme.

It is possible either that the isolate was endemic in two units in the hospital or that that a common endemic plasmid was present.

The absence of apparent plasmid DNA in 25% of the isolates may have been due to technical factors in the plasmid extraction method. With regard to the fairly wide diversity of plasmid profiles consideration could be given to a number of possibilities. Technical factors in the extraction method and variations in the conditions of electrophoresis may cause loss of antibiotic resistance plasmids and the distinction between isolates reduces when numerous bands are present especially with small differences in molecular size of bands. Other factors include differences in plasmid patterns following conjugation and molecular rearrangement or deletion resulting in a difference in molecular size (Pfaller, 1991).

The transfer of gentamicin resistance with a reduction in the degree of resistance to tobramicin and the lack of transfer of netilmicin resistance in the transconjugant *Escherichia coli* may have been due to inefficient transcription of resistance genes. The aminoglycoside modifying enzymes are, however, constitutive enzymes and are produced regardless of whether the antibiotic is present or not (Davies, 1986) and the presence of the *aac(3)-Va* genes was demonstrated in transconjugant *Escherichia coli* in DNA/DNA hybridization tests.

The intracellular location of the aminoglycoside-modifying enzymes may have played a role in determining the level of resistance of the organisms. If these enzymes were located in the cytoplasm a certain percentage of aminoglycoside molecules would escape modification and inhibit bacterial protein synthesis (Shaw *et al*, 1993).

An additional consideration was the possibility that species-related phenotypic differences may have occurred in which the same enzyme may have determined a lower level of resistance to tobramycin and netilmicin in the transconjugant *Escherichia coli* (Davies, 1986).

Levels may also vary depending on the copy number of the genes (Davies, 1986).

There are a number of possibilities for the lack of correlation in plasmid profiles of donor isolates and transconjugants.

Plasmids may have been transferred but, during electrophoresis, not migrated from the wells because of their relatively large molecular size or may have been contained in the chromosomal DNA.

Another possibility is that the plasmids containing resistance genes were of low copy number and were therefore not visible in gel electrophoresis.

It should also be borne in mind that transposons carrying resistance genes are capable of inserting into unrelated plasmids or other extrachromosomal DNA elements which, following their transfer to recipient bacteria, could then encode resistance (O'Brien *et al*, 1982). Elwell (1986) suggests that antibiotic resistance can be conjugatively transferred in the apparent absence of plasmid DNA through transpositional events. Another possible mediator of antibiotic resistance could have been a bacteriophage acting as a temporary host to a transposon.

Plasmids are usually stable in bacteria but the loss of plasmids must also be considered. Apart from the possible loss of plasmids during culture or storage of isolates, the breakage of covalent bonds in plasmids during the extraction procedure may also have caused their loss (Hawkey, 1987; Mayer, 1988; Pfaller, 1991). Hawkey (1987) suggests that molecular changes may occur in plasmid profiles over a period of time. As a result the use of plasmid profiles in determining the relatedness of isolates should preferably be made over a relatively short period of time.

CHAPTER 5

CONCLUSION

CONCLUSION

Of the four aminoglycosides tested tobramycin resistance was the most frequently occurring in the four species of Enterobacteriaceae tested among which there was a predominance of *Klebsiella pneumoniae*.

The most frequently occurring resistance mechanisms were the AAC(3)-V and the AAC(6')-1 modifying enzymes. Although the specific aminoglycoside usage in the hospitals studied was not obtained in this study it would be useful, in future studies, to relate it to the occurrence of the most frequently occurring enzyme resistance mechanisms.

The absence of the ANT(2")-1 enzyme in any of the isolates which is unlike the results obtained in other studies in South Africa and in other countries is difficult to explain. Although the phenotype overlaps with the phenotypes of the AAC(3)-V and AAC(6')-1 enzymes and would therefore be masked in isolates tested for resistance to gentamicin, tobramycin, netilmicin and amikacin the gene probe tests were negative in all 43 isolates tested. Another unexplained occurrence was the high frequency of negative probe results for the AAC(3)-V enzyme in isolates with an AAC(3)-V phenotype in combination with an AAC(6')-1 phenotype which was always positive. Furthermore, in all but one isolate, the AAC(3)-V phenotype in combination with the APH(3")-1 phenotype yeilded negative probe results for both. These factors suggest that certain of the enzymes are coded by additional genes or that an altered DNA sequence results in certain enzyme combinations.

The relatively high frequency of combinations in this study is suggestive of the increased usage of a number of a number of aminoglycoside antibiotics.

The predominance of the AAC(3)-V modifying enzyme in all isolates, and particularly *Klebsiella pneumoniae* isolates, from King Edward V111 Hospital needs to be further investigated to determine whether the enzyme is present as a result of selective pressure brought about by the use of a particular aminoglycoside antibiotic or due to the presence of a resident bacterial strain harbouring and disseminating the resistance genes. Isolates with similar resistance profiles could be collected and more intensive typing performed, including full aminoglycoside antibiogram and plasmid and chromosomal DNA analysis using specific probes.

Generally, with a few exceptions, plasmid profiles in this sample population were not found to be similar in a particular species or in unrelated species and could not be related to patterns of resistance or resistance mechanisms.

One exception was the group of isolates from Addington Hospital which carried similar or identical plasmids, demonstrated the same resistance phenotype and possessed the AAC(6')-1 aminoglycoside modifying enzyme.

These isolates, from two units in the hospital, could form the basis for further studies to determine whether one or more of the plasmids or the strain of *Klebsiella pneumoniae* could be endemic in these units. This would neccessitate the collection of isolates with the same aminoglycoside resistance profiles. The isolates would then be subjected to plasmid profile analysis, electroporation of specific plasmid DNA and plasmid mapping.

It was confirmed in the *in vitro* study on conjugation that aminoglycoside resistance mechanisms could be transferred between different species of bacteria conferring resistance on previously susceptible bacteria.

Plasmid transfer between different species of bacteria *in vitro* was also confirmed but the direct role of plasmids in the transfer of resistance was not shown in this study. It is possible that other DNA vectors mediated the transfer.

Although the incidence of resistance to the most commonly used aminoglycoside antibiotics and the enzyme resistance mechanisms encountered in the Greater Durban area were generally similar to the findings in other studies and, at the time of the study, relatively few resistant isolates were shown to have common properties, it would be advisable to continually monitor these aspects. Resistant bacteria should be collected and the source units recorded. This data could then be compared with aminoglycoside usage figures. Other factors which should also be taken into account include the number of patients in particular units and facilities available such as isolation wards. It is possible that by restricting the usage of a particular aminoglycoside antibiotic resistance brought about by selective pressure could be reduced, thereby improving its efficacy when used selectively.

Should the newer aminoglycoside antibiotic, isepamicin, be or have been introduced to the antibiotic regimen, it would be interesting to monitor the possible development of resistance in view of earlier findings that it is less susceptible to modifying enzymes than gentamicin, tobramycin, netilmicin and amikacin.

APPENDIX A

Aminoglycoside resistant isolates : *Klebsiella* species, *Enterobacter* species, *Serratia* species and *Escherichia coli*.

(Table A1 : all isolates, Tables A2 to A5 : by genus)

KEY :

HOSPITALS :

Add	- Addington
KE	- King Edward V111
RKK	- R K Khan
Went	- Wentworth

ANTIBIOTICS :

G	- gentamicin
T	- tobramycin
N	- netilmicin
A	- amikacin

SUSCEPTIBILITY :

S	- susceptible
R	- resistant
I	- intermediate

SPECIMENS :

Asc.f	- ascitic fluid
Asp	- aspirate
BC	- blood culture
Caes	- caesarian section
CSF	- cerebrospinal fluid
CVP	- venous catheter
Ear/s	- ear swab
ETT	- endotracheal tube
MSU	- mid-stream urine
Pus/s	- pus swab
Spt	- sputum
Surg	- surgical swab
Swab	- swab, variety of sites
Ulcer	- ulcer swab
Urin.cath	- urinary catheter
Woun	- wound swab

Table A.1

NO	ISOLATE	SPEC	HOS	UNIT	G T N A	DATE
1	*S.marcescens*	Swab	Add	Burns	S R R S	4/11
2	*E.cloacae*	BC	Add	3B	S I S I	8/11
3	*E.cloacae*	Surg	Add	6A	R R R S	2/11
4	*K.pneumoniae*	BC	Add	5C	R R R S	15/11
5	*E.cloacae*	Surg	Add	3B	R R R S	14/11
6	*K.pneumoniae*	Surg	Add	4B	R R R R	16/11
7	*K.pneumoniae*	Caes	Add	Surg	R R I S	18/11
8	*E.cloacae*	MSU	Add	9A	R R R S	21/11
9	*K.pneumoniae*	CSF	Went	OPD	R R R R	27/11
10	*K.pneumoniae*	Per.f	Add	2B	S R R R	28/11
11	*K.pneumoniae*	CSF	Went	Neuro	R R R S	27/1
12	*E.cloacae*	Surg	Went	Thor	R R R R	27/1
13	*E.cloacae*	Surg	Went	Thor	S R R R	27/1
14	*K.pneumoniae*	Spt	Went	Neuro	S R R R	27/1
15	*E.cloacae*	Spt	Went	Thor	I R R R	27/1
16	*E.coli*	ETT	KE	ICU	R R I I	3/3
17	*S.marscescens*	CVP	KE	ICU	S R R R	3/3
18	*S.liquefaciens*		KE	ICU	S R R S	3/3
19	*K.pneumoniae*	ETT	KE	ICU	R R R S	3/3
20	*E.cloacae*	CVP	KE	ICU	R R I S	3/3
21	*K.pneumoniae*	ETT	KE	ICU	R R I S	3/3
22	*K.pneumoniae*	MSU	KE	ICU	R R R S	3/3
23	*E.coli*	Asc.f	Add	OPD	R R R S	25/2
24	*E.cloacae*	MSU	Add	3A	R R R S	20/2
25	*K.pneumoniae*	MSU	Add	3A	R R R S	20/2
26	*E.cloacae*	Woun	Add	14A	R R R I	25/2
27	*K.pneumoniae*	Woun	Add	14A	S R R I	25/2
28	*E.cloacae*	CVP	Add	2A	R R R I	24/2
29	*E.cloacae*	BC	Add	11A	R R R R	26/2

Table A.1 continued

NO	ISOLATE	SPEC	HOS	UNIT	G T N A	DATE
30	*E.coli*	Ulcer	Add	2B	R R I S	20/2
31	*E.coli*	Ulcer	Add	2B	S R S S	20/2
32	*K.pneumoniae*	Spt	Add	5B	S R I I	20/2
33	*K.pneumoniae*	Spt	Add	5B	S R R I	20/2
34	*K.oxytoca*	Asp	Add	3B	R R R S	20/2
35	*K.pneumoniae*	BC	Add	OPD	R R I S	20/2
36	*E.cloacae*	Stool	Add	13A	R R I S	20/2
37	*K.pneumoniae*	Woun	Add	2A	S R R I	20/2
38	*E.cloacae*		Add		R R R S	20/2
39	*K.pneumoniae*	BC	KE		R R R S	22/3
40	*K.pneumoniae*	BC	KE		R R R S	22/3
41	*K.pneumoniae*	ETT	KE	ICU	R I S S	22/3
42	*K.pneumoniae*	ETT	KE	Resp	R R R S	22/3
43	*S.marcescens*	Woun	Add	6A	S I I S	7/4
44	*K.oxytoca*	BC	Add	10B	S R S S	7/4
45	*K.pneumoniae*	CVP	Add	3B	S R R I	7/4
46	*E.coli*	Woun	Add	OPD	I R I I	7/4
47	*K.pneumoniae*	Ear/s	Add	3B	S R R R	7/4
48	*K.pneumoniae*	Woun	Add	HDU	S R R R	7/4
50	*E.coli*	BC	Add	12B	R R I I	12/4
51	*K.pneumoniae*	BC	Add	5B	S R R R	19/4
52	*E.cloacae*		KE		R R R R	25/4
53	*E.cloacae*	Pus/s	RKK		R R S S	12/5
54	*E.cloacae*	Pus/s	RKK		R R R S	12/5
55	*K.pneumoniae*	Pus/s	RKK		S R R S	12/5
56	*K.pneumoniae*	Pus/s	RKK		R R S S	12/5

Table A.1 continued

NO	ISOLATE	SPEC	HOS	UNIT	G T N A	DATE
57	*E.coli*	MSU	RKK		R I S S	12/5
58	*S.marcescens*	BC	Add	11A	R R R R	17/5
59	*K.pneumoniae*	Aspir	Add	5B	S R R I	17/5
60	*K.pneumoniae*		Add	5B	S R R S	17/5
61	*K.pneumoniae*	BC	Add		R R R R	17/5
63	*E.cloacae*	MSU	RKK		R R S S	12/6
64	*E.cloacae*		RKK		R R R S	12/6
65	*K.pneumoniae*		RKK		R R R S	12/6
66	*E.cloacae*	Bile	Add		R R R S	14/6
67	*S.marcescens*	Spt	Add	11B	S R R S	30/6
68	*E.aerogenes*	BC	Add	5C	R R R R	30/6
69	*E.cloacae*	Asp	Add	2A	R R R S	30/6
70	*E.cloacae*	Pus/s	Add	4B	R R R I	30/6
72	*S.marcescens*		Add		R R R S	10/8
73	*E.cloacae*		Add		R R R S	10/8
74	*K.pneumoniae*	Spt	KE	ICU	R R R S	31/8
79	*K.pneumoniae*	CVP	KE	ICU	R R R S	31/8
80	*K.pneumoniae*	BC	KE	ICU	R R R S	31/8
82	*K.pneumoniae*	BC	KE	Paed	R R R S	31/8
83	*K.pneumoniae*	BC	KE	Paed	R R R S	31/8
84	*K.pneumoniae*	ETT	KE	ICU	R R R S	31/8
85	*K.pneumoniae*	ETT	KE	ICU	R R I S	31/8
86	*K.pneumoniae*	ETT	KE	ICU	R R R S	31/8
88	*K.pneumoniae*	ETT	KE	ICU	R R R S	31/8
89	*K.pneumoniae*		KE	ICU	R R S S	31/8
90	*E.aerogenes*		KE	ICU	R R R S	31/8
93	*E.coli*		KE	Gynae	R R R S	31/8
95	*K.pneumoniae*	BC	KE	Paed	R R R S	31/8
96	*K.pneumoniae*	Pus/s	KE	Surg	R R R S	31/8

Table A.1 continued

NO.	ISOLATE	SPEC	HOS	UNIT	G T N A	DATE
97	*K.pneumoniae*		KE		R R R S	31/8
98	*K.pneumoniae*		KE	Surg	R R R S	31/8
100	*K.pneumoniae*	MSU	KE	Surg	R R R S	31/8
101	*K.pneumoniae*	BC	KE	D2	R R R S	31/8
102	*K.pneumoniae*	BC	KE	D2	R R R S	31/8
103	*E.coli*	Urin. Cath.	KE	Paed	R R I S	31/8
106	*K.pneumoniae*	Pus/s	KE	ICU	R R R S	31/8
107	*K.pneumoniae*	BC	KE		R R R S	31/8
108	*E.coli*		KE	M1B	R R I S	31/8
109	*K.pneumoniae*		KE	Paed	R R R S	31/8
110	*E.coli*	Urine	KE	Paed	R R I S	31/8
112	*K.pneumoniae*	ETT	KE	Paed	R R S S	31/8
113	*E.coli*		KE	Paed OPD	R R I S	31/8
115	*K.pneumoniae*		KE	Paed OPD	R R I S	31/8
116	*K.pneumoniae*		KE	Paed OPD	R R R S	31/8
117	*K.pneumoniae*		KE	Paed OPD	R R R S	31/8

Table A.2 *Klebsiella* species

NO	ISOLATE	SPEC	HOS	UNIT	G T N A	DATE
19	*K.pneumoniae*	ETT	KE	ICU	R R R S	3/3
21	*K.pneumoniae*	ETT	KE	ICU	R R S S	3/3
22	*K.pneumoniae*	MSU	KE	ICU	R R R S	3/3
39	*K.pneumoniae*	BC	KE		R R R S	22/3
40	*K.pneumoniae*	BC	KE		R R R S	22/3
41	*K.pneumoniae*	ETT	KE	ICU	R I S S	22/3
42	*K.pneumoniae*	ETT	KE	Resp	R R R S	22/3
74	*K.pneumoniae*	Spt	KE	ICU	R R R S	31/8
79	*K.pneumoniae*	CVP	KE	ICU	R R R S	31/8
80	*K.pneumoniae*	BC	KE	ICU	R R R S	31/8
82	*K.pneumoniae*	BC	KE	Paed	R R R S	31/8
83	*K.pneumoniae*	BC	KE	Paed	R R R S	31/8
84	*K.pneumoniae*	ETT	KE	ICU	R R R S	31/8
85	*K.pneumoniae*	ETT	KE	ICU	R R I S	31/8
86	*K.pneumoniae*	ETT	KE	ICU	R R R S	31/8
88	*K.pneumoniae*	ETT	KE	ICU	R R R S	31/8
89	*K.pneumoniae*		KE	ICU	R R S S	31/8
95	*K.pneumoniae*	BC	KE	Paed	R R R S	31/8
96	*K.pneumoniae*	P/S	KE	Surg	R R R S	31/8
97	*K.pneumoniae*		KE		R R R S	31/8
98	*K.pneumoniae*		KE	Surg	R R R S	31/8
100	*K.pneumoniae*	Urine	KE	Surg	R R R S	31/8
101	*K.pneumoniae*	BC	KE	D2	R R R S	31/8
102	*K.pneumoniae*	BC	KE	D2	R R R S	31/8
106	*K.pneumoniae*	P/S	KE	ICU	R R R S	31/8
107	*K.pneumoniae*	BC	KE		R R R S	31/8
109	*K.pneumoniae*		KE	Paed	R R R S	31/8
112	*K.pneumoniae*	ETT	KE	Paed	R R S S	31/8
115	*K.pneumoniae*		KE	Paed OPD	R R I S	31/8
116	*K.pneumoniae*		KE	Paed OPD	R R R S	31/8
117	*K.pneumoniae*		KE	Paed OPD	R R R S	31/8

Table A.2 continued

NO	ISOLATE	SPEC	HOS	UNIT	G T N A	DATE
4	*K.pneumoniae*	BC	Add	5C	R R R S	15/11
6	*K.pneumoniae*	Surg	Add	4B	R R R R	16/11
10	*K.pneumoniae*	Per.f	Add	2B	S R R R	28/11
25	*K.pneumoniae*	MSU	Add	3A	R R R S	20/2
27	*K.pneumoniae*	Woun	Add	14A	S R R I	25/2
32	*K.pneumoniae*	Spt	Add	5B	S R I I	20/2
33	*K.pneumoniae*	Spt	Add	5B	S R R I	20/2
35	*K.pneumoniae*	BC	Add	OPD	R R I S	20/2
37	*K.pneumoniae*	Woun	Add	2A	S R R I	20/2
45	*K.pneumoniae*	CVP	Add	3B	S R R I	7/4
47	*K.pneumoniae*	Ear/s	Add	3B	S R R R	7/4
48	*K.pneumoniae*	Woun	Add	HDU	S R R R	7/4
51	*K.pneumoniae*	BC	Add	5B	S R R R	19/4
59	*K.pneumoniae*	Aspir	Add	5B	S R R I	17/5
60	*K.pneumoniae*		Add		S R R S	17/5
61	*K.pneumoniae*	BC	Add	3B	R R R R	17/5
7	*K.pneumoniae*	Caes.	Add	Mater	R R I S	18/11
34	*K.oxytoca*	Aspir	Add	3B	R R R S	20/2
44	*K.oxytoca*	BC	Add	10B	S R S S	7/4

Table A.2 continued

NO	ISOLATE	SPEC	HOS	UNIT	G T N A	DATE
9	*K.pneumoniae*	CSF	Went	OPD	R R R R	27/11
11	*K.pneumoniae*	CSF	Went	Neuro	R R R S	27/1
14	*K.pneumoniae*	Spt	Went	Neuro	S R R R	27/1

Table A.2 continued

55	*K. pneumoniae*	Pus/s	RKK		S R R S	12/5
56	*K. pneumoniae*	Pus/s	RKK		R R S S	12/5
65	*K. pneumoniae*		RKK		R R R S	12/6

Table A.3 *Enterobacter* species

NO	ISOLATE	SPEC	HOS	UNIT	G T N A	DATE
2	*E.cloacae*	BC	Add	3B	S I S I	8/11
3	*E.cloacae*	Surg	Add	6A	R R R S	2/11
5	*E.cloacae*	Surg	Add	3B	R R R S	14/11
8	*E.cloacae*	MSU	Add	9A	R R R S	21/11
24	*E.cloacae*	MSU	Add	3A	R R R S	20/2
26	*E.cloacae*	Woun	Add	14A	R R R I	25/2
28	*E.cloacae*	CVP	Add	2A	R R R I	24/2
29	*E.cloacae*	BC	Add	11A	R R R R	26/2
36	*E.cloacae*	Stool	Add	13A	R R I S	20/2
38	*E.cloacae*		Add		R R R S	20/2
66	*E.cloacae*	Bile	Add		R R R S	14/6
69	*E.cloacae*	Aspir	Add	2A	R R R S	30/6
70	*E.cloacae*	Pus/s	Add	4B	R R R I	30/6
73	*E.cloacae*		Add		R R R S	10/8
68	*E.aerogenes*	BC	Add	5C	R R R R	30/6

Table A.3 continued

12	*E.cloacae*	Surg	Went	Thor	R R R R	27/1
13	*E.cloacae*	Surg	Went	Thor	S R R R	27/1
15	*E.cloacae*	Spt	Went	Thor	I R R R	27/1

Table A.3 continued

53	*E.cloacae*	Pus/s	RKK		R R S S	12/5
54	*E.cloacae*	Pus/s	RKK		R R R S	12/5
63	*E.cloacae*	MSU	RKK		R R S S	12/6
64	*E.cloacae*		RKK		R R R S	12/6

Table A.3 continued

20	*E.cloacae*	CVP	KE	ICU	R R I S	3/3
52	*E.cloacae*		KE		R R R R	25/4
90	*E.aerogenes*		KE	ICU	R R R S	

Table A.4 *Escherichia coli*

NO	ISOLATE	SPEC	HOS	UNIT	G T N A	DATE
23	*E.coli*	Asc.f	Add	OPD	R R R S	25/2
30	*E.coli*	Ulcer	Add	2B	R R I S	20/2
31	*E.coli*	Ulcer	Add	2B	S R S S	20/2
46	*E.coli*	Woun	Add	OPD	I R I I	7/4
50	*E.coli*	BC	Add	12B	R R I I	12/4

Table A.4 continued

57	*E.coli*	MSU	RKK		R I S S	12/5

Table A.4 continued

16	*E.coli*	ETT	KE	ICU	R R I I	3/3
93	*E.coli*		KE	Gynae	R R R S	31/8
103	*E.coli*	Urin cath	KE	Paed	R R I S	31/8
108	*E.coli*		KE	M1B	R R I S	31/8
110	*E.coli*	Urine	KE	Paed	R R I S	31/8
113	*E.coli*		KE	Paed	R R I S	31/8

Table A.5 *Serratia* species

NO	ISOLATE	SPEC	HOS	UNIT	G T N A	DATE
1	*S.marcescens*	Swab	Add	Burns	S R R S	4/11
17	*S.marcescens*	CVP	KE	ICU	S R R R	3/3
18	*S.liquefaciens*		KE	ICU	S R R S	3/3
43	*S.marcescens*	Woun	Add	6A	S I I S	7/4
58	*S.marcescens*	BC	Add	11A	R R R R	15/5
67	*S.marcescens*	Spt	Add	11B	S R R S	30/6
72	*S.marcescens*		Add		R R R S	10/8

APPENDIX B

Aminoglycoside resistance mechanisms assigned according to disc diffusion susceptibility tests with gentamicin, tobramycin, netilmicin and amikacin.

KEY :

ANTIBIOTICS :
G - gentamicin
T - tobramycin
N - netilmicin
A - amikacin

SUSCEPTIBILITY :
S - susceptible
R - resistant
I - intermediate

Table B.1 *Klebsiella* species

KING EDWARD

No.	ISOLATE	G T N A	ASSIGNED RESISTANCE MECHANISM
19	*K.pneumoniae*	R R R S	AAC(3)-V
21	*K.pneumoniae*	R R I S	AAC(3)-V
22	*K.pneumoniae*	R R R S	AAC(3)-V
39	*K.pneumoniae*	R R R S	AAC(3)-V
40	*K.pneumoniae*	R R R S	AAC(3)-V
41	*K.pneumoniae*	R I S S	ANT(2")-1
42	*K.pneumoniae*	R R R S	AAC(3)-V
74	*K.pneumoniae*	R R R S	AAC(3)-V
79	*K.pneumoniae*	R R R S	AAC(3)-V
80	*K.pneumoniae*	R R R S	AAC(3)-V
82	*K.pneumoniae*	R R R S	AAC(3)-V
83	*K.pneumoniae*	R R R S	AAC(3)-V
84	*K.pneumoniae*	R R R S	AAC(3)-V
85	*K.pneumoniae*	R R I S	AAC(3)-V
86	*K.pneumoniae*	R R R S	AAC(3)-V
88	*K.pneumoniae*	R R R S	AAC(3)-V
89	*K.pneumoniae*	R R S S	AAC(3)-V
95	*K.pneumoniae*	R R R S	AAC(3)-V
96	*K.pneumoniae*	R R R S	AAC(3)-V
97	*K.pneumoniae*	R R R S	AAC(3)-V
98	*K.pneumoniae*	R R R S	AAC(3)-V
100	*K.pneumoniae*	R R R S	AAC(3)-V
101	*K.pneumoniae*	R R R S	AAC(3)-V
102	*K.pneumoniae*	R R R S	AAC(3)-V
106	*K.pneumoniae*	R R R S	AAC(3)-V
107	*K.pneumoniae*	R R R S	AAC(3)-V
109	*K.pneumoniae*	R R R S	AAC(3)-V
112	*K.pneumoniae*	R R S S	AAC(3)-V
115	*K.pneumoniae*	R R I S	AAC(3)-V
116	*K.pneumoniae*	R R R S	AAC(3)-V
117	*K.pneumoniae*	R R R S	AAC(3)-V

Table B.1 continued

ADDINGTON

4	*K.pneumoniae*	R R R S	AAC(3)-V
6	*K.pneumoniae*	R R R R	AAC(3)-V + AAC(6')-1
7	*K.pneumoniae*	R R I S	AAC(3)-V
10	*K.pneumoniae*	S R R R	AAC(6')-1
25	*K.pneumoniae*	R R R S	AAC(3)-V
27	*K.pneumoniae*	S R R I	AAC(6')-1
32	*K.pneumoniae*	S R I I	AAC(6')-1
33	*K.pneumoniae*	S R R I	AAC(6')-1
35	*K.pneumoniae*	R R I S	AAC(3)-V
37	*K.pneumoniae*	S R R I	AAC(6')-1
45	*K.pneumoniae*	S R R I	AAC(6')-1
47	*K.pneumoniae*	S R R R	AAC(6')-1
48	*K.pneumoniae*	S R R R	AAC(6')-1
51	*K.pneumoniae*	S R R R	AAC(6')-1
59	*K.pneumoniae*	S R R I	AAC(6')-1
60	*K.pneumoniae*	S R R S	AAC(6')-1
61	*K.pneumoniae*	R R R S	AAC(3)-V
34	*K.pneumoniae*	R R R S	AAC(3)-V
44	*K.pneumoniae*	S R S S	AAC(6')-1

Table B.1 continued

WENTWORTH

9	*K.pneumoniae*	R R R R	AAC(3)-V + AAC(6')-1
11	*K.pneumoniae*	R R R S	AAC(3)-V
14	*K.pneumoniae*	S R R R	AAC(6')-V

Table B.1 continued

RK KHAN

55	*K.pneumoniae*	S R R S	AAC(6')-1
56	*K.pneumoniae*	R R R S	AAC(3)-V
65	*K.pneumoniae*	R R R S	AAC(3)-V

Table B.2 *Enterobacter* species

ADDINGTON

NO	ISOLATE	G T N A	ASSIGNED RESISTANCE MECHANISM
2	*E.cloacae*	S I S I	AAC(6')-1
3	*E.cloacae*	R R R S	AAC(3)-V
5	*E.cloacae*	R R R S	AAC(3)-V
8	*E.cloacae*	R R R S	AAC(3)-V
24	*E.cloacae*	R R R S	AAC(3)-V
26	*E.cloacae*	R R R I	AAC(3)-V + AAC(6')-1
28	*E.cloacae*	R R R I	AAC(3)-V + AAC(6')-1
29	*E.cloacae*	R R R R	AAC(3)-V + AAC(6')-1
36	*E.cloacae*	R R I S	AAC(3)-V
38	*E.cloacae*	R R R S	AAC(3)-V
66	*E.cloacae*	R R R S	AAC(3)-V
69	*E.cloacae*	R R R S	AAC(3)-V
70	*E.cloacae*	R R R I	AAC(3)-V + AAC(6')-1
73	*E.cloacae*	R R R S	AAC(3)-V
68	*E.cloacae*	R R R R	AAC(3)-V + AAC(6')-1

Table B.2 continued

WENTWORTH

12	*E.cloacae*	R R R R	AAC(3)-V + AAC(6')-1
13	*E.cloacae*	S R R R	AAC(6')-1
15	*E.aerogenes*	I R R R	AAC(3)-V + AAC(6')-1

Table B.2 continued

RK KHAN

53	*E.cloacae*	R R S S	AAC(3)-V
54	*E.cloacae*	R R R S	AAC(3)-V
63	*E.cloacae*	R R S S	AAC(3)-V
64	*E.cloacae*	R R R S	AAC(3)-V

Table B.2 continued

KING EDWARD

20	*E.cloacae*	R R I S	AAC(3)-V
52	*E.cloacae*	R R R R	AAC(3)-V + AAC(6')-1
90	*E.aerogenes*	R R R S	AAC(3)-V

Table B.3 *Escherichia coli*

ADDINGTON

NO	ISOLATE	G T N A	ASSIGNED RESISTANCE MECHANISM
23	*E.coli*	R R R S	AAC(3)-V
30	*E.coli*	R R I S	AAC(3)-V
31	*E.coli*	S R S S	AAC(3)-V
46	*E.coli*	I R I I	AAC(3)-V + AAC(6')-1
50	*E.coli*	R R I I	AAC(3)-V + AAC(6')-1

Table B.3 continued

RK KHAN

57	*E.coli*	R I S S	ANT(2")-1

Table B.3 continued

KING EDWARD

16	*E.coli*	R R I I	AAC(3)-V + AAC(6')-1
93	*E.coli*	R R R S	AAC(3)-V
103	*E.coli*	R R I S	AAC(3)-V
108	*E.coli*	R R I S	AAC(3)-V
110	*E.coli*	R R I S	AAC(3)-V
113	*E.coli*	R R I S	AAC(3)-V

Table B.4 *Serratia* species

NO	ISOLATE	G T N A	ASSIGNED RESISTANCE MECHANISM
	KING EDWARD		
17	*S.marcescens*	S R R R	AAC(6')-1
18	*S.marcescens*	S R R S	AAC(6')-1
	ADDINGTON		
1	*S.marcescens*	S R R S	AAC(6')-1
43	*S.marcescens*	S I I S	AAC(6')-1
58	*S.marcescens*	R R R R	AAC(3)-V + AAC(6')-1
67	*S.marcescens*	S R R S	AAC(6')-1
72	*S.marcescens*	R R R S	AAC(3)-V

Table B.5 Transconjugant *Escherichia coli*

NO	TRANSCONJUGANT	G T N A	ASSIGNED RESISTANCE MECHANISM
C11	*E.coli* (J53)	R R S S	AAC(3)-V
C39	*E.coli* (J53)	R I S S	AAC(3)-V
C40	*E.coli* (J53)	R S S S	AAC(3)-V
C42	*E.coli* (J53)	R I S S	AAC(3)-V
C82	*E.coli* (J53)	R I S S	AAC(3)-V

Table B.6 Control/recipient *Escherichia coli*

NO	CONTROL (RECIPIENT)	G T N A	ASSIGNED RESISTANCE MECHANISM
J53	*E.coli*	S S S S	NONE

APPENDIX C

Assigned resistance mechanisms according to changes in resistance to apramycin, fortimicin, 6'-N-ethyl-netilmicin, 2'-N-ethyl-netilmicin, gentamicin, tobramycin, amikacin, isepamicin, netilmicin, 5-epi-sisomicin, kanamycin and neomycin.

KEY:

AP Apramycin (100μg)
AS Fortimicin (100μg)
6N 6'-N-ethyl-netilmicin (100μg)
2N 2'-N-ethyl-netilmicin (100μg)
G Gentamicin (10μg)
T Tobramycin (10μg)
A Amikacin (30μg)
I Isepamicin (30μg)
NT Netilmicin (30μg)
5E 5-epi-sisomicin (10μg)
K Kanamycin (30μg)
NY Neomycin (30μg)

Average zone sizes (mm) in susceptible isolates

	AP	AS	6N	2N	G	T	A	I	NT	5E	K	NY
E-K	24	24	26	25	21	21	22	24	24	21	20	20
Ec	22	24	26	25	21	20	20	22	24	21	20	18
S	27	29	29	24	23	20	24	26	25	24	24	22

E-K - *Enterobacter* species/*Klebsiella* species
Ec - *Escherichia coli*
S - *Serratia* species

(Miller G.H., F.J.Sabatelli, P.Mann *et al.* Schering-Plough Research Institute.)

Table C.1 Sizes of zones of inhibition (mm) of a range of aminoglycoside antibiotics in 38 random isolates, 5 transconjugants and their respective donor organisms and a control *Escherichia coli*.

NO			6N	2N	G	T	A	I	NT	5E	K	NY	MECHANISM
41	24	24	15	14	<6	<6	22	24	18	19	18	20	AAC(3)-V
79	23	23	6	6	<6	<6	21	22	11	12	10	17	AAC(3)-V
80	22	24	9	10	<6	9	20	21	10	12	10	19	AAC(3)-V
83	20	22	9	10	<6	8	20	20	11	15	12	17	AAC(3)-V
84	22	27	9	9	<6	8	22	22	9	16	11	19	AAC(3)-V
85	22	22	9	9	<6	<6	19	21	16	15	14	17	AAC(3)-V
86	24	26	9	6	<6	8	22	22	12	16	12	20	AAC(3)-V
88	22	23	9	9	<6	11	22	22	12	17	12	20	AAC(3)-V
89	21	21	13	12	8	9	21	23	17	19	17	19	AAC(3)-V
35	22	25	10	10	<6	8	20	22	15	17	<6	<6	AAC(3)-V + APH(3')-1
61	23	22	19	<6	<6	<6	12	20	8	<6	<6	<6	AAC(3)-V + AAC(6')-1 + APH(3')-1
9	23	28	9	<6	<6	<6	14	19	8	<6	<6	19	AAC(3)-V + AAC(6')-1
56	23	28	15	15	<6	12	21	24	18	18	16	16	AAC(3)-V
65	21	24	10	10	<6	9	22	22	12	15	<6	<6	AAC(3)-V + APH(3')-1
7	22	21	10	11	<6	8	21	24	15	17	<6	<6	AAC(3)-V = APH(3')-1
3	24	24	9	<6	<6	<6	17	22	8	<6	<6	<6	AAC(3)-V + AAC(6')-1 + APH(3')-1
5	22	22	9	9	<6	8	20	20	13	15	12	15	AAC(3)-V
26	22	22	9	<6	<6	<6	16	21	8	<6	<6	<6	AAC(3)-V + AAC(6')-1 APH(3')-1
28	22	22	11	<6	8	<6	15	18	9	<6	<6	18	AAC(3)-V + AAC(6')-1
29	20	20	13	<6	8	<6	14	19	9	<6	<6	17	AAC(3)-V + AAC(6')-1
36	24	24	9	9	<6	<6	20	19	14	17	11	20	AAC(3)-V
38	23	23	9	10	<6	8	18	21	12	17	12	19	AAC(3)-V
68	30	30	< 6	<6	<6	<6	9	17	<6	<6	<6	19	AAC(3)-V + AAC(6')-1
53	23	23	11	12	<6	<6	20	21	16	16	16	18	AAC(3)-V
20	23	24	9	9	<6	8	22	23	14	17	14	17	AAC(3)-V
52	23	23	9	<6	<6	<6	13	20	<6	<6	<6	<6	AAC(3)-V + AAC(6')-1 APH(3')-1
90	23	23	9	9	<6	8	18	20	9	17	11	19	AAC(3)-V
30	19	22	13	13	<6	10	17	20	16	17	14	20	AAC(3)-V

Table C.1 continued

NO			6N	2N	G	T	A	I	NT	5E	K	NY	MECHANISM
50	18	18	13	13	<6	11	16	16	16	16	15	15	AAC(3)-V + PERMEABILITY
57	18	20	18	21	<6	13	17	18	19	15	15	16	AAC(3)-V + PERMEABILITY
93	19	19	12	11	<6	8	17	19	13	15	<6	10	AAC(3)-V
103	23	25	10	9	<6	8	22	24	14	15	12	18	AAC(3)-V
108	20	23	9	9	<6	<6	18	18	15	15	<6	<6	AAC(3)-V + APH(3')-1
110	19	23	12		<6	10	18	21	15	15	<6	10	AAC(3)-V
113	21	21	10	10	<6	9	19	21	15	17	<6	<6	AAC(3)-1 + APH(3')-1
17	18	20	22	<6	17	<6	13	15	11	15	16	19	AAC(6')-1 + PERMEABILITY
43	22	23	22	13	21	14	18	22	16	20	17	20	AAC(6')-1
51	22	22	22	<6	18	8	14	20	10	<6	<6	17	AAC(3)-V
11	19	20	<6	<6	<6	<6	18	19	<6	<6	17	19	AAC(3)-V
39	18	23	8	8	<6	8	21	17	13	13	10	16	AAC(3)-V
40	20	21	9	10	<6	8	18	19	12	17	11	19	AAC(3)-V
42	24	24	10	11	<6	10	21	23	12	22	14	20	AAC(3)-V
82	21	22	9	8	<6	8	22	22	10	14	11	19	AAC(6')-1
J53	26	28	28	28	23	20	22	24	26	22	20	20	NONE
C11	25	26	14	13	<6	12	22	22	20	20	18	21	AAC(3)-V
C39	25	20	14	15	<6	13	24	24	18	19	18	22	AAC(3)-V
C40	26	28	20	24	7	16	21	23	22	20	20	20	AAC(3)-V
C42	28	28	14	15	7	13	23	24	18	20	18	22	AAC(3)-V
C82	26	26	14	12	7	13	22	21	19	20	20	22	AAC(3)-V

APPENDIX D Summary of assigned aminoglycoside resistance mechanisms in 48 isolates

KEY :

1 Aminoglycoside resistance mechanisms assigned according to :

1.1 Susceptibility tests with gentamicin, tobramycin, netilmicin and amikacin.

1.2 Susceptibility to a wider range of aminoglycoside antibiotics (gentamicin, tobramycin, netilmicin, amikacin, kanamycin and neomycin, apramicin, fortimicin, 6'-N-ethyl-netilmicin, 2'-N-ethyl-netilmicin, isepamicin and 5-epi-sisomicin).

2 Resistance genes detected in hybridization tests.

3 Final mechanism assigned.

Table D.1 *Klebsiella* species

KING EDWARD

NO.	ISOLATE	1.1	1.2	2	3
39	*K.pneumoniae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
40	*K.pneumoniae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
41	*K.pneumoniae*	ANT(2")-1	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
42	*K.pneumoniae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
79	*K.pneumoniae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
80	*K.pneumoniae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
82	*K.pneumoniae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
83	*K.pneumoniae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
84	*K.pneumoniae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
85	*K.pneumoniae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
86	*K.pneumoniae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
88	*K.pneumoniae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
89	*K.pneumoniae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V

Table D.1 continued

ADDINGTON

7	*K.pneumoniae*	AAC(3)-V	AAC(3)-V + APH(3')-1	Negative	AAC(3)-V + APH(3')-1
35	*K.pneumoniae*	AAC(3)-V	AAC(3)-V + APH(3')-1	Negative	AAC(3)-V + APH(3')-1
51	*K.pneumoniae*	AAC(6')-1	AAC(6')-1	*aac(6')-1b*	AAC(6')-1
61	*K.pneumoniae*	AAC(3)-V	AAC(3)-V + AAC(6')-1 + APH(3')-1	*aac(6')-1b* (weak)	AAC(3)-V + AAC(6')-1 + APH(3')-1

Table D.1 continued

WENTWORTH

9	*K.pneumoniae*	AAC(3)-V + AAC(6')-1	AAC(3)-V + AAC(6')-1	*aac(6')-1b* (weak)	AAC(3)-V + AAC(6')-1
11	*K.pneumoniae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V

Table D.1 continued

RK KHAN

56	*K.pneumoniae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
65	*K.pneumoniae*	AAC(3)-V	AAC(3)-V + APH(3')-1	Negative	AAC(3)-V + APH(3')-1

Table D.2 *Enterobacter* species

ADDINGTON

NO	ISOLATE	1.1	1.2	2	3
3	*E.cloacae*	AAC(3)-V	AAC(3)-V + AAC(6')-1 + APH(3')-1	*aac(3)-Va* + *aac(6')-1b* + *aac(6')-11b*	AAC(3)-V + AAC(6')-1 + APH(3')-1
5	*E.cloacae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
26	*E.cloacae*	AAC(3)-V + AAC(6')-1	AAC(3)-V + AAC(6')-1 + APH(3')-1	*aac(6')-1b* + *aac(6')-11b*	AAC(3)-V + AAC(6')-1 + APH(3')-1
28	*E.cloacae*	AAC(3)-V + AAC(6')-1	AAC(3)-V + AAC(6')-1	*aac(6')-1b*	AAC(3)-V + AAC(6')-1
29	*E.cloacae*	AAC(3)-V + AAC(6')-1	AAC(3)-V + AAC(6')-1	*aac(6')-1b*	AAC(3)-V + AAC(6')-1
36	*E.cloacae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
38	*E.cloacae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
68	*E.aerogenes*	AAC(3)-V + AAC(6')-1	AAC(3)-V + AAC(6')-1	*aac(6')-1b*	AAC(3)-V + AAC(6')-1

Table D.2 continued

RK KHAN

53	*E.cloacae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V

Table D.2 continued

KING EDWARD

20	*E.cloacae*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
52	*E.cloacae*	AAC(3)-V + AAC(6')-1	AAC(3)-V + AAC(6')-1 + APH(3')-1	*aac(6')-1b* + *aac(6')-11b*	AAC(3)-V + AAC(6')-1 + APH(3')-1
90	*E.aerogenes*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V

Table D.3 *Escherichia coli*

ADDINGTON

NO	ISOLATE	1.1	1.2	3	4
30	*E.coli*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
50	*E.coli*	AAC(3)-V + AAC(6')-1	AAC(3)-V + PERMEABILITY	Negative	AAC(3)-V + PERMEABILITY

Table D.3 continued

RK KHAN

57	*E.coli*	ANT(2")-1	AAC(3)-V + PERMEABILITY	Negative	AAC(3)-V + PERMEABILITY

Table D.3 continued

KING EDWARD

93	*E.coli*	AAC(3)-V	AAC(3)-V + APH(3')-1	Negative	AAC(3)-V + APH(3')-1
103	*E.coli*	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
108	*E.coli*	AAC(3)-V	AAC(3)-V + APH(3')-1	Negative	AAC(3)-V + APH(3')-1
110	*E.coli*	AAC(3)-V	AAC(3)-V + APH(3')-1	Negative	AAC(3)-V + APH(3')-1
113	*E.coli*	AAC(3)-V	AAC(3)-V + APH(3')-1	Negative	AAC(3)-V + APH(3')-1

Table D.4 *Serratia* species

NO	ISOLATE	1.1	1.2	2	3
	KING EDWARD				
17	*S.marcescens*	AAC(6')-1	AAC(6')-1 + PERMEABILITY	*aac(6')-1c*	AAC(6')-1 + PERMEABILITY
	ADDINGTON				
43	*S.marcescens*	AAC(6')-1	AAC(6')-1	*aac(6')-1c*	AAC(6')-1

Table D.5 Transconjugant *Escherichia coli*

NO	TRANS-CONJUGANT	1.1	1.2	2	3
C11	*E.coli* (J53)	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
C39	*E.coli* (J53)	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
C40	*E.coli* (J53)	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3)-V
C42	*E.coli* (J53)	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC(3-V
C82	*E.coli* (J53)	AAC(3)-V	AAC(3)-V	*aac(3)-Va*	AAC3-V

Table D.6 Control/recipient *Escherichia coli*

NO.	CONTROL (RECIPIENT)	1.1	1.2	2	3
J53	*E.coli*	Susceptible	Susceptible	Negative	None

APPENDIX E

Final aminoglycoside resistance mechanisms assigned to all isolates, transconjugants and control *Escherichia coli* on the basis of either resistance to gentamicin, tobramycin, netilmicin and amikacin **or** relative resistance to gentamicin, tobramycin, netelmicin, amikacin, apramycin, fortimicin, 6′-N-ethyl-netilmicin, 2′-N-ethyl-netilmicin, isepamicin, 5-epi-sisomicin, kanamycin and neomycin in conjunction with DNA/DNA hybridization tests.

Table E.1 *Klebsiella* species

KING EDWARD

NO.	ISOLATE	RESISTANCE MECHANISM
19	*K.pneumoniae*	AAC(3)-V
21	*K.pneumoniae*	AAC(3)-V
22	*K.pneumoniae*	AAC(3)-V
39	*K.pneumoniae*	AAC(3)-V
40	*K.pneumoniae*	AAC(3)-V
41	*K.pneumoniae*	AAC(3)-V
42	*K.pneumoniae*	AAC(3)-V
74	*K.pneumoniae*	AAC(3)-V
79	*K.pneumoniae*	AAC(3)-V
80	*K.pneumoniae*	AAC(3)-V
82	*K.pneumoniae*	AAC(3)-V
83	*K.pneumoniae*	AAC(3)-V
84	*K.pneumoniae*	AAC(3)-V
85	*K.pneumoniae*	AAC(3)-V
86	*K.pneumoniae*	AAC(3)-V
88	*K.pneumoniae*	AAC(3)-V
89	*K.pneumoniae*	AAC(3)-V
95	*K.pneumoniae*	AAC(3)-V
96	*K.pneumoniae*	AAC(3)-V
97	*K.pneumoniae*	AAC(3)-V
98	*K.pneumoniae*	AAC(3)-V
100	*K.pneumoniae*	AAC(3)-V
101	*K.pneumoniae*	AAC(3)-V
102	*K.pneumoniae*	AAC(3)-V
106	*K.pneumoniae*	AAC(3)-V
107	*K.pneumoniae*	AAC(3)-V
109	*K.pneumoniae*	AAC(3)-V
112	*K.pneumoniae*	AAC(3)-V
115	*K.pneumoniae*	AAC(3)-V
116	*K.pneumoniae*	AAC(3)-V
117	*K.pneumoniae*	AAC(3)-V

Table E.1 continued

ADDINGTON

4	*K.pneumoniae*	AAC(3)-V
6	*K.pneumoniae*	AAC(3)-V + AAC(6')-1
7	*K.pneumoniae*	AAC(3)-V + APH(3')-1
10	*K.pneumoniae*	AAC(6')-1
25	*K.pneumoniae*	AAC(3)-V
27	*K.pneumoniae*	AAC(6')-1
32	*K.pneumoniae*	AAC(6')-1
33	*K.pneumoniae*	AAC(6')-1
35	*K.pneumoniae*	AAC(3)-V + APH(3')-1
37	*K.pneumoniae*	AAC(6')-1
45	*K.pneumoniae*	AAC(6')-1
47	*K.pneumoniae*	AAC(6')-1
48	*K.pneumoniae*	AAC(6')-1
51	*K.pneumoniae*	AAC(6')-1
59	*K.pneumoniae*	AAC(6')-1
60	*K.pneumoniae*	AAC(6')-1
61	*K.pneumoniae*	AAC(3)-V + AAC(6')-1 + APH(3')-1
34	*K.oxytoca*	AAC(3)-V
44	*K.oxytoca*	AAC(6')-1

Table E.1 continued

WENTWORTH

9	*K.pneumoniae*	AAC(3)-V + AAC(6')-1
11	*K.pneumoniae*	AAC(3)-V
14	*K.pneumoniae*	AAC(6')-1

Table E.1 continued

RK KHAN

55	*K.pneumoniae*	AAC(6')-1
56	*K.pneumoniae*	AAC(3)-V
65	*K.pneumoniae*	AAC(3)-V + APH(3')-1

Table E.2 *Enterobacter* species

ADDINGTON

NO	ISOLATE	RESISTANCE MECHANISM
2	*E.cloacae*	AAC(6')-1
3	*E.cloacae*	AAC(3)-V + AAC(6')-1 + APH(3')-1
5	*E.cloacae*	AAC(3)-V
8	*E.cloacae*	AAC(3)-V
24	*E.cloacae*	AAC(3)-V
26	*E.cloacae*	AAC(3)-V + AAC(6')-1 + APH(3')-1
28	*E.cloacae*	AAC(3)-V + AAC(6')-1
29	*E.cloacae*	AAC(3)-V + AAC(6')-1
36	*E.cloacae*	AAC(3)-V
38	*E.cloacae*	AAC(3)-V
66	*E.cloacae*	AAC(3)-V
69	*E.cloacae*	AAC(3)-V
70	*E.cloacae*	AAC(3)-V
73	*E.cloacae*	AAC(3)-V
68	*E.aerogenes*	AAC(3)-V + AAC(6')-1

Table E.2 continued

WENTWORTH

12	*E.cloacae*	AAC(3)-V + AAC(6')-1
13	*E.cloacae*	AAC(6')-1
15	*E.cloacae*	AAC(6')-1

Table E.2 continued

RK KHAN

53	*E.cloacae*	AAC(3)-V
54	*E.cloacae*	AAC(3)-V
63	*E.cloacae*	AAC(3)-V
64	*E.cloacae*	AAC(3)-V

Table E.2 continued

KING EDWARD

20	*E.cloacae*	AAC(3)-V
52	*E.cloacae*	AAC(3)-V + AAC(6')-1 + APH(3')-1
90	*E.aerogenes*	AAC(3)-V

Table E.3 *Escherichia coli*

ADDINGTON

NO	ISOLATE	RESISTANCE MECHANISM
23	*E.coli*	AAC(3)-V
30	*E.coli*	AAC(3)-V
31	*E.coli*	AAC(3)-V
46	*E.coli*	AAC(6')-1
50	*E.coli*	AAC(3)-V + PERMEABILITY

Table E.3 continued

RK KHAN

57	*E.coli*	AAC(3)-V + PERMEABILITY

Table E.3 continued

KING EDWARD

16	*E.coli*	AAC(3)-V
93	*E.coli*	AAC(3)-V + APH(3')-1
103	*E.coli*	AAC(3)-V
108	*E.coli*	AAC(3)-V + APH(3')-1
110	*E.coli*	AAC(3)-V + APH(3')-1
113	*E.coli*	AAC(3)-V + APH(3')-1

Table E.4 *Serratia* species

NO	ISOLATE	RESISTANCE MECHANISM
	KING EDWARD	
17	*S.marcescens*	AAC(6')-1 + PERMEABILITY
18	*S.liquefaciens*	AAC(6')-1
	ADDINGTON	
1	*S.marcescens*	AAC(6')-1
43	*S.marcescens*	AAC(6')-1
58	*S.marcescens*	AAC(3)-V + AAC(6')-1
67	*S.marcescens*	AAC(6')-1
72	*S.marcescens*	AAC(3)-V

Table E.5 Transconjugant *Escherichia coli*

NO	TRANS CONJUGANT	RESISTANCE MECHANISM
C11	*E.coli* (J53)	AAC(3)-V
C39	*E.coli* (J53)	AAC(3)-V
C40	*E.coli* (J53)	AAC(3)-V
C42	*E.coli* (J53)	AAC(3)-V
C82	*E.coli* (J53)	AAC(3)-V

Table E.6 Control/recipient *Escherichia coli*

NO.	CONTROL (RECIPIENT)	RESISTANCE MECHANISM
J53	*E.coli*	None

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