# Molecular Methods for the Detection of TEM- and SHV-Related Beta Lactamase Genes in Members of the *Enterobacteriaceae*

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Thesis submitted in fulfilment of the requirements for the degree Master of Science in the Faculty of Medicine, University of Cape Town.

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## Part I

# DEDICATION

*To my parents*

# DECLARATION

I, Andrew Christopher Whitelaw, hereby declare that the work on which this thesis is based is my own original work (except where acknowledgements indicate otherwise), and neither the whole work, or any part thereof, has been, is being or will be submitted for another degree in this or any other university.

I empower the University of Cape Town to reproduce for the purposes of research, the whole or any part of this work in any form whatsoever.

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Date:

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# ABBREVIATIONS

| | |
|---|---|
| A | Adenine |
| bp | Base pairs |
| C | Cytosine |
| °C | Degrees centigrade |
| CFU | Colony forming units |
| CFU/ml | Colony forming units per millilitre |
| CsCl | Cesium Chloride |
| CTAB | Hexadecyl trimethyl ammonium bromide |
| dATP | Deoxy adenosine triphosphate |
| dCTP | Deoxy cytidine triphosphate |
| dGTP | Deoxy guanosine triphophate |
| dTTP | Deoxy thymidine triphosphate |
| dNTP | Deoxy nuleotide triphosphate |
| ddATP | Dideoxy adenosine triphosphate |
| ddCTP | Dideoxy cytidine triphosphate |
| ddGTP | Dideoxy guanosine triphophate |
| ddTTP | Dideoxy thymidine triphosphate |
| DNA | Deoxyribonucleic acid |
| dsDNA | Double stranded DNA |
| EDTA | Ethylene diamine tetra-acetic acid |
| ESBL | Extended spectrum beta lactamase |
| EtBr | Ethidium bromide |
| G | Guanine |
| ***g*** | Gravity (centrifugal force) |
| g | Grams |
| GIT | Gastro-intestinal tract |
| HCl | Hydrochloric acid |
| ICU | Intensive care unit |
| IPTG | Isopropyl-D-thio-galactopyranoside |
| kb | Kilobases |
| LiCl | Lithium chloride |

| | |
|---|---|
| M | Molar (moles per litre) |
| mg/ml | Milligrams per millilitre |
| MH | Mueller Hinton |
| MIC | Minimum inhibitory concentration |
| mg | Milligrams |
| ml | Millilitres |
| mm | Millimetres |
| NaCl | Sodium chloride |
| Nal | Nalidixic acid |
| NaOH | Sodium hydroxide |
| NCCLS | National Committee on Clinical Laboratory Standards |
| ng | Nanograms |
| $OD_{600}$ | Optical density at 600nm wavelength |
| $^{32}P$ | Radioactive phosphorous |
| PBP | Penicillin binding protein |
| pDNA | Plasmid DNA |
| pg | Picograms |
| pI | Isoelectric point |
| psi | Pounds per square inch |
| rpm | Revolutions per minute |
| $^{35}S$ | Radioactive sulphur |
| s | Seconds |
| SDS | Sodium dodecyl sulphate |
| SSC | Salt sodium citrate |
| T | Thymine |
| TAE | Tris-acetate-EDTA |
| TE | Tris-EDTA |
| TEMED | NNN'N' – tetramethylethylene-diamine |
| µg | Micrograms |
| µg/ml | Micrograms per millilitre |
| µl | Micrlitre |
| µM | Micromolar |

| | |
|---|---|
| UV | Ultraviolet |
| V/cm | Volts per centimeter |
| W | Watts |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D galactoside |
| YT | Yeast-tryptone |

# AMINO ACID ABBREVATIONS

| | | | |
|---|---|---|---|
| **Ala** | Alanine | **Leu** | Leucine |
| **Arg** | Arginine | **Lys** | Lysine |
| **Asn** | Asparagine | **Met** | Methionine |
| **Asp** | Aspartate | **Phe** | Phenylalanine |
| **Cys** | Cysteine | **Pro** | Proline |
| **Gln** | Glutamine | **Ser** | Serine |
| **Glu** | Glutamate | **Thr** | Threonine |
| **Gly** | Glyceine | **Trp** | Tryptophan |
| **His** | Histidine | **Tyr** | Tyrosine |
| **Ile** | Isoleucine | **Val** | Valine |

# ABSTRACT

Bacterial resistance to antibiotics is a common and important clinical problem. Beta lactam resistance in Gram negative bacilli is mediated predominantly by beta lactamases, enzymes able to hydrolyse the beta lactam ring. The commonest plasmid mediated beta lactamases in the *Enterobacteriaceae* are those related to either TEM-1 or SHV-1. Although TEM-1, TEM-2 and SHV-1 do not have activity against extended spectrum beta lactams, their derivatives (TEM-3 and SHV-2 onwards) are able to confer resistance to one or more of these antibiotics. A problem encountered in clinical microbiology laboratories is the lack of a reliable method for the detection of ESBLs, along with the lack of a quick, reliable method of differentiating TEM-related genes from SHV-related genes. The primary aim of this study was to evaluate two molecular techniques for the detection of SHV and TEM-related genes in clinical isolates.

The study sample consisted of 209 clinical isolates of enteric Gram negative bacilli, isolated at Groote Schuur Hospital microbiology laboratory. The isolates had all been selected on the basis of resistance to one or more of the extended spectrum beta lactams. These isolates were all identified, and the susceptibility of each to a variety of beta lactam antibiotics determined. Using this information, 45 isolates, belonging to different genera and with differing antimicrobial sensitivity patterns, were selected for this pilot study. These 45 isolates consisted of 24 *Klebsiella* spp., 14 *Enterobacter* spp., 3 *Citrobacter* spp., 2 *Salmonella* spp., 1 *Pantoea agglomerans* and 1 *Serratia marcescens*.

ESBL production was determined in these 45 isolates using both the double disc diffusion test and the Etest ESBL test. Of the 45 isolates, 27 were shown to be ESBL producers – 24 by the double disc test and 26 by the Etest ESBL test. All 23 of the *K. pneumoniae* isolates produced ESBLs, in addition, 2 *Salmonella* spp. and 2 *E. cloacae* isolates produced ESBLs.

A probe specific for TEM-related genes, prepared by PCR, was hybridised to genomic DNA of all the isolates that had been transferred to a nylon membrane using a slot blot apparatus. These hybridisations showed that 12 of the 45 isolates contained TEM-related genes. These 12 consisted of 8 strains of *K. pneumoniae*, 2 *Salmonella* spp., 1 *S. marcescens* and 1 *P. agglomerans*. Hybridisaton of the probe to colony blots of the isolates was also carried out, but the results did not correlate with the hybridisation to genomic DNA.

Two probes for SHV-related genes were used – an oligoprobe and a probe prepared by PCR amplification from an SHV-1 producing control strain. The results of hybridisation of both probes to genomic DNA showed that all the *K. pneumoniae* isolates contained SHV-related genes. This was felt to be due to the fact that *K. pneumoniae* contains a chromosomal beta lactamase gene with a high degree of homology to the plasmid mediated SHV-1 gene. SHV-related genes were detected in 4 other isolates - 2 *E. cloacae* and 2 *Salmonella* spp. Hybridisation of the colony blots again resulted in no correlation with the hybridisation to genomic DNA.

PCR assays, using templates prepared by boiling colonies in water, were designed to detect either SHV- or TEM-related genes. The assays using the TEM specific primers detected TEM-related genes in all of the isolates shown by hybridisation to contain TEM-related genes. This assay was reproducible for all the isolates except one, where the TEM-related gene was detected in only 2 of the 3 assays. Contamination was found to be a problem with this assay, and was overcome by predigesting the PCR mixture with *Ava*II.

The assay for the detection of the SHV-related genes was not entirely successful, for two reasons. Firstly, conditions compatible with a reliable, reproducible assay could not be determined and secondly the assay, when it did work, detected SHV-related genes in all the *K. pneumoniae* isolates. The assay did, however, detect the SHV-related genes that had been demonstrated by hybridisation in the non-*Klebsiella* isolates.

Conjugation studies, using *E. coli* J53 ($Nal^R$) as a recipient were performed on all the isolates that had been shown to contain either SHV- or TEM-related genes. Resistance to ampicillin was transferred from all of the 29 donors, although 9 of the transconjugants could not be cultured further in selective media. Antibiotic susceptibilities were performed on the remaining 20 transconjugants. ESBL activity, determined using the Etest ESBL test, was detected in 9 of the transconjugants. Plasmids were extracted from 19 of the transconjugants and TEM- and SHV-related genes detected in these plasmids using Southern hybridisation of *Bam*HI digested plasmid DNA. The results of the hybridisation to the plasmid and the original donor were discrepant for one of the transconjugants, and the results pertaining to this transconjugant were ignored. TEM- and SHV-related genes were detected in 7 and 13 of the plasmids, respectively: both genes were detected in 2 of the plasmids.

This study shows that TEM- and SHV-related genes are present in clinical isolates of Gram negative bacilli at Groote Schuur Hospital, and are most common in, but not confined to, isolates of *K. pneumoniae*. PCR as an epidemiological tool for the detection of TEM- and SHV-related genes shows promise, but more work needs to be done on the PCR assay for SHV-related genes. The transmissibility of resistance from all the isolates is worrying, although probably not surprising, in light of the already monumental problem of antibiotic resistance dissemination.

# CHAPTER ONE

# LITERATURE REVIEW

## 1.1 INTRODUCTION

The discovery of antimicrobial agents has been arguably one of the most significant events, and the development of antibiotics one of the most important avenues of research, in the field of medicine. These agents have made a significant contribution to the reduced mortality and morbidity associated with infectious disease (Sabath, 1980).

Among the multitude of antibiotics currently in use world-wide, the largest group consists of the beta lactam compounds, comprising the penicillins, cephalosporins, carbapenems and monobactams. There are well over a hundred antibiotics in this group; their spectrum of activity includes Gram positive, Gram negative, aerobic and anaerobic bacteria, making beta lactam antibiotics one of the most commonly used groups of drugs (Neu, 1992). Although resistance to antibiotic agents can be due to either active efflux of the antibiotic, reduction in permeability or alterations in the target site (Dever, 1991; Livermore, 1991), the most common cause of bacterial resistance to beta lactams is mediated by the production of beta lactamase enzymes, which inactivate beta lactam compounds (Jacoby & Archer, 1991; Sanders, 1992; Livermore & Yuan, 1996; Livrelli *et al*, 1996).

## 1.2 BETA LACTAMS

Penicillin, the first of the beta lactams, was described by Alexander Fleming in 1929 and it was effective against almost all staphylococcal infections when first used clinically. Shortly after the introduction of penicillin resistance to this antibiotic emerged, resulting in a decrease in efficacy of this drug against staphylococci. This led to the development of compounds such as cloxacillin, which is resistant to the effects of the staphylococcal penicillinase. Penicillinase mediated resistance to penicillin has resulted in the majority of staphylococci world-wide being resistant to penicillin at present (Waldvogel, 1985).

Similarly, the emergence of antibiotic resistance in other bacterial species resulted in the development of various penicillin derivatives, such as the antipseudomonal drug piperacillin and amoxycillin, used to treat infections caused by *Haemophilus influenzae*. Although penicillin and its derivatives are used predominantly for the treatment of Gram positive infections, they do remain effective against most species of the *Neisseriaceae* as well as strains of *Pseudomonas* and *Haemophilus* (Nathwani & Wood, 1993).

The cephalosporins, a group of beta lactam antibiotics derived from the organism *Cephalosporium acremonium*, were first developed in the mid-1960's (Quintiliani *et al*, 1982). The first cephalosporins were more effective than penicillins against Gram negative organisms. Subsequent generations of cephalosporins (Table1.1) have increased clinical efficacy against Gram negative organisms (Neu, 1990a). This has however been associated with some reduction in their usefulness in treating infections caused by Gram positive bacteria, (Quintiliani *et al*, 1982) although this is not an absolute rule. The antibacterial activity of the various cephalosporins is a complex issue, which will not be dealt with in any great detail in this discussion.

Table 1.1

Examples of cephalosporins in the various "generations" of this class of antibiotic

| 1st Generation | 2nd Generation | 3rd Generation | 4th Generation |
|---|---|---|---|
| Cephalothin | Cefoxitin | Cefotaxime | Cefepime * |
| Cefazolin | Cefuroxime | Ceftriaxone | |
| Cefalexin | Cefamandole | Ceftazidime | |
| Cephradine | Cefaclor | | |

*Some may consider cefepime to be a 3rd generation cephalosporin

One of the reasons for the improved efficacy against Gram negative bacteria is that the cephalosporins are more resistant to the beta lactamases of Gram negative bacteria. The third generation cephalosporins in particular were considered to be resistant to the effects of the beta lactamases that had rendered penicillins useless against many of the *Enterobacteriaceae* (Cunha & Ristuccia, 1982; Neu, 1990a).

The monobactams and carbapenems are the most recent additions to the beta lactam class of antibiotics. Aztreonam, a monobactam, is effective against Gram negative aerobic bacteria only, while the carbapenems, imipenem and meropenem, are active against both Gram positive and Gram negative organisms. While resistance has been reported to the carbapenems it is fortunately still uncommon (Drusano, 1998; Greenhalgh & Edwards, 1998).

### 1.2.1 Structure of Beta Lactams

All beta lactams consist of a central ring structure (the beta lactam ring) which is essential to their activity (Fig 1.1). The different properties of the various beta lactam antibiotics are the result of a variety of side chains and ring structures that have been added to the beta lactam ring. These may either alter the antibacterial range of the drug or render it less susceptible to enzymatic inactivation, or both (Sykes & Matthew, 1976; Collatz *et al*, 1984).

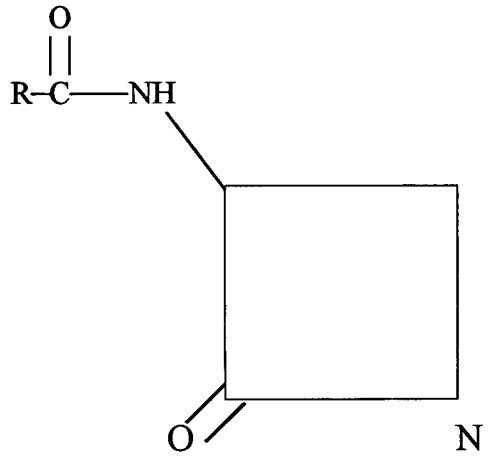
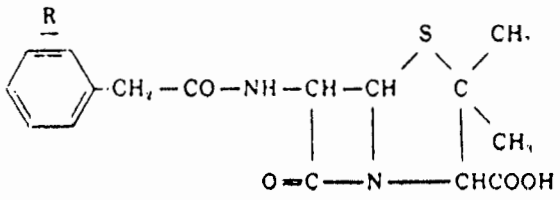



Fig 1.1 Basic structure of the beta lactam ring.

Penicillins:

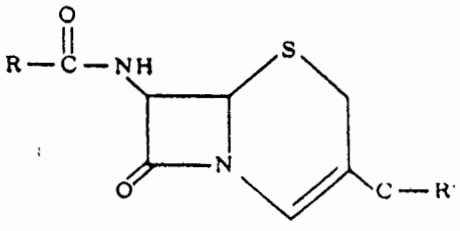


Use: Gram positive cocci

Limited Gram negative use

*T. pallidum*, anaerobes

Cephalosporins:

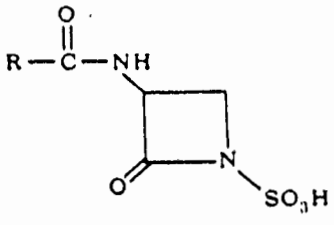


Use: Gram positive cocci [1]

Gram negative bacilli [2]

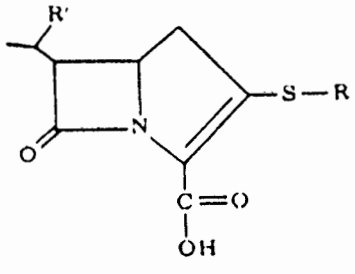
Less anaerobic cover

Monobactams:



Use: Most Gram negative aerobes[3]

Carbapenems



Use: Gram positive and Gram negative aerobes and anaerobes

*Key: 1 = not Enterococci*

*2 = not Pseudomonas*

*3 = exceptions - some Enterobacter spp, Pseudomonas spp, Legionella, Acinetobacter spp*

Fig 1.2 Basic structures of the four major classes of beta lactam (Condemi & Sheehan, 1996).

The diagrams on the previous page illustrate the major structural differences between the penicillins, cephalosporins, monobactams and carbapenems. A detailed discourse on the structure of each individual antibiotic is beyond the scope of this review, and the information provides a general background only.

## 1.2.2 Mechanism of Action of Beta Lactams

The construction of bacterial cell walls involves the linking of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAMA) residues. These amino sugars are arranged in a latticework and held together by beta 1,4 linkages. These bonds occur between amino groups attached to adjacent NAMA molecules, or between adjacent NAG and NAMA molecules (Boyd & Marr, 1980a). A number of membrane bound proteins - transpeptidases and carboxypeptidases - are involved in the process of linking the residues. These enzymes are the targets of beta lactam antibiotics and for this reason they have been called penicillin binding proteins (PBPs) (Richmond, 1978; Malouin & Bryan, 1986; Stratton, 1988). The high molecular weight PBPs (eg PBPs I, II and III) are usually present at lower concentrations than the lower MW PBPs. It is the higher MW PBPs however, that are more essential for cell growth and viability (Waxman & Strominger, 1983). Beta lactam molecules bind to PBPs to form acyl enzyme intermediates that are relatively long lived with resultant inactivation of the PBPs, disruption of cell wall synthesis and death of the organism (Sanders, 1992; Stratton, 1996a).

It is known that different PBPs play different roles in cell wall synthesis and the inhibition of specific PBPs has characteristic effects on the cell wall (Curtis *et al.*, 1980). Inhibition of PBP I results in cell wall lysis, while inhibition of PBP II leads to disruption of the cell shape with the formation of round forms. PBP III is involved in aspects of cell division and septum formation and inhibition of this PBP causes the formation of filamentous forms (Spratt, 1975; Waxman & Strominger, 1983). What is also interesting is that certain beta lactams have affinities for specific PBPs which may influence the effect the antibiotic has on the organism. For example, cefotaxime binds strongly to PBPs 1B and II, while cefoxitin binds preferentially to PBPs V and VI (Richmond 1981, Neu 1985). Mecillinam,

## 1.3.1 Mechanism of Action of Beta Lactamases

Beta lactamases hydrolyse the beta lactam ring at the cyclic amide bond, rendering the antibiotic irreversibly inactive via the formation of an acyl intermediate. This is analogous to the inactivation of PBPs by beta lactams, with one important difference: during beta lactam hydrolysis the beta lactamase-beta lactam acyl intermediate is short lived resulting in the beta lactamase being released to act on other beta lactam molecules. This is in contrast to the PBP-beta lactam acyl intermediate, which is long lived, thereby preventing the PBP from carrying out its role in cell wall synthesis (Stratton, 1988; Sanders, 1992; Livermore, 1995). With this in mind, it is interesting that sequence similarities have been found between at least some of the genes encoding PBPs and beta lactamase genes (Waxman *et al.*, 1982).

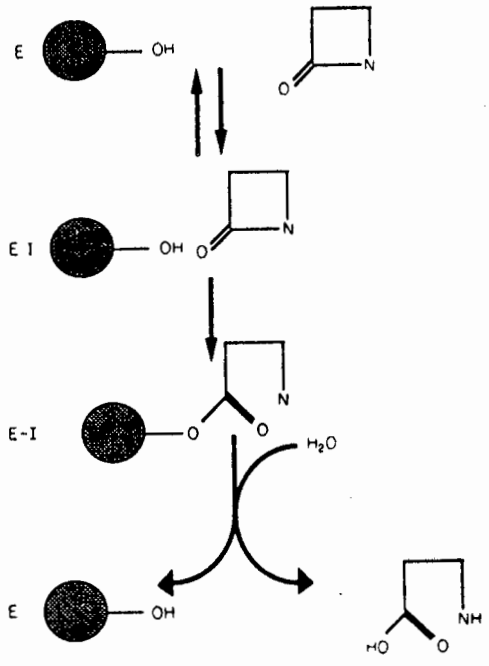



Fig 1.3 Hydrolysis of a beta lactam ring by a beta lactamase enzyme, showing the steps of binding of the enzyme to the beta lactam, acylation of the complex and ultimate hydrolysis of the beta lactam (Livermore, 1993).

a penicillin derivative, binds exclusively to PBP II (Spratt, 1975) while cephaloridine has a high affinity for PBP I (Waxman & Strominger, 1983).

The concentration of PBP and the affinity of the PBPs for beta lactams can play a role in beta lactam resistance in both Gram positive and Gram negative bacteria and are important considerations in the battle between bacterium and antibiotic. If the concentration of a given PBP is increased, more antibiotic may be required for bactericidal activity, and resistance may ensue (Richmond, 1978; Collatz *et al*, 1984). Resistance may also be brought about by a decreased affinity of the PBP for the beta lactam compound - usually a result of a structural change in the PBP itself. This is the primary mechanism of resistance of pneumococci to penicillin (Dever & Dermody, 1991).

Despite the continued development of beta lactam compounds, resistance remains an important clinical problem. Resistance to the newer compounds, cephalosporins and carbapenems, is becoming a significant concern.

## 1.3 BETA LACTAMASES

Bacterial resistance to the beta lactams can result from alteration of the PBPs, reduced access of the beta lactam to the target, or inactivation of the beta lactam, or from a combination of these mechanisms. Inactivation of the antibiotic is the commonest mechanism of resistance to beta lactams and is due to the action of beta lactamases. As this discussion focuses on beta lactamases, the other mechanisms will not be discussed.

As early as 1940, the year that penicillin underwent its first clinical trials, the first beta lactamase was described (Abraham & Chain, 1940). By 1944 clinical isolates of *Staphylococcus aureus* with beta lactamase mediated penicillin resistance had been described (Moellering, 1993). Despite the development of the newer beta lactams described previously, resistance to this group of antibiotics continues to pose a problem, with beta lactamases being responsible for the majority of this resistance.

### *1.3.1.1 Beta Lactamases in Gram Positive Bacteria*

Beta lactamases produced by Gram positive bacteria are released into the surrounding medium and act on the beta lactam before it reaches the bacterium. To be effective, the enzyme must be produced in large quantities. The greater the concentration of bacteria the greater the amount of enzyme produced, and hence the greater the resistance. This phenomenon is also known as the inoculum effect, and it plays an important role in clinical determination of sensitivity and resistance profiles (Sykes & Matthew, 1976). In effect, if an inoculum of $10^8$ CFU/ml of an organism is used in sensitivity testing, as is used in clinical laboratories according to NCCLS (National Committee for Clinical Laboratory Satnadards) criteria (NCCLS, 1997), the organisms may appear sensitive to a particular beta lactam. However, if a larger inoculum of the same organism is used, it could appear resistant to the same beta lactam by virtue of the greater amount of enzyme produced.

### *1.3.1.2 Beta Lactamases in Gram Negative Bacteria*

In Gram negative organisms the enzyme is located primarily in the periplasmic space. Since it is located in a confined space, a smaller quantity of enzyme is required to attain an effective concentration. There is thus a relationship in Gram negative bacteria between the rate of influx of the antibiotic and the rate of inactivation. A faster rate of influx necessitates more beta lactamase in the periplasmic space. If the rate of influx exceeds the rate of inactivation, beta lactam molecules will escape hydrolysis and be able to bind the PBPs (Sanders, 1992; Stratton, 1996a). Figure 1.4 illustrates the differences between Gram positive and Gram negative organisms with respect to the site of action of the beta lactamase.

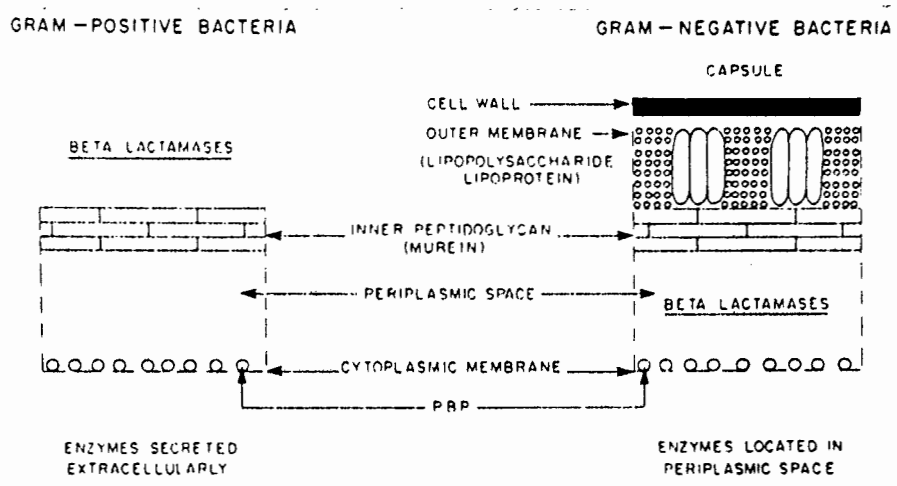



Fig 1.4 Structure of the Gram positive and Gram negative cell walls. The beta lactamase is concentrated in the periplasmic space of the Gram negative organisms while the enzyme is released extracellularly from Gram positive bacteria (Grieco, 1982).

## 1.3.2 Beta Lactamase Inhibitors

One of the strategies employed to combat beta lactamase mediated resistance has been the development of compounds that inhibit beta lactamases. To date, three compounds have been developed that are in use clinically - clavulanate, tazobactam and sulbactam (Livermore, 1993). The method of action of all three is similar in that they irreversibly bind to the beta lactamase, rendering it ineffective by means of suicide inactivation. The sequence of events starts with the binding of the inhibitor to the enzyme which then catalyses the fragmentation of the inhibitor. The resultant fragments contain active groups which react with additional residues on the enzyme to render it inactive (Bush & Sykes, 1983). These three inhibitors have all been combined with beta lactam antibiotics and have been used to treat infections with beta lactamase producing organisms.

Other substances are also able to inhibit beta lactamases, although they have not yet found a clinical niche in this capacity. Antibiotics such as cloxacillin, cefoxitin and aztreonam can

act as competitive substrates of certain beta lactamases. As the name implies, these compounds act as very poor substrates for the enzyme, and a long time is required for hydrolysis of the substrate. The compound *p*-chloromercuribenzoate (pCMB) acts as an amino acid modifier of enzymes such as lactic dehydrogenase as well as certain beta lactams (Bush & Sykes, 1983).

### 1.3.3 Classification of Beta Lactamases

Numerous beta lactamases have been described and much confusion exists in the literature over their classification. Over the years many schemes have been proposed, based on a variety of criteria, common being molecular structure or phenotypic properties of the beta lactamase. A drawback of many of these schemes is that as more and more beta lactamases are described, the classifications become increasingly complex, and oftentimes unwieldy.

Table 1.2 provides a summary of some of the more commonly used schemes and provides examples of representative enzymes. Not all classifications are represented, firstly to avoid confusion and secondly because some, such as the Amyes/Payne scheme (1991), deal only with plasmid mediated extended spectrum beta lactamases (ESBLs) and do not readily fit into the more general schemes described.

In 1973, Richmond and Sykes proposed a classification based on substrate and inhibitor profiles. This was extended in 1976 by Sykes and Matthew. The scheme proposed by the latter authors classifies the enzymes according to the location (chromosome or plasmid) of the beta lactamase resistance genes. The enzymes encoded by chromosomally located genes are then subdivided into cephalosporinases, penicillinases and broad-spectrum beta lactamases. Similarly, three groups of plasmid mediated enzymes were proposed, based on the spectrum of substrates hydrolysed by the beta lactamase. The first group consists of enzymes that do not hydrolyse isoxazoyl beta lactam substrates, such as cloxacillin. Enzymes with activity against methicillin and isoxazoyl beta lactam substrates comprise a second group, while miscellaneous beta lactamases constitute a third group.

Table 1.2

| Richmond/ Sykes (1973) | Ambler Molecular (1980) | Bush (1989b,c) | Bush/ Jacoby/ Medeiros (1995) | Substrate | Inhibited by clavulanate | Example *either enzyme, or organism* |
|---|---|---|---|---|---|---|
| Not incl | A | 2 | 2a | Penicillins | Yes | Enzymes from Gram positive organisms |
| III | A | 2b | 2b | Penicillins, cephalosporins | Yes | TEM-1&2; SHV-1 |
| IV (K1) | A | 2b' | 2be | Penicillins, narrow & broad spectrum cephalosporins | Yes | **ESBL's** ***TEM and SHV derived enzymes*** |
| Not incl | A | Not incl | 2br | Penicillins | ? | TEM 30-36 |
| II, V | A | 2c | 2c | Penicillins, carbapenems | Yes | PSE-1,3,4 |
| Ic | A | 2e | 2e | Cephalosporins | Yes | *P. vulgaris* |
| Not incl | A | Not incl | 2f | Penicillins, cephalosporins, carbapenems | Yes | Chromosomal enzymes from *Serratia marcescens* & *Enterobacter cloacae* |
| Not incl | B | 3 | 3 | Most beta lactams | No | L1, CerA |
| Ia, Ib, Id | C | 1 | 1 | Cephalosporins | No | AmpC, MIR-1 |
| V | D | 2d | 2d | Penicillins, cloxacillin | ? | OXA1-11 |
| Not incl | Unknown | 4 | 4 | Penicillins | No | *Pseudomonas cepacia* |

The Ambler scheme, proposed in 1980, classified beta lactamases into four groups - A to D. The scheme is structurally based: groups A, C and D are evolutionary distinguishable groups of enzymes and contain a serine residue at the active site. Beta lactamases, including the penicillinases of some Gram positive bacteria (e.g. *S. aureus*) as well as various plasmid encoded enzymes (such as TEM-1 and -2) from Gram negative bacilli, are

included in Class A. Enzymes which contain zinc at the active site are grouped in class B, and are referred to as metallo enzymes. One of the characteristics of these class B enzymes is the ability to hydrolyse carbapenems. In addition, these enzymes are not inhibited by beta lactamase inhibitors. Chromosomally encoded enzymes from Gram negative bacilli, which are not inhibited by beta lactamase inhibitors such as clavulanic acid, are located in class C while class D contains enzymes that preferentially hydrolyse cloxacillin as well as penicillin.

Burgeoning data on beta lactamases ushered in further classifications. Based on rates of hydrolysis, preferred substrates and susceptibility of the enzyme to beta lactamase inhibitors, the Bush classification scheme of 1989(b,c) placed beta lactamases into four groups. In this classification, group 1 type enzymes hydrolyse cephalosporins and are not inhibited by clavulanic acid, while enzymes in group 2 include most of those that are inhibited by clavulanate. In order to accommodate a large number of similar yet different enzymes, six subdivisions (2a, 2b, 2b', 2, 2d, 2e) were created within group 2. Group 3 contains the previously mentioned metallo-enzymes, and penicillinases not inhibited by clavulanate are placed in group 4.

Another proposal was made by Payne and Amyes in 1991. This classification was based on the differences in rates of hydrolysis of ceftazidime and cefotaxime by different transferable beta lactamases. Enzymes in group 1 hydrolyse both these beta lactams with poor efficiency whereas those in group 2 hydrolyse ceftazidime more readily than cefotaxime. Although group 3 contains three subgroups, a common feature of the enzymes within this group is that they all confer greater resistance to ceftazidime than to cefotaxime. Paradoxically the hydrolysis rates of cefotaxime are greater than for ceftazidime, and the greater susceptibility to cefotaxime is thought to be due to the fact that cefotaxime enters the periplasmic space more efficiently than ceftazidime [1.3.1.2]. The group 4 enzymes confer resistance to all cephalosporins, and are resistant to inhibition by clavulanate.

The most recent scheme, proposed by Bush *et al.* (1995) is probably the most comprehensive and is a modification of the previous classification proposed by Bush (1989b,c). While it retains the four classes of this scheme, two subdivisions were added to the existing six in group 2 to accommodate the increasing number of new TEM and SHV

related ESBLs. One of these subdivisions - 2br - was added to include enzymes with less affinity for beta lactamase inhibitors but still related to their counterparts in group 2. The other additional subdivision - 2bf - contains carbapenemases. While this is probably the most detailed classification to date, the numerous subdivisions in group 2 can cause confusion to both initiated and uninitiated alike.

In this review, I will confine myself to the Ambler molecular classification. In addition, as this study is concerned with the detection of TEM and SHV related beta lactamases in Gram negative bacilli, the remainder of the review will focus on these enzymes.

## 1.4 TEM- AND SHV-RELATED BETA LACTAMASES

In 1983 the first plasmid mediated beta lactamase able to hydrolyse extended spectrum cephalosporins (such as cefuroxime, cefotaxime, cetriaxone) as well as penicillins was reported (Knothe *et al.*, 1983). In the ensuing decade and a half, extended spectrum beta lactamases have flourished and are now a significant clinical problem, world-wide. The majority of ESBLs have been derived from the enzymes TEM-1, TEM -2, or SHV-1 (Philippon *et al.*, 1989). While these three may be progenitors of ESBLs they do not in themselves have the activity against the newer extended spectrum cephalosporins associated with ESBLs.

### 1.4.1 Structure and Regulation of TEM- and SHV-Related Beta Lactamases

TEM and SHV related beta lactamases consist of an alpha helical domain and beta pleated sheets. The active site containing a serine at amino acid position 70 (ser-70) is located in one of the alpha helices, and an oxyanion pocket is formed between this alpha helix and one of the beta pleated sheets. Ser-70 combined with a glutamine at position 166 (Glu-166) are the prime catalytic amino acids. A beta lactam molecule is bound in the oxyanion pocket resulting in the formation of an acyl enzyme with the carbonyl of the beta lactam ring, followed shortly thereafter by deacylation and release of the inactive hydrolysed beta lactam (Bush & Sykes, 1983; Knox, 1995).

Point mutations at certain sites in the structural TEM and SHV resistance genes and the concomitant amino acid changes result in enzymes that hydrolyse some or all of the second and third generation cephalosporins. This can be illustrated by comparing MIC (minimum inhibitory concentration) profiles of some of the cephalosporins for various ESBL producing organisms (Table 1.3) (Du Bois *et al.*, 1995).

Table 1.3

MIC profiles (μg/ml) of selected SHV and TEM derived beta lactamases (Du Bois *et al.*, 1995)

| Enzyme | Ampicillin/Penicillin* | Ceftazidime | Cefotaxime |
|---|---|---|---|
| **SHV-1** | 1024 | 0,125 | 0,03 |
| **SHV-2** | >2048 | 4 | 4 |
| **SHV-3** | 4096 | 2 | 4 |
| **SHV-4** | >2048 | 128 | 16 |
| **SHV-5** | >2048 | 64 | 8 |
| **TEM-1** | >128 | 0,25 | 0,03 |
| **TEM-5** | >128 | 128 | 4 |
| **TEM-9** | >128 | 128 | 2 |
| **TEM-10** | >128 | 32 | 0,25 |
| **TEM-12** | >128 | 2 | 0,03 |
| **TEM-17** | >128 | 0,03 | 0,25 |
| **TEM-26** | >128 | 64 | 0,25 |

* All SHV MICs are for ampicillin and TEM MICs for penicillin

As can be seen, the MICs can vary greatly depending on which ESBL is present. In some cases, such as TEM-17, the MICs may still be below the breakpoint required to label the organism resistant. However the MICs for the cephalosporin are in all cases higher than those obtained when testing organisms producing enzymes without extended spectrum activity, and the differences in activity are due to the different amino acid changes in the various enzymes.

As few as three amino acid changes in the TEM-1 structural protein are sufficient to increase its spectrum of activity to include many third generation cephalosporins. To date, twelve SHV and more than forty different TEM derivatives have been described world-wide (Mabilat & Courvalin, 1990; Mercier & Levesque, 1990; Petit *et al.*, 1990; Jacoby &

Medeiros, 1991; Payne & Amyes, 1991; Moellering, 1993; Nuesch-Inderbinen *et al.*, 1997)

The point mutations and amino acid changes found in ESBLs that enable them to hydrolyse the cephalosporins do not affect either of the two critical residues (serine and glutamine) or the less critical Lys-73, Ser-130 or Lys-234 residues. The amino acid changes that result in substrate changes have been reviewed before (Knox, 1995) and have been described both in the vicinity of and remote from the active site. Suffice it to say that the various changes induce either conformational changes in the oxyanion pocket, changes in thermodynamic factors, changes in electrostatic factors and in some cases the reason underlying the effect seen with a change in amino acid remains to be elucidated. The amino acid changes present in some extended spectrum beta lactamases are represented in Table 1.4 (Sutcliffe, 1978; Mabilat & Courvalin, 1990; Mabilat *et al.*, 1990; Ambler *et al.*, 1991; Jacoby & Medeiros, 1991; Naumovski *et al.*, 1992; Palzkill & Botstein, 1992; Davies, 1994; Jacoby, 1994; Palzkill *et al.*, 1994; Knox, 1995; Bradford *et al.*, 1996; Farzaneh *et al.*, 1996; Poyart *et al.*, 1998; Yang *et al.*, 1998).

Table 1.4

Amino acid substitutions conferring extended spectrum activity in selected TEM- and SHV-related enzymes

| Amino acid Site | 39 | 69 | 104 | 164 | 182 | 205 | 237 | 238 | 240 | 244 | 265 | 276 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Beta lactamase** | | | | | | | | | | | | |
| **TEM-1** | Gln | Met | Glu | Arg | Met | Gln | Ala | Gly | Glu | Arg | Thr | Asn |
| **TEM-2** | Lys | | | | | | | | | | | |
| **TEM-3** | Lys | | Lys | | | | | Ser | | | | |
| **TEM-4** | | | Lys | | | | | Ser | | | Met | |
| **TEM-5** | | | | Ser | | | Thr | | Lys | | | |
| **TEM-6** | | | Lys | His | | | | | | | | |
| **TEM-7** | Lys | | | Ser | | | | | | | | |
| **TEM-8** | Lys | | Lys | Ser | | | | Ser | | | | |
| **TEM-9** | | | Lys | Ser | | | | | | | Met | |
| **TEM-10** | | | | Ser | | | | | Lys | | | |
| **TEM-11** | Lys | | | His | | | | | | | | |
| **TEM-12** | | | | Ser | | | | | | | | |
| **TEM-13** | Lys | | | | | | | | | | Met | |
| **TEM-14** | Lys | | Lys | | | | | Ser | | | Met | |
| **TEM-15** | | | Lys | | | | | Ser | | | | |
| **TEM-16** | Lys | | Lys | His | | | | | | | | |
| **TEM-17** | | | Lys | | | | | | | | | |
| **TEM-18** | Lys | | Lys | | | | | | | | | |
| **TEM-19** | | | | | | | | Ser | | | | |
| **TEM-20** | | | | | Thr | | | | | | | |
| **TEM-24** | Lys | | Lys | Ser | | | Thr | | Lys | | | |
| **TEM-26** | | | Lys | Ser | | | | | | | | |
| **TEM-27** | | | | His | | | | | Lys | | Met | |
| **TEM-30** | | | | | | | | | | Ser | | |
| **TEM-31** | | | | | | | | | | Cys | | |
| **TEM-32** | | Ile | | | Thr | | | | | | | |
| **TEM-33** | | Leu | | | | | | | | | | |
| **TEM-34** | | Val | | | | | | | | | | |
| **TEM-35** | | Leu | | | | | | | | | | Asp |
| **TEM-36** | | Val | | | | | | | | | | Asp |
| **TEM-43** | | | Lys | His | Thr | | | | | | | |
| **TEM-52** | | | Lys | | Thr | | | Ser | | | | |
| **SHV-1** | Gln | Met | Asp | Arg | | Arg | Ala | Gly | Glu | Arg | Leu | Asn |
| **SHV-2** | | | | | | | | Ser | | | | |
| **SHV-3** | | | | | | Leu | | Ser | | | | |
| **SHV-4** | | | | | | Leu | | Ser | Lys | | | |
| **SHV-5** | | | | | | | | Ser | Lys | | | |
| **SHV-7** | | | | | | | | Ser | Lys | | | |

The amino acid abbreviations can be found on page xii

Despite the greater spectrum of activity conferred by the mutations, it has been shown that ESBLs have reduced catalytic activity compared to the parent TEM or SHV enzymes (Bush & Singer, 1989; Jacoby & Carreras, 1990). Purified TEM -3, -5, -9 and -10 enzymes have between 0,4 and 2,2% of the catalytic activity against amoxycillin of TEM-1 (Jacoby, 1994). This can be partly countered by the fact that plasmid mediated beta lactamases are often produced in larger quantity. An important determinant of the resistance profile is also the level of expression of the beta lactamase, particularly in the case of the ESBLs, given the lower catalytic activity of these enzymes compared to the "parent" enzymes. Increased production of beta lactamases has been implicated in the emergence of ESBLs resistant to inhibition by beta lactamase inhibitors such as clavulanic acid (Sanders *et al.*, 1988; Roy *et al.*, 1989; Thomson *et al.*, 1990). Specific mutations in beta lactamase structural genes have also been linked to resistance to beta lactamase inhibitors (Henquell *et al.*, 1994; Cormican *et al.*, 1998)

By and large, the level of expression of a resistance gene is determined by the number of copies of the gene and/or the regulation of transcription of the structural gene (Stratton, 1988). The number of copies of a resistance gene can be increased if the gene is located on a high copy number plasmid. Although most ESBL genes to date have been described on large plasmids, which are usually low copy number, the presence of even a small number of these plasmids may allow for some increase in the expression of the particular beta lactamase.

The regulation of transcription is a more complex issue, but mutations in some of the promoters of ESBL genes are thought to be responsible for increasing the level of expression of these genes. The gene encoding TEM-2 has a more efficient promoter that encoding TEM-1, which may explain why TEM-2 derivatives are more commonly encountered than their TEM-1 counterparts. Chen and Clowes (1987) showed a single base pair difference between the promoter regions of the TEM-1 and TEM-2 genes. This mutation results in two overlapping promoters for TEM-2. RNA studies showed that both promoters were recognised resulting in increased transcription of the gene and consequently up to ten times the ampicillin resistance in TEM-2 as compared to TEM-1. It has been shown subsequently that many of the enzymes derived from TEM-1 are regulated by two promoters which have presumably arisen by natural mutation (Mabilat *et al.*, 1990).

In TEM-6, an insertion sequence related to IS1, containing a –35 region, has inserted between the –10 and –35 regions of the prototype promoter. The insertion of this 116bp sequence results in a hybrid promoter consisting of the prototype –10 region of the TEM-1 promoter P3, and the –35 sequence contained in the insertion sequence. This –35 region shows perfect homology to the consensus –35 sequence for E. coli (TTGACA) (Hawley & McClure, 1983), resulting in a more efficient transcription of TEM-6 (Goussard et al., 1991). Studies have shown that SHV related ESBLs are also regulated by different promoters. In 1991 Podbielski *et al.*(b) showed with RNA studies that the different promoters of SHV-2 and SHV-2a were responsible for different levels of cefotaxime resistance conferred by the two enzymes

## 1.4.2 Location of TEM and SHV Related Beta Lactamase Resistance Genes

Plasmid encoded beta lactamases of all classes have been described. The first plasmid mediated beta lactamases, TEM-1 and -2, SHV-1 and OXA-1, were described in the mid 1960's (Bush, 1989d; Moellering, 1993) and the first plasmid mediated ESBL was described in 1983 (Knothe *et al.,* 1983). This was shown to be a derivative of SHV-1, and was called SHV-2. Subsequently there has been a veritable deluge of reports on plasmid mediated extended spectrum beta lactamases (Kliebe *et al.*, 1985; Bure *et al.*, 1988; Gutmann *et al.,* 1989; Petit *at al.*, 1990; Payne & Amyes, 1991; Mugnier *et al.*, 1996).

Transposons, genetic elements capable of migrating from one site in the genome to another, are obviously important in the spread of resistance genes. Integrons, a component of one class of transposon, could also play an important role in resistance transfer. These elements have flanking regions and a central region which is capable of acquiring and rearranging so called "gene cassettes" which can code for a variety of genes, such as resistance genes (Hall & Collis, 1995). The fact that these cassettes are capable of moving around within the integron and thus within the transposon opens up frightening possibilities for antibiotic resistance transfer and dissemination.

It is known that a number of plasmid mediated beta lactamases are transposon determined - TEM-1 and -2, SHV-1, OXA-1, and PSE-1 and -4, among others (Richmond *et al.*,

1980; Medeiros, 1984). ESBLs have also been identified on transposable elements (Sirot *et al.*, 1991; Heritage *et al.*, 1992). Many chromosomally encoded enzymes are thought to be on transposons, especially in *Klebsiella* species (Frere, 1995), and this may well be one of the mechanisms of the escape of chromosomal genes to plasmids. Although no TEM- or SHV-related ESBL genes have yet been described on integrons, other beta lactamase genes, particularly those related to the OXA "family" of beta lactamases, have been described on integrons (Danel *et al.*, 1997; Vila *et al.*, 1997; Naas *et al.*, 1998). More recently, an integron located ESBL gene (VEB-1) was identified from a clinical isolate of *Escherichia coli* (Poirel *et al.*, 1999).

## 1.4.3 Incidence and Spread of Beta Lactamases

Although there are a great number of beta lactamases as can be seen from Table 1.2, it is the ESBLs that are emerging as one of the more clinically important types (Jones, 1996). They are found almost exclusively in members of the family *Enterobacteriaceae*, including *Klebsiella* spp., *Salmonella* spp. and *Escherichia* spp. In a study of Gram negative infections in 396 Intensive Care Units (ICUs) in the USA, *E. coli* was shown to be the commonest pathogen (21,3%), followed by *P. aeruginosa* (19,7%), *Enterobacter* spp. (16,1%) and *Klebsiella* spp. (15,3%) (Itokazu *et al.*, 1996). Respiratory tract infections accounted for 47% of the infections caused by these organisms, followed by urinary tract infections (UTIs), bacteraemias and wound infections.

### *1.4.3.1 Incidence of Beta Lactamase Mediated Resistance*

Although beta lactamase resistance genes may be located on transposons, integrons, plasmids or in the chromosome of bacteria, all TEM and SHV related enzymes are plasmid mediated. A disturbing rise in ESBL mediated resistance in Gram negative pathogens has been documented over the last 5 - 10 years. The emergence and spread of these enzymes has been linked to the transfer of conjugative plasmids between bacterial strains.

National Nosocomial Infection Surveillance (NNIS) data from the USA show that the resistance to extended spectrum beta lactams amongst *K. pneumoniae* has risen from 1,5% in 1986 to nearly 13% in 1993 (Archibald *et al.*, 1997). This increase is not confined to

*Klebsiella* spp; ceftazidime resistant *Enterobacter* spp have increased from 30,8 % in 1990 to 38,3% in 1993 (Itokazu *et al.*, 1996). Beta lactam resistance in *E. coli* rose from 8 - 28 % from 1984 - 1987 (Sanders & Sanders, 1992). A study of ampicillin resistant *E. coli* isolates in 1990 showed that the majority of beta lactamases in these organisms were TEM-related (Cooksey *et al.*, 1990). Beta lactam resistance in *E. coli* and *K. pneumoniae* isolates is usually due to the production of TEM- or SHV-related beta lactamases, whereas beta lactam resistance in *Enterobacter* spp. is more likely to be on the basis of chromosomal beta lactamase production – the so-called AmpC beta lactamase (Livermore, 1995).

In 1986, none of the isolates of *K. pneumoniae* from a hospital in Chicago (Rush-Presbyterian-St Luke's) were ceftazidime resistant. By 1993, 27% of them displayed ceftazidime resistance (Schiappa *et al.*, 1996). Similarly, at Mercy Hospital in Chicago, the percentage of *K. pneumoniae* isolates resistant to ceftazidime increased from about 2% to 15% between 1988 and 1990. This resistance was felt to be predominantly due to ESBL production by the isolates. It is interesting to note that the use of Ceftazidime in Mercy Hospital had increased nearly six-fold from 1987 to 1991 (1000g in 1987 and 5888g in 1991) (Bradford *et al.*, 1994).

In 1990, a study in the United States showed approximately 3,6% of klebsiella isolates to be resistant to ceftazidime. This figure quadrupled to 14,4% in 3 years. While it is probable that the resistance rates will plateau and not rise at this rate indefinitely, it is still a cause for concern in clinical and microbiological circles (Itokazu *et al.*, 1996). In 1994, 60% of *E. coli* isolates in New York City were resistant to ceftazidime. Similar studies have shown similar increases in Europe: of 1000 *Klebsiella* spp. from ICU's throughout Europe, 23% were shown to be resistant to ceftazidime due to ESBL production (Livermore & Yuan, 1996).

It can be seen that the problem is world-wide and while some countries may have slightly higher or lower incidences of ESBLs in Gram negative bacilli, no country would appear to be immune. No large scale studies of the problem have been carried out in South Africa, but it is thought that SHV-2 and -5, as well as TEM 26 are the commonest ESBLs in this country (Pitout, 1996).

Resistance rates have been found to be the highest in Intensive Care Units, and it is thought that resistance often emerges in an ICU, and spreads to the hospital and the community (De Champs *et al.*, 1991; Sirot *et al.*, 1992; Burwen *et al.*, 1994). Risk factors for colonisation or infection by ESBL producing Gram negatives include length of stay in an ICU, recent surgery, instrumentation, prolonged stay in hospital and recent antibiotic exposure, particularly to extended spectrum beta lactam agents.

There is a clear link between these factors and colonisation by resistant organisms. In terms of the focus of this dissertation, what deserves more consideration is the extent to which the use of the newer third generation cephalosporins has encouraged the emergence of resistance.

A number of authors have linked the use of these agents to the emergence of resistance (Stratton 1988; Bush, 1989d; Neu, 1990b; Peña *et al.*, 1998). As previously mentioned, 75% of nursing home patients were found to be colonised by ceftazidime resistant Gram negative organisms soon after admission to a hospital. Ceftazidime was extensively used in these nursing homes (Quinn, 1994). It has been shown that use of a third generation cephalosporin within 2 weeks of an *Enterobacter* infection is associated with a significantly greater risk of subsequent infection by a cephalosporin resistant isolate. Infection by resistant *Enterobacter* organisms is also associated with a higher mortality rate - 32% vs 15% (Chow *et al.*, 1991). Although this resistance may be due to chromosomal beta lactamases (AmpC) rather than ESBLs, the principle of a resistant organism being associated with higher morbidity and mortality would hold true for ESBL producing isolates as well.

#### *1.4.3.2 Spread of Beta Lactamase Mediated Resistance*

The spread of antibiotic resistance is always cause for concern, particularly in the hospital setting. As detailed previously, since their first description in 1983 plasmid mediated ESBLs have been reported from many countries, and in many different species of *Enterobacteriaceae* (Tenover, 1991; Payne & Amyes, 1991; Jacoby & Medeiros, 1991).

Plasmids encoding ESBLs have been transferred from strain to strain in vitro, and have also proven to be responsible for spread of beta lactam resistance within and between

wards. In 1990 a plasmid containing a gene encoding for TEM-3 was shown to be self transmissible, and was thought to have spread from an isolate of *S. marcescens* to seven different bacterial genera over 4 years in an area of France (Petit *et al.*, 1990). In the early 1990's, a study of multi-drug resistant *K. pneumoniae* isolates in a paediatric ward in France was undertaken. The result of ribotyping and plasmid profile studies showed genetically unrelated strains of the organism with the same plasmid content. Although these plasmids were not shown to contain the genes coding for the ESBL, all the isolates involved in the outbreak were ESBL producers and the possibility was raised that at least some of the plasmids contained ESBL genes (Bingen *et al.*, 1993). In 1995, a Tunisian isolate of *Salmonella enteritidis* was shown to have acquired a resistance plasmid encoding an ESBL *in vivo* following cefotaxime therapy. This resistance plasmid was thought to have originated in another intestinal organism (Barguellil *et al.*, 1995).

In institutions such as nursing homes patients are in close contact with each other and may be exposed to antibiotics more frequently than other community members. In this setting development of resistance and subsequent transfer of resistance plasmids to other organisms can lead to clinical problems. The problem is compounded when patients from the nursing home require hospitalisation and thereby import resistant organisms to the hospital. In a Chicago hospital it was noted that up to 75% of ceftazidime resistant Gram negative isolates originated from patients who came from nursing homes. Most of this resistance appeared to be due to the production of the TEM-10 beta lactamase, suggesting transfer of a common resistance plasmid from organism to organism within the home (Quinn, 1994).

Direct spread of organisms from patient to patient has also been demonstrated in similar situations. A study of ESBL producing *Klebsiella pneumoniae* strains in a geriatric ward showed that the outbreak was due to a single clone that had spread within the ward (Gouby *et al.*, 1994). Another study in France showed spread of an ESBL producing *K. pneumoniae* strain from one hospital to another with subsequent spread within the respective hospitals. The organism was thought to have been carried by an infected patient who was transferred between the hospitals (Bure *et al.*, 1988). Two other outbreaks of ESBL producing *K. pneumoniae* strains were also shown to be due to a single clone of the organism (Gazouli *et al.*, 1997; Peña *et al.*, 1998).

Resistance spread within institutions is thus a complex and common problem, with both organism and plasmid transfer taking place. These data reinforce the need for strict infection control measures, particularly in cases where a multi-drug resistant organism is implicated in the infection.

Spread of ESBLs by the expedient of international travel has been reported. Strains of *K pneumoniae* producing SHV type ESBLs (SHV-2 and -5) have been reportedly imported to the UK from Egypt and Greece (Shannon *et al.*, 1990). Similar imported cases have been reported in France, Egypt and other European and African countries (Philippon *et al.*, 1994). While the clinical importance of this mode of "transfer" is not known, it is unlikely to be as significant as plasmid transfer between organisms.

It is not only pathogens that constitute a problem when considering the spread of ESBLs. Plasmids encoding resistance genes may remain cryptic in certain bacteria such as commensals in the gastro-intestinal tract (GIT). The introduction of an antibiotic into this environment would place selective pressure on organisms harbouring resistance genes, allowing them to flourish and increasing the relative proportions of resistant organisms within the GIT and possibly also within the general community.

A study in Finland showed that 38% of Gram negative enteric bacilli from healthy people were resistant to ampicillin although no data was presented on transmissibility of this resistance (Leistevuo *et al.*, 1996). A study in South Africa showed that amoxicillin resistance was present in 88% of the faecal flora of healthy people (adults and children) and 26% of this resistance was carried on self-transferable plasmids. While it was found that the majority of this resistance was mediated by TEM-1, which is not an ESBL, it still lends weight to the theory that faecal flora can act as a reservoir for resistance plasmids which may harbour ESBLs. Another concern is that many of these plasmids can carry multiple resistance genes, coding for resistance to commonly used antibiotics such as tetracycline and sulphonamides (Shanahan *et al.*, 1995). With the increasing use of oral antibiotics it is easy to see how selective pressure could result in the proliferation of these commensals allowing them to act as a potential source of resistance determinants.

Given the increasing spread and incidence of ESBL mediated resistance, detection of ESBLs is increasingly important. Reliable detection of these enzymes is however difficult.

Allied to this is the fact that an isolate harbouring an ESBL may appear sensitive *in vitro* despite being resistant *in vivo*. This can result in isolates being incorrectly reported as sensitive to cephalosporin antibiotics. Subsequent treatment failure, with its attendant higher morbidity and mortality, has been described both experimentally and in the clinical setting (Rice *et al.*, 1991; Karas *et al.*, 1996).

### 1.4.4 Detection and Characterisation of Beta Lactamase Mediated Resistance

The importance of detecting beta lactamases, especially ESBLs, was alluded to previously. For epidemiological purposes it is important to characterise ESBLs. This information may allow determination of whether a particular beta lactamase has arisen as a result of a new mutation or is a previously characterised enzyme in a hithertofore unsuspected organism or location. This section will deal firstly with some of the tests commonly used to characterise the enzymes and then address the problem of detection in more detail.

A number of laboratory tests, including isoelectric focussing and determination of both substrate and inhibition profiles, have been used for the detection and characterisation of beta lactamses (Sykes & Matthew, 1976; Bush, 1989a). Isoelectric focusing was first used in 1975 (Mathew *et al.*, 1975) for the detection and characterisation of beta lactamases. While this technique has proved to be useful in characterising these enzymes it has its drawbacks. It is a technically demanding test and suffers from the disadvantage that many related beta lactamases, especially ESBLs, cluster at the same pI. For example, most of the TEM related enzymes (of which there are more than forty) have pI's clustered around 5.5 - 6.3 (Mabilat & Courvalin, 1990; Jacoby & Medeiros, 1991) making differentiation difficult. The information is often combined with the results of other investigations and may be useful as supportive or confirmatory evidence when investigating an outbreak of beta lactamase producing organisms (Nouvellon *et al.*, 1994).

The rates at which different beta lactamases hydrolyse various antibiotics has also been used as a means of characterising enzymes. A number of antibiotics are used to determine the substrate profile of an enzyme. Benzylpenicillin, ampicillin, carbenicillin and cloxacillin are the penicillin compounds traditionally used. Of the cephalosporins, cephaloridine and

cephalothin were used initially. However, with the advent of the ESBLs, it was necessary to include the extended spectrum cephalosporins cefotaxime and ceftazidime in the substrate profile analysis. Other studies have also included antibiotics such as cefoxitin, ceftriaxone and imipenem (Bush, 1989c,d; Liu *et al.*, 1992; Bauernfeind *et al.*, 1996a). Both maximal hydrolysis rates ($V_{max}$) and enzyme affinities (Km) should be included in the characterisation of a new beta lactamase, although in many instances only the $V_{max}$ is determined.

Various substances, including beta lactamase inhibitors, are known to inhibit the action of beta lactamases by irreversibly inactivating the enzyme and different enzymes are affected to varying degrees by these inhibitors. Cephalosporinases are thought to be more strongly inhibited by aztreonam than by clavulanic acid, whereas the reverse is true for penicillinases, and this can be used as a means of distinguishing cephalosporinases from penicillinases (Bush, 1989a).

Detection of the presence of an ESBL based on *in vitro* susceptibility tests has proven to be a problem (Meyer *et al.*, 1993). In the laboratory many of the clinical strains that produce ESBLs do not appear resistant to cephalosporins, or at best they appear resistant to only some of the cephalosporins. Vercauteren *et al.* (1997) showed that the standard disc diffusion test using ceftazidime detected only 48% of the ESBL producers. Allied to this problem is the consideration that with so many different cephalosporins available it is not practical to test every one of them. Representative antibiotics are often tested and different antibiotics chosen for testing depending on the suspected organism. The deduction of the resistance mechanism in the organism from these results has been called "interpretative reading" and allows prediction of the expected *in vivo* resistance phenotype (Courvalin, 1992).

The presence of an ESBL in an organism may not lead to a high enough level of resistance to the antibiotic being tested to appear resistant on current criteria (either zone size or MIC). It has been reported that as few as 23% of ESBL producing *K. pneumoniae* isolates will be reported as resistant using NCCLS criteria on disc diffusion testing with cefotaxime (Jacoby & Han, 1996). This problem has also been noted for aztreonam, cefotaxime, ceftriaxone and ceftazidime in different studies (Bure *et al.*, 1988; Katsanis *et*

*al.*; 1994; Livermore & Yuan, 1996; Sanders *et al.*, 1996), and incidences of treatment failure using a supposedly appropriate antibiotic have been reported (Karas *et al.*, 1996).

The above problem is related to the inoculum effect. If the inoculum tested *in vitro* is smaller than the inoculum *in vivo*, the MIC of the antibiotic for that organism may rise dramatically, with ensuing treatment failure (Rice *et al.*, 1991; Katsanis *et al.*, 1994). This is a result of the increased amount of beta lactamase produced by the larger number of organisms. This led to the recommendation by the NCCLS that any organism which is shown to produce an ESBL should be regarded as resistant to all cephalosporins, with the possible exception of cefepime (NCCLS, 1998).

Since susceptibility testing on its own is often inadequate for determining the presence of ESBLs, and given the importance of detecting the enzymes, various strategies for overcoming the difficulties in detecting ESBL production have been devised. The following section will deal with some of the more promising methods.

#### *1.4.4.1 Phenotypic Methods*

##### *i) Double Disc Diffusion Test*

ESBLs are inhibited (albeit sometimes to different degrees) by beta lactamase inhibitors (clavulanate, sulbactam, tazobactam). A disc containing one of the cephalosporins (often ceftazidime) is placed proximal to a disc containing clavulanate on agar inoculated with an isolate. If an ESBL is produced by the isolate an increased zone of inhibition of growth will occur between the two antibiotic discs, often giving rise to a dumbbell shaped zone of inhibition (Fig 1.5). This occurs because the clavulanate inhibits the action of the ESBL and the cephalosporin can then act in the absence of the beta lactamase.

Although some authors have reported great success with this method (Mabilat & Courvalin, 1990; Karas *et al.*, 1996), others have found it to be unreliable and that inter-laboratory variation may occur. This is due to the fact that the discs need to be placed an appropriate distance apart (a distance that has not to date been reliably determined). The determination of whether this "zone of inhibition" is present is also subject to inter-observer differences (Cormican *et al.*, 1996; Jacoby & Han, 1996; Sanders *et al.*, 1996).

Notwithstanding these problems, this test is still widely used because of the ease with which it can be incorporated into a laboratory's routine susceptibility testing.

Figure 1.5 Double disc test on an ESBL producing isolate of *K. pneumoniae*, showing the characteristic zone of extension between the cefotaxime and clavulanic acid discs. Note the absence of such a zone between the ceftazidime and clavulanate discs. Left-hand disc contains cefotaxime, middle disc contains amoxicillin/clavulanic acid, lower right-hand disc contains ceftazidime.

*ii) Vitek ESBL Test*

This test also utilises clavulanic acid. Isolates are inoculated into 4 wells, two containing cefotaxime and ceftazidime (0,5 µg/ml) alone and two wells containing each of the antibiotics plus clavulanic acid (4 µg/ml). After incubation, a predetermined reduction in growth in the wells with antibiotic plus clavulanic acid as compared to the wells with antibiotic alone is taken to indicate the presence of an ESBL. A study conducted in 1996 (Sanders *et al.*) compared this test with the double disc diffusion test, using organisms containing previously characterised beta lactamases, or by determining isoelectric points and substrate profiles on the beta lactamase. The organisms tested were *K. pneumoniae*

and *E. coli,* as well as a smaller number of *Enterobacter* species and miscellaneous Gram negative bacilli.

The results showed that the Vitek ESBL test was 99,5% sensitive and 100% specific in detecting ESBLs, compared to 98% and 99,4% sensitivity and specificity respectively for the double disc test. The test was also shown to be better able to distinguish between an ESBL and the chromosomal beta lactamase of *K. oxytoca*. Whether this method will stand up to further testing and become widely used to detect ESBLs remains to be seen.

*iii) Etest ESBL Screen*

This is similar to the Vitek ESBL test although instead of inoculating the organism into wells containing antibiotic, plastic strips impregnated with the antibiotic are placed on agar inoculated with the organism. One side of the strip is impregnated with a gradient of ceftazidime (32 - 0,12µg/ml) while the other side contains the same ceftazidime gradient plus a fixed concentration of clavulanate (2µg/ml). The strips are placed on agar inoculated with the strain to be tested, incubated, and the MICs for the antibiotic and the antibiotic plus inhibitor determined from where the zone of inhibition meets the plastic strip. The criteria for a positive result (i.e. ESBL present) are reduction of the ceftazidime MIC by more than two log dilution steps when clavulanate is present. Using this criterion, the Etest has been shown to be more sensitive than the double disc test (100% vs 87% sensitivity) (Cormican *et al.*, 1996). A major drawback to the Etest is the cost, a consideration particularly important in South Africa.

*iv) Other Tests for Detection of ESBLs*

Apart from the above methods, a number of other strategies for the detection of ESBLs have been proposed. It has been reported that a disc with 5µg of ceftazidime and a different breakpoint is more sensitive in detecting resistance than the usual 30µg ceftazidime disc (Jacoby & Han, 1996). Alternatively, results of disc diffusion tests using cefpodoxime have been shown to correlate very well with the presence of an ESBL (Coudron *et al.*, 1997). Broth microdilution panels have also shown some promise in detecting ESBL production (Moland *et al.*, 1998).

Genes coding for ESBL production are often present on large plasmids which contain other antibiotic resistance genes. This may allow one to infer that an organism with phenotypic resistance to another class of antibiotic (aminoglycosides for example) may also produce an ESBL despite this not being apparent on routine sensitivity testing. However this is very non-specific and resistance to non beta-lactam antibiotics is obviously not enough to unequivocally assume to presence of an ESBL (Katsanis *et al.*, 1994).

#### *1.4.4.2 Molecular Techniques*

Molecular techniques have proved valuable tools for the characterisation and detection of beta lactamase resistance genes. DNA sequencing has allowed phylogenetic relationships to be explored, and has opened up the way for the use of DNA-DNA hybridisation in the detection and identification of beta lactamase genes. Amino acid sequences, whether deduced from DNA or determined primarily, also provide phylogenetic information and facilitate the analysis of structure-function relationships (Mabilat & Courvalin, 1990; Knox, 1995; Bauernfeind *et al.*, 1996a,b).

##### *i) DNA-DNA Hybridisation*

The use of DNA probes for the detection and characterisation of beta lactamases has been well described. A number of studies have used this technique in epidemiological surveys of beta lactamases (Mabilat & Courvalin, 1990; Ling *et al.*, 1994; Huovinen *et al.*, 1988). In addition, it allows for more rapid analysis of samples than isoelectric focusing (Ouellette *et al.*, 1988). The probes and techniques can be varied to either specifically identify an ESBL gene or to broadly categorise the gene into either TEM or SHV related groups. The use of DNA-DNA hybridisation techniques in the identification and characterisation of TEM and SHV related ESBLs will be discussed in subsequent chapters.

##### *ii) Polymerase Chain Reaction (PCR)*

Knowledge of DNA sequences of the beta lactamase resistance genes has allowed PCR to be used both for the detection and characterisation of beta lactamases. Using primers complementary to conserved sequences in, for example, TEM-related genes, the presence of a TEM related gene can be ascertained using PCR. Similarly, SHV related genes (or

any other beta lactamase gene) could be detected using appropriate primers (Leung *et al.*, 1997; Payne & Thomson, 1998). As with hybridisation, the use of PCR for detecting ESBL genes will also be discussed in more detail in subsequent chapters.

## 1.5 AIM OF STUDY

Beta lactamases are an ongoing and growing problem in clinical and laboratory circles. While the incidence of ESBLs in South Africa is not accurately known, SHV-2, SHV-5 and TEM 26 are though to be prevalent in this country (Pitout, 1996). A study examining isolates from four major medical centres throughout South Africa (Cape Town, Durban, Johannesburg and Pretoria) found these ESBLs to be present in various isolates of *Enterobacteriaceae* (Pitout *et al.*, 1998b).

The incidence of ESBL activity may be underestimated because of the problems associated with detecting this activity. It is suggested that the ESBL incidence in some areas of the world may be as high as 74% (Sanders & Sanders, 1992). There is little information pertaining to large hospitals in the Western Cape – it is in large teaching hospitals that beta lactamases are known to proliferate – and none directly pertaining to Groote Schuur Hospital.

The aims of this study were firstly to investigate the molecular epidemiology of TEM- and SHV-related beta lactamases at Groote Schuur Hospital, secondly to compare and contrast various methods of detecting ESBLs and ESBL activity, and thirdly to compare various molecular methods of detecting the presence of TEM- or SHV-related genes.

# CHAPTER TWO

# BACTERIAL ISOLATES AND ANTIBIOTIC SENSITIVITY TESTING

## 2.1 –IDENTIFICATION OF BACTERIAL ISOLATES

All media and solutions mentioned in this and subsequent chapters are to be found in Appendix A. The sample used in this study was selected from 209 clinical isolates of Gram negative bacilli collected between January 1991 and June 1995 by the clinical microbiology laboratory at Groote Schuur Hospital, Cape Town. Bacterial strains had been either freeze dried or stored on Dorset's egg agar. Following inoculation onto MacConkey agar and incubation aerobically at 37°C overnight, the organisms were identified using standard biochemical tests (Collee & Miles, 1989) used in the Groote Schuur hospital clinical laboratory.

## 2.2 ANTIBIOTIC SENSITIVITY TESTING

Antibiotic sensitivity tests were carried out using the Kirby Bauer disc diffusion test on Mueller Hinton agar incubated aerobically at 37°C for 18 hours as recommended by the NCCLS (NCCLS, 1997). A variety of beta lactam antibiotics and beta lactam/beta lactamase-inhibitor combinations were tested. Other antibiotic sensitivities were not determined since the focus of this research is on beta lactamase production. Discs contained the following antibiotics - amoxicillin/clavulanic acid, ceftriaxone, ceftazidime, cefoxitin, cefuroxime, imipenem, piperacillin/tazobactam. All discs were supplied by Oxoid (Hampshire, UK) except piperacillin/tazobactam which was supplied by Mast diagnostics (Merseyside, UK). The zone size criteria (Table 2.1) used to determine sensitive, intermediate and resistant strains of bacteria were those recommended by the NCCLS (NCCLS, 1995).

Table 2.1

Zone size criteria (mm) recommended by the NCCLS for sensitivity testing (NCCLS, 1995)

| | Amount (µg) | Resistant | Intermediate | Sensitive |
|---|---|---|---|---|
| **Amoxicillin/ Clavulanic acid** | 20/10 | ≤ 13 | 14 - 17 | ≥18 |
| **Piperacillin/ Tazobactam** | 100/10 | ≤ 17 | 18 - 20 | ≥ 21 |
| **Ceftazidime** | 30 | ≤ 14 | 15 - 17 | ≥ 18 |
| **Ceftriaxone *** | 30 | ≤ 14 | 15 - 17 | ≥ 18 |
| **Cefoxitin** | 30 | ≤ 14 | 15 - 17 | ≥ 18 |
| **Cefuroxime** | 30 | ≤ 14 | 15 - 17 | ≥ 18 |
| **Imipenem** | 10 | ≤ 13 | 14 - 15 | ≥ 16 |

* In the 1998 NCCLS Standards the criteria relating to ceftriaxone, ceftizoxime and cefotaxime have changed, with larger zones now required in order for an organism to be sensitive to these antibiotics. (NCCLS, 1998).

Minimum inhibitory concentrations (MICs) of aztreonam, cefotaxime, ceftazidime and ceftazidime/clavulanic acid were determined using E-strips (AB Biodisk, Solna, Sweden). A single colony of the isolate was inoculated into 0,5ml Mueller Hinton broth to make a 0,5 McFarland standard (NCCLS, 1997). A swab was dipped into the inoculum, excess medium was squeezed out against the side of the test tube and the inoculum spread onto Mueller Hinton agar in 150mm diameter Petri dishes using the method recommended by the manufacturer. The inoculum was allowed to dry for 15 minutes and the E-strips were placed on the inoculum and incubated at 37°C overnight. MICs were determined by assessing which concentration of antibiotic along the gradient had inhibited bacterial growth (Fig 2.1).

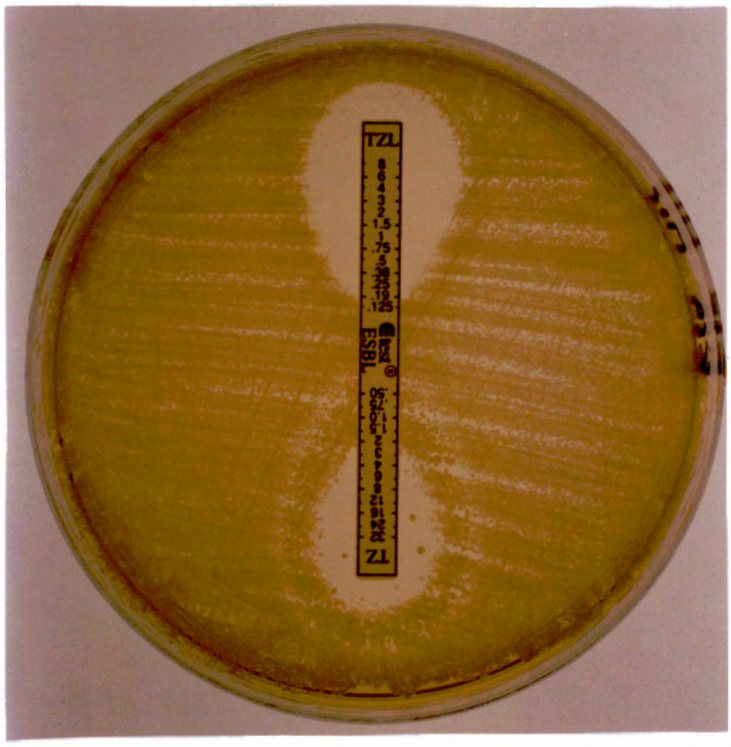



Fig 2.1 MIC determination using a ceftazidime - ceftazidime/clavulanate E-strip. The MIC is determined by visually assessing which antibiotic concentration along the gradient of the E-strip inhibits bacterial growth. Note also the presence of mutant colonies around the ceftazidime containing half, which may interfere with assessment of the MIC. TZL represents ceftazidime/clavulanate and TZ represents ceftazidime. MICs are read as μg/ml.

## 2.3 DETECTION OF ESBL PRODUCTION

Two methods were used to detect the presence of ESBLs. Firstly, the double disc diffusion test [1.4.4.1] was used to detect the presence of these enzymes. An amoxicillin/clavulanic acid disc was placed between ceftazidime and ceftriaxone discs (20mm between adjacent edges of the discs). Any dumb-bell shaped extension of the zone of inhibition around either of these discs towards the clavulanate disc was taken as indicative of the presence of ESBL activity. In certain cases, where the results were equivocal or negative, the test was repeated and the ceftazidime and ceftriaxone discs were placed 10 mm and 15 mm from the clavulanic acid containing disc and incubated overnight at 37°C.

E-strips were also used to test for ESBL production. Strips with a range of ceftazidime concentrations (0,5 - 32μg/ml) along one side and ceftazidime (0,125 – 8μg/ml) plus a fixed amount of clavulanate (4μg/ml) along the other side were used for this purpose. A ratio of the ceftazidime MIC to the ceftazidime plus clavulanate MIC of greater than four (i.e >2log dilutions) was taken to be indicative of the presence of an ESBL (Cormican *et al.*, 1996).

# 2.4 RESULTS

## 2.4.1 Identification and Antibiotic Susceptibility

The identity of 160 of the 209 isolates was established although in some instances it was not possible to speciate an organism using the standard biochemical tests used in our laboratory. The remaining 49 isolates were either non-viable or yielded mixed growth on McConkey agar. Based on the identity and antibiotic susceptibility profiles of the 160 isolates, 45 were chosen for further study. The 45 isolates selected represented a variety of organisms as well as a representative of each antibiotic susceptibility pattern. For example, all *K. pneumoniae* isolates with differing antibiograms were included. The identity and antibiotic susceptibility profile of these 45 isolates is shown in Table 2.2 and the MICs of aztreonam, cefotaxime, ceftazidime and ceftazidime/clavulanic acid for the isolates are detailed in Table 2.3.

Table 2.2 Identities and antibiotic resistance profiles of the 45 selected isolates.

| Isolate | Identity | Zone (Amc – Cro) | Cro | Amc | Zone (Amc – Caz) | Caz | Fox | Cxm | Tzp |
|---|---|---|---|---|---|---|---|---|---|
| K4 | *K. pneumoniae* | + | 17 I | 20 S | + | 20 S | 24 S | 15 I | 24 S |
| K8 | *K. pneumoniae* | +* | NZ R | 15 I | -* | 11 R | NZ R | NZ R | 17 R |
| K11 | *K. pneumoniae* | + | 16 I | 16 I | + | 22 S | 26 S | 24 S | 25 S |
| K16 | *K. pneumoniae* | + | 17 I | 20 S | + | 19 S | 11 R | 9 R | 20 I |
| K17 | *K. pneumoniae* | - | 12 R | 17 I | + | 14 R | 24 S | 13 R | 21 S |
| K18 | *K. pneumoniae* | - | 17 I | 17 I | + | 20 S | 22 S | 15 I | 21 S |
| K29 | *K. pneumoniae* | + | 15 I | 19 S | + | 14 R | 17 I | 10 R | 20 I |
| K35 | *K. pneumoniae* | - | 10 R | 15 I | + | 15 I | 25 S | 10 R | 18 I |
| K36 | *K. pneumoniae* | + | 14 R | 17 I | + | 18 S | 24 S | 14 R | 21 S |
| K39 | *K. pneumoniae* | - | 9 R | 15 I | + | 13 R | 25 S | 9 R | 15 R |
| K42 | *K. pneumoniae* | - | 19 S | 16 I | + | 7 R | 25 S | 22 S | 20 I |
| K43 | *K. pneumoniae* | - | 15 I | 17 I | + | 19 S | 25 S | 13 R | 19 I |
| K46 | *K. pneumoniae* | +* | 11 R | 18 S | +* | NZ R | 27 S | 10 R | 18 I |
| K50 | *K. pneumoniae* | - | 14 R | 15 I | + | 20 S | 25 S | 15 I | 18 I |
| K52 | *K. oxytoca* | - | 11 R | 12 R | - | 24 S | 22 S | NZ R | NZ R |
| K54 | *K. pneumoniae* | +* | NZ R | 13 R | +* | NZ R | 12 R | NZ R | NZ R |
| K58 | *K. pneumoniae* | + | 7 R | 15 I | + | 10 R | 15 I | NZ R | 13 R |
| K63 | *K. pneumoniae* | + | 15 I | 16 I | + | NZ R | 26 S | 15 I | 24 S |
| K68 | *K. pneumoniae* | + | 17 I | 15 I | + | 20 S | 24 S | 15 I | 19 I |
| K69 | *K. pneumoniae* | + | 13 R | 14 I | + | 20 S | 24 S | 14 R | 20 I |
| M9 | *C. freundii* | - | 10 R | 10 R | - | NZ R | NZ R | NZ R | 17 R |
| M10 | *C.* sp. | - | 8 R | 10 R | - | 8 R | NZ R | NZ R | 21 S |
| M20 | *C.* sp. | - | 8 R | 8 R | - | 8 R | NZ R | NZ R | 18 I |
| M25 | *K. pneumoniae* | -* | NZ R | 10 R | -* | NZ R | 15 I | 7 R | 13 R |
| M26 | *K. pneumoniae* | + | 20 S | 20 S | + | 24 S | 27 S | 20 S | 26 S |
| M28 | *K. pneumoniae* | + | 15 I | 18 I | + | 16 I | 26 S | 14 I | 21 S |
| M30 | *K. pneumoniae* | -* | 17 I | 19 S | -* | 20 S | 24 S | 17 I | 24 S |
| M37 | *E. cloacae* | - | NZ R | 11 R | - | 12 R | NZ R | NZ R | 19 I |
| M40 | *E. cloacae* | -* | 16 I | 18 S | -* | 10 R | 24 S | 13 R | 22 I |
| M43 | *P. agglomerans* | -* | NZ R | 9 R | -* | 12 R | NZ R | NZ R | 16 I |
| M44-1 | *Salmonella* sp. | +* | 15 I | 21 S | +* | NZ R | 28 S | 13 R | 22 I |
| M44-2 | *Salmonella* sp. | +* | 15 I | 21 S | +* | NZ R | 28 S | 13 R | 22 I |
| M46 | *S. marscecens* | - | 12 R | NZ R | - | NZ R | 16 I | NZ R | 16 R |
| E1 | *E. cloacae* | - | 9 R | 11 R | - | 11 R | 13 R | NZ R | 20 I |
| E5 | *E. cloacae* | - | NZ R | 12 R | - | 7 R | NZ R | NZ R | 18 I |
| E7 | *E. cloacae* | - | 8 R | 10 R | - | 15 I | NZ R | NZ R | 24 S |
| E8 | *E.* sp. | - | 17 I | 15 I | - | 15 I | NZ R | NZ R | 20 I |
| E10 | *E.* sp. | - | 15 I | 14 I | - | 16 I | NZ R | NZ R | 20 I |
| E12 | *E. cloacae* | - | 16 I | 8 R | - | 15 I | NZ R | NZ R | 20 I |
| E13 | *E. cloacae* | - | NZ R | 10 R | - | 11 R | NZ R | NZ R | 21 I |
| E16 | *E. cloacae* | - | 13 R | 12 R | - | 7 R | 7 R | NZ R | 22 S |
| E17 | *E. cloacae* | - | 28 S | 10 R | - | 29 S | NZ R | 24 S | 27 S |
| E21 | *E.* sp. | - | 15 I | 13 R | - | 15 I | NZ R | 7 R | 19 I |
| E37 | *E.* sp. | - | 15 I | 15 I | - | 13 R | NZ R | NZ R | 18 I |
| E49 | *E. cloacae* | + | 20 S | 11 R | + | 25 S | 10 R | 17 I | 26 S |

**Zone** '+' represents a zone of inhibition towards a BLI (beta lactamase inhibitor) '-' represents no zone of inhibition towards a BLI '*' organism tested twice for zone of inhibition . Zone sizes in mm. NZ = growth up to disc; S = sensitive, I = intermediate, R = resistant. **Cro** = Ceftriaxone, **Amc** = amoxicillin/clavulanic acid, **Caz** = ceftazidime, **Fox** = cefoxitin, **Cxm** = cefuroxime, **Tzp** = piperacillin/tazobactam . Imipenem not listed - all isolates were sensitive to imipenem

Table 2.3

MICs (μg/ml) of the 45 selected isolates

| *ISOLATE* | *IDENTITY* | *CEFOTAXIME* *R≥64 S≤8μg/ml* | *AZTREONAM* *R≥32 S≤8μg/ml* | *CEFTAZIDIME* *R≥32 S≤8μg/ml* | *CEFTAZ/CLAV* | *ESBL* |
|---|---|---|---|---|---|---|
| K4 | *K. pneumoniae* | 1,5 | 1,5 | 8 | 0,75 | Y |
| K8 | *K. pneumoniae* | >256 | >25 | >32 | >8 | N |
| K11 | *K. pneumoniae* | 3 | 1 | 12 | 0,75 | Y |
| K16 | *K. pneumoniae* | 12 | 2 | 6 | 0,75 | Y |
| K17 | *K. pneumoniae* | 8 | 1,5 | >32 | 0,5 | Y |
| K18 | *K. pneumoniae* | 4 | 3 | >32 | 1,5 | Y |
| K29 | *K. pneumoniae* | 2 | 32 | >32 | 0,38 | Y |
| K35 | *K. pneumoniae* | 12 | 3 | >32 | 0,75 | Y |
| K36 | *K. pneumoniae* | 4 | 1,0 | 8 | 0,38 | Y |
| K39 | *K. pneumoniae* | 16 | 4,0 | >32 | 0,5 | Y |
| K42 | *K. pneumoniae* | 0,5 | 6 | >32 | 0,5 | Y |
| K43 | *K. pneumoniae* | 12 | 1,5 | 12 | 0,125 | Y |
| K46 | *K. pneumoniae* | 24 | >256 | >32 | 0,75 | Y |
| K50 | *K. pneumoniae* | 4 | 2 | >32 | 1,5 | Y |
| K52 | *K. oxytoca* | 3-4 | >256 | 3 | 1 | N |
| K54 | *K. pneumoniae* | >256 | 64 | >32 | 0,75 | Y |
| K58 | *K. pneumoniae* | >25 | 16 | >32 | 3 | Y |
| K63 | *K. pneumoniae* | 3 | 48 | >32 | 0,5 | Y |
| K68 | *K. pneumoniae* | 2 | 1 | 24 | 1,5 | Y |
| K69 | *K. pneumoniae* | 6-8 | 4 | >32 | 0,5 | Y |
| M9 | *C. freundii* | 32 | 16 | >32 | >8 | N |
| M10 | *C.* sp. | 64 | 24 | >32 | >8 | N |
| M20 | *C.* sp. | 48 | 24 | >32 | >8 | N |
| M25 | *K. pneumoniae* | 48 | 6 | >32 | 0,5 | Y |
| M26 | *K. pneumoniae* | 1 | 0,5 | 2 | 0,38 | Y |
| M28 | *K. pneumoniae* | 4 | 1,5 | >32 | 0,5 | Y |
| M30 | *K. pneumoniae* | 8 | 1,5 | 12 | 0,5 | Y |
| M37 | *E. cloacae* | 48 | >256 | >32 | >8 | N |
| M40 | *E. cloacae* | 4 | >256 | >32 | 1,0 | Y |
| M43 | *P. agglomerans* | >256 | 96 | >32 | >8 | N |
| M44-1 | *Salmonella* sp. | 16 | >256 | >32 | 0,75 | Y |
| M44-2 | *Salmonella* sp. | 16 | >256 | >32 | 0,75 | Y |
| M46 | *S. marscecens* | 64 | 8,0 | 12 | >8 | N |
| E1 | *E. cloacae* | >256 | 24 | >32 | >8 | N |
| E5 | *E. cloacae* | >256 | 32 | >32 | >8 | N |
| E7 | *E. cloacae* | 64 | 8 | >32 | >8 | N |
| E8 | *E.* sp. | 8 | 4 | >32 | >8 | N |
| E10 | *E.* sp. | 6 | 4 | >32 | >8 | N |
| E12 | *E. cloacae* | 24 | 12 | >32 | >8 | N |
| E13 | *E. cloacae* | 192 | 16-24 | >32 | >8 | N |
| E16 | *E. cloacae* | >256 | 24 | >32 | >8 | N |
| E17 | *E. cloacae* | 0,125 | 0,47 | <0,5 | 0,5 | N |
| E21 | *E.* sp. | 4 | 4 | >32 | >8 | N |
| E37 | *E.* sp. | 1 | 4 | >32 | >8 | N |
| E49 | *E. cloacae* | 4 | 0,75 | 6-8 | 0,25 | Y |

Breakpoints for the antibiotics are given under the respective headings. None exist for ceftazidime/clavulanate. Under ESBL, Y = Yes, N = No, based on the ratio of ceftazidime MIC to ceftazidime/clavulanate MIC (see text).

In instances where a significant difference between the ceftazidime MIC and ceftazidime disc diffusion result was present, both the disc diffusion test and ceftazidime MIC determination were repeated. A significant difference was taken as one where there was a sensitive/resistant discrepancy between the two results. Table 2.4 below shows the results of the repeat testing on the three isolates. It should be noted that interpretation of the second set of MIC results was made difficult due to the presence of mutant colonies growing alongside the E-strip. In these cases (K18 & K50) the MIC was taken as the highest concentration which inhibited the growth of mutant colonies.

Table 2.4

Results of repeat disc diffusion and E-test MICs on three isolates

| Isolate | Ceftazidime size – 1st test | z | Ceftazidime – 1st test | | Ceftazidime size –2nd test | z | Ceftazidime MI 2nd test | |
|---|---|---|---|---|---|---|---|---|
| **K18** | 20 | *S* | >32 | *R* | 17 | *I* | >32 | *R* |
| **K50** | 20 | *S* | >32 | *R* | 18 | *S* | >32 | *R* |
| **K69** | 20 | *S* | >32 | *R* | 18 | *S* | 8 | *S* |

Zone size in mm, MIC in μg/ml. S= sensitive, I= intermediate, R = resistant

## 2.4.2 Detection of ESBL Activity

ESBL activity was detected using the double disc diffusion test and the Etest ESBL test. Of the 45 isolates, 27 (60%) were shown to produce these enzymes by either one or both of the methods. The remaining 18 isolates showed no evidence of the presence of ESBL activity. It should be noted that the double disc test detected ESBL activity in five of the isolates (K8, K46, K54, M44-1 and M44-2) only when the test was performed using discs separated by both 10 and 15mm but not when the discs were 20mm apart.

The double disc test detected 24 of the 27 ESBL producers and the Etest 26 of the 27. Of the 24 ESBL producers detected by the double disc test, 16 were identified using both the ceftriaxone/clavulanate discs and ceftazidime/clavulanate discs. Of the remaining eight, seven were detected only by the ceftazidime/clavulanate combination, and one (K8) was only detected using the ceftriaxone/clavulanate discs but not by the ceftazidime/clavulanate combination.

Three additional isolates (M25, M30 and M40) were shown to have ESBL activity using the Etest but this activity was not demonstrated by the double disc test, even when the test was repeated. Conversely, ESBL activity was demonstrated in isolate K8 by the double disc diffusion test but not by the Etest ESBL test. A comparison of the results of the double disc tests and Etests is shown in Table 2.5.

Table 2.5

Results of ESBL detection tests comparing Etest to double disc test

| Isolate | Identity | Ceftriaxone/ Clavulanate 20mm apart | Ceftriaxone/ Clavulanate 10/15mm apar | Ceftazidime/ Clavulanate 20mm apart | Ceftazidime/ Clavulanate 10/15mm apar | E-tes |
|---|---|---|---|---|---|---|
| K4 | *K. pneumoniae* | Pos | | Pos | | Pos |
| K8 | *K. pneumoniae* | Neg | Pos | Neg | Neg | Neg |
| K11 | *K. pneumoniae* | Pos | | Pos | | Pos |
| K16 | *K. pneumoniae* | Pos | | Pos | | Pos |
| K17 | *K. pneumoniae* | Neg | | Pos | | Pos |
| K18 | *K. pneumoniae* | Neg | | Pos | | Pos |
| K29 | *K. pneumoniae* | Pos | | Pos | | Pos |
| K35 | *K. pneumoniae* | Neg | | Pos | | Pos |
| K36 | *K. pneumoniae* | Pos | | Pos | | Pos |
| K39 | *K. pneumoniae* | Neg | | Pos | | Pos |
| K42 | *K. pneumoniae* | Neg | | Pos | | Pos |
| K43 | *K. pneumoniae* | Neg | | Pos | | Pos |
| K46 | *K. pneumoniae* | Neg | Pos | Neg | Pos | Pos |
| K50 | *K. pneumoniae* | Neg | | Pos | | Pos |
| K54 | *K. pneumoniae* | Neg | Pos | Neg | Pos | Pos |
| K58 | *K. pneumoniae* | Pos | | Pos | | Pos |
| K63 | *K. pneumoniae* | Pos | | Pos | | Pos |
| K68 | *K. pneumoniae* | Pos | | Pos | | Pos |
| K69 | *K. pneumoniae* | Pos | | Pos | | Pos |
| M25 | *K. pneumoniae* | Neg | Neg | Neg | Neg | Pos |
| M26 | *K. pneumoniae* | Pos | | Pos | | Pos |
| M28 | *K. pneumoniae* | Pos | | Pos | | Pos |
| M30 | *K. pneumoniae* | Neg | Neg | Neg | Neg | Pos |
| M40 | *E. cloacae* | Neg | Neg | Neg | Neg | Pos |
| M44-1 | *Salmonella* sp. | Neg | Pos | Neg | Pos | Pos |
| M44-2 | *Salmonella* sp. | Neg | Pos | Neg | Pos | Pos |
| E49 | *E. cloacae* | Pos | | Pos | | Pos |

Neg = No ESBL detected Pos = ESBL detected

## 2.5 DISCUSSION

The antibiotic susceptibilities of 45 isolates of Gram negative bacilli to ceftriaxone, ceftazidime, cefuroxime, cefoxitin, imipenem, amoxicillin/clavulanate and piperacillin/clavulanate were determined using disc diffusion tests, while the MICs of cefotaxime, ceftazidime and aztreonam of the isolates were determined using Etests. The 45 isolates comprised 24 *Klebsiella* spp., 14 *Enterobacter* spp., 3 *Citrobacter* spp., 1 *Serratia marcescens*, 2 *Salmonella* spp. and 1 *Pantoea agglomerans*. The presence of ESBL activity was determined using both the double disc diffusion test and Etest MICs.

For 11 of the isolates there were differences in the results of the Etest MICs and the disc diffusion tests for ceftazidime. These consisted of sensitive/intermediate or intermediate/resistant discrepancies and were not considered to be significant. A number of factors could account for these inconsistencies such as inoculum effect and visual error. Standardisation of the inoculum is done visually and differences in inoculum size can lead to the above-mentioned discrepancies. The disc diffusion tests and Etests were carried out at different times and the inoculum size used for each test may subsequently have differed. The difference between an "intermediate" reading and either "sensitive" or "resistant" can be a matter of one or two millimetres and inaccuracies in visual measurement may also account for the discrepancies.

The discrepancies between the MICs and the disc diffusion results for the three isolates K18, K50 and K69 were more disturbing given the significant differences between the two sets of results. All three isolates initially produced zones 20mm in diameter on the disc diffusion test, a result compatible with susceptibility to ceftazidime. The MICs of ceftazidime for all three isolates were >32μg/ml, above the resistance breakpoint for this antibiotic. These discrepancies could not be easily disregarded or explained and consequently the tests were repeated.

While the results after repeating the tests did not eliminate the discrepancies, they did go some way to explaining them. Firstly, it should be noted that the two sets of ceftazidime zone sizes differed by 2-3mm. This may not appear significant but it does lend weight to part of the explanation of the "insignificant" discrepancies described earlier. The

ceftazidime MIC of isolate K69 was 8μg/ml on repeated testing, compatible with both of the disc diffusion results for this isolate. Although the ceftazidime MICs of the other two isolates remained >32μg/ml, these MICs were determined on the basis of mutant colonies, such as those shown in Fig 2.1, which may not have been detected by the disc diffusion test, and this is one possible explanation for the discrepancies in the results.

ESBL activity was demonstrated in 27 of the 45 isolates using one or both of the tests. Of the 27 ESBL producing organisms, the majority (23) were *Klebsiella pneumoniae*, two were *Salmonella* species and two were *Enterobacter cloacae.* This is in agreement with previous work (De Champs *et al.,* 1989; Liu *et al.,* 1992) where *K. pneumoniae* isolates have been frequently shown to produce ESBLs. The presence of ESBLs in *E. cloacae* and *Salmonella* spp. has been described but this activity in these organisms is unusual (Barguellil *et al.,* 1995; Pitout *et al.,* 1997).

The Etest failed to detect ESBL activity in only one isolate (K8). This isolate had a ceftazidime MIC of >32ug/ml and a ceftazidime/clavulanate MIC of >8ug/ml. Both of these figures represent the upper limits of the MIC determination for these antibiotics using Estrips. The actual MICs are thus unknown and if they were determined (using broth dilution techniques for example) the ratio may in fact indicate the presence of an ESBL.

Eight of the 27 ESBL producing isolates (K8, K46, K54, M25, M30, M40, M44-1 and M44-2) did not initially show ESBL activity using the double disc diffusion tests. In five of these isolates ESBL activity was detected only by when the discs were placed closer together (either 10 or 15mm). Since the double disc test relies on clavulanate diffusing through agar sufficiently to inhibit the organism's beta lactamase in an area where the cephalosporin will still be active, it is perhaps not surprising that placing the discs closer together would increase the sensitivity of this test. The problem is that the zones created by doing this sometimes overlap, which could make interpretation equally difficult for isolates which have a "positive" double disc test using discs 20mm apart. Assuming that a clinical laboratory would neither repeat sensitivity testing, nor vary the distance at which the discs are placed on routine isolates, undetected ESBL activity may be present in nearly 30% of Gram negative bacilli. This figure corresponds with previous work in which the false negative rate of the double disc test has been estimated to be 20% (Cormican *et al.,* 1996).

What is also interesting is that despite testing these eight isolates twice by the double disc test, ESBL activity could still only be demonstrated in three isolates using the Etest. This again highlights the difficulties in detecting extended spectrum beta lactamase activity in a clinical laboratory. To date no satisfactory alternative to the double disc test has been widely accepted, although a number of other strategies have been tested [1.4.4].

As mentioned previously, 7 of the 24 ESBL producers, detected using the double disc test, only showed ESBL activity between ceftazidime and clavulanate. The significance of this finding is that in a clinical laboratory ESBL production may be missed in up to 30% of isolates if a ceftazidime disc is not placed adjacent to a clavulanate containing disc. This correlates with the recommendation that ceftazidime should be used alongside a clavulanate disc in the double disc diffusion test (Jacoby & Han, 1996). Contrast this, however, with the finding that the ESBL of one isolate was only detected between ceftriaxone and clavulanate. This suggests that the clavulanate disc should probably be placed between two extended spectrum cephalosporins to maximise the efficiency of ESBL detection, a practice that is routinely carried out in the Groote Schuur Hospital diagnostic microbiology laboratory.

# CHAPTER THREE

# DNA - DNA HYBRIDISATION STUDIES

## 3.1 - INTRODUCTION

A number of antibiotic resistance gene probes, including probes specific for TEM or SHV genes, have been used in hybridisation experiments to detect the presence of the corresponding gene in DNA prepared from resistant bacterial strains. Both oligoprobes and longer probes have been used to identify and characterise beta lactamase genes from clinical isolates (Seetulsingh *et al.*, 1991; Liu *et al.*, 1992, Ling *et al.*, 1994; Hibbert-Rogers *et al.*, 1995; Huovinen *et al.*, 1988; Ouellette *et al.*, 1988). A similar approach was adopted in this study. Since TEM- and SHV-related enzymes are the most commonly identified enzymes in clinical isolates (Jacoby & Medeiros, 1991), probes specific for SHV and TEM related genes were used in hybridisation experiments to detect their counterparts in the isolates selected in chapter 2.

Oligoprobes have also been used to accurately type beta lactamases [1.4.4.2]. Mabilat and Courvalin (1990) described a technique whereby oligoprobes were used to determine the "subtype" of the TEM beta lactamase gene present in the organism. The technique relies on the fact that a limited number of areas in the progenitor TEM gene contain "hotspots" where point mutations occur, resulting in the altered spectrum of activity of the enzyme. Oligoprobes were designed around the areas containing point mutations, with the potentially mutated nucleotide centred in the oligoprobe. If the isolate under investigation contained a structural gene with the same point mutation, the probe would anneal and a hybridisation signal would be obtained. However, if the mutation was not present, the probe would not anneal and no signal would be obtained. Using combinations of oligoprobes, the various point mutations present in the gene were identified and the identity of the TEM-related beta lactamase could consequently be deduced from this information. However, the increased number of TEM-related enzymes described since 1990 would make this technique far more difficult to apply at present.

A factor complicating hybridisations for the detection of SHV-related genes is the presence of chromosomal genes (such as LEN-1) with a high degree of homology to the plasmid mediated SHV-1 in isolates of *K. pneumoniae*. It is believed that SHV-1 was derived from LEN-1 and they share approximately 89% homology (Garbarg-Chenon *et al.*, 1990). Some authors have suggested that other chromosomal beta lactamase genes in strains of *K. pneumoniae* may be more closely related to the plasmid mediated SHV-1 (Hægmann et al., 1997; Leung *et al.*, 1997). To date, however, LEN-1 is the only chromosomal beta lactamase gene in *K. pneumoniae* that has been sequenced (Arakawa *et al.*, 1986).

During the course of this study a number of hybridisation experiments were carried out to detect the presence of TEM or SHV related genes. Probes specific for these genes were hybridised either to DNA prepared from colonies on a nylon membrane (colony blot) or to DNA that had been transferred to a nylon membrane using a slot blot apparatus.

# 3.2 MATERIALS AND METHODS

## 3.2.1 Bacterial Isolates and Plasmids

Control strains of *E. coli* containing plasmids encoding SHV-1 and -2 beta lactamases were kindly donated by Dr C. Thomson, University of Edinburgh. The plasmid vector pUC19, containing the TEM-1 gene (Yanisch-Perron *et al.*, 1985), was introduced into *E. coli* DH5α by transformation and used as a TEM positive control strain.

### *3.2.1.1 Preparation of Competent Cells*

Competent cells, capable of taking up DNA, were prepared as follows (Chung & Miller, 1988). An aliquot (0,5ml) of an overnight broth culture of *E. coli* DH5α was inoculated into 50mls of fresh YT (Yeast Tryptone) broth and cultured aerobically at 37°C until early log phase ($OD_{600}$ between 0,2 and 0,4). The cells were harvested by centrifugation at 5 000x***g*** for five minutes in a Beckman GS-6 centrifuge (Beckman Instruments, California) and resuspended in one tenth the volume of ice cold TSB broth.

### *3.2.1.2 Transformation*

50 – 100pg of DNA was added to 100µl of competent cells, which were left on ice for 10 minutes to allow the DNA to adhere to the cells. The cells were heat shocked at 42°C for 2 minutes to facilitate uptake of the DNA. After addition of YT broth (0,9ml) the cells were incubated at 37°C for 30 - 60 minutes to allow for antibiotic resistance gene expression. An aliquot (100 – 200µl) of the transformation mix was then inoculated onto YT agar containing ampicillin (100µg/ml) and incubated at 37°C for 18 hrs.

### *3.2.1.3 Extraction of Genomic DNA*

Genomic DNA of the isolates and controls was extracted as follows (Ausubel *et al.*, 1987). Organisms were cultured overnight at 37°C in 3 – 4ml of tryptone water. The cells were harvested by centrifugation in a microfuge (Eppendorf 5415C) at 12 000 rpm for one to two minutes and the cell pellet resuspended in 10% SDS (30µl) to lyse the cells and proteinase K (3µl of 20 mg/ml) (Boehringer Mannheim, Germany). The mixture was incubated at 37°C for one hour to allow the proteinase K to break down proteinaceous products after which 100µl of 5M NaCl and 80µl of CTAB/NaCl were added. Cell wall debris, denatured protein and polysaccharides are complexed to the CTAB while the presence of NaCl at concentrations greater than 0,5M prevents similar complexing of nucleic acids. The mixture was incubated at 65°C for ten minutes. An equal volume of chloroform was added to remove CTAB/protein/polysaccharide complexes, the mixture was centrifuged at 14 000 rpm in a microfuge and the aqueous phase extracted to a clean eppendorf tube. The DNA was purified further by at least three extractions with phenol/chloroform until the white proteinaceous interface had disappeared. The use of tryptone water, a relatively nutritionally poor culture medium (compared to YT broth), produced cells with less capsular material and thus reduced the number of phenol/chloroform extractions required. The DNA was precipitated by adding 0,6 volumes of isopropanol, standing the mixture at room temperature for ten minutes and then centrifuging it at room temperature at 12 000 rpm for 10 minutes. The DNA pellet was washed with 70% ethanol, centrifuged briefly in a microfuge and any remaining liquid removed with a pipette. The dry pellet was then suspended in TE buffer. An aliquot of the DNA was subjected to agarose gel electrophoresis alongside standards of known

concentration (Boehringer Mannheim molecular weight marker X), and the concentration of DNA estimated visually.

## 3.2.2 Transfer of DNA to a Stable Matrix

### *3.2.2.1 Slot Blots*

Genomic DNA [3.2.1.3] was transferred to nylon membranes (Hybond $N^+$, Amersham, England) using a slot – blot apparatus (Shleicher and Sheuell, West Germany). Approximately one microgram of DNA in 50µl of distilled water was heated in a water bath at 95°C for five minutes to separate the DNA strands after which they were immediately placed on ice to prevent reannealing of the DNA. In order to increase the ionic content of the mixture and facilitate binding of the DNA to the membrane, 50µl of 20xSSC was added to each sample. An appropriately sized sheet of nylon membrane was soaked in 10xSSC, placed on two sheets of filter paper (Whatman) also saturated with 10xSSC and all three sheets then placed in the slot-blot apparatus. The samples were loaded into the wells in the slot blot apparatus and transferred to the membrane by a vacuum, which was applied until the samples had been transferred from the wells to the membrane. The membrane was removed and placed on filter paper soaked in a solution of 1,5M NaCl, 0,5M NaOH for five minutes to denature the DNA. Neutralisation was achieved by placing the membrane onto filter paper soaked in a solution of 1,5M NaCl, 0,5M Tris at pH 7,4 for at least five minutes. After air drying, the DNA was cross linked to the membrane by exposing it to UV light (254nm) in a UV Crosslinker (Hoefer Scientific, San Francisco).

### *3.2.2.2 Colony Blots*

A number of procedures were used to transfer colonies to the membrane prior to lysis and transfer of DNA. First, bacteria were inoculated onto YT agar and incubated at 37°C for approximately six hours - ideally until colonies were about 2 mm in diameter. A nylon membrane was then placed onto the colonies on the agar and peeled off after 1 minute with the colonies adhering to the membrane. Alternatively, bacteria were inoculated directly onto the nylon membrane on YT agar and incubated at 37°C until the colonies on

the membrane had reached a size of approximately 2mm in diameter. The membrane was then removed from the agar. A third procedure involved inoculating isolates into 200µl of YT broth in a microtitre plate and incubating them overnight at 37°C. These were then transferred by means of a multi-pronged "hedgehog" device to either plain YT agar or onto a membrane that had been placed on YT agar, which were then incubated as before.

DNA was released from the bacterial colonies by placing the membrane onto filter paper saturated with 0,5M NaOH for five minutes. Bacterial cell wall debris was removed by vigorously washing the membrane in 5xSSC for one minute. After two such washes the membrane was air dried on clean filter paper.

## 3.2.3 Methods Used in the Preparation of the TEM Probe

### *3.2.3.1 PCR Assay*

The probe used for the detection of TEM genes was obtained by amplifying a portion of the pUC19 located TEM-1 gene by the polymerase chain reaction (PCR). PCR consists of cyclical replications of DNA using a thermostable DNA polymerase enzyme in the presence of dNTPs and primers. Primers are annealed to denatured sample DNA and synthesis of a complementary strand of DNA is initiated from these primers by the polymerase enzyme (*Taq*) in the presence of dNTPs. The newly synthesised strands are once again denatured by heat and when the temperature drops the primers anneal and another cycle of synthesis commences.

With each cycle, the amount of dsDNA produced increases exponentially, since each new fragment of dsDNA synthesised serves as a template in subsequent cycles. If sufficient nucleotides and primers are provided in the reaction mixture the process can be repeated 30 - 40 times and a large quantity of a specific DNA sequence can be synthesised. This process has been summarised in Figure 3.1.

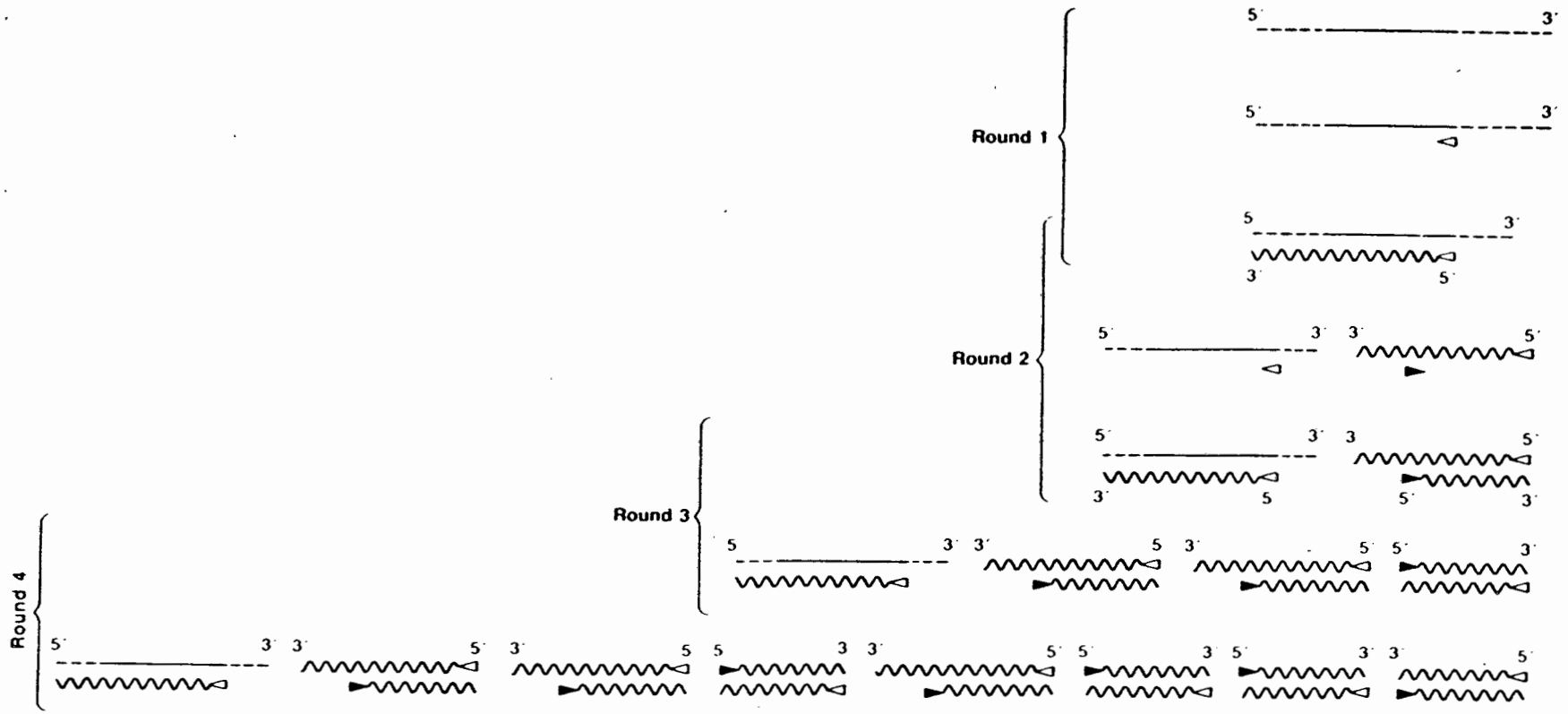



Fig 3.1 PCR consists of repeated cycles of denaturation, primer annealing and extension as illustrated above. In the first round, heterogenous sized products are formed, but with subsequent rounds, the segment of DNA that lies between the two primers (the open and closed arrows) is amplified preferentially, and becomes the major product of the reaction. The original template in this diagram is shown by a solid line, and newly synthesised DNA shown by a wavy line. If the original template is double stranded, both strands, once denatured in the initial denaturation step, would undergo the same process (Sambrook *et al.*, 1989).

The primers (TEM-A 5' -CCC CGA AGA ACG TTT TC and TEM-B 5' -ATC AGC AAT AAA CCA GC) described by Mabilat and Courvalin (1990) were used in a PCR assay to amplify a 517bp fragment of the TEM-1 gene on pUC19 (Fig 3.2). Boehringer Mannheim *Taq* polymerase, PCR buffer and dNTP's were used with primers synthesised by the Department of Medical Biochemistry at UCT. PCR assays were carried out in a Perkin Elmer Thermocycler (Perkin Elmer GeneAmp 2400) under the following conditions. Initial denaturing was at 94°C for 5 minutes, followed by cycles of 94°C for 30 seconds, 60°C for 50 seconds and 72°C for 60 seconds. After 40 cycles final extension was carried out at 72°C for seven minutes.

```
ATGCTTCAAT AATATTGAAA AAGGAAGAGT ATGAGTATTC AACATTTCCG TGTCGCCCTT   60
                                 ->

ATTCCCTTTT TTGCGGCATT TTGCCTTCCT GTTTTTGCTC ACCCAGAAAC GCTGGTGAAA  120
GTAAAAGATG CTGAAGATCA GTTGGGTGCA CGAGTGGGTT ACATCGAACT GGATCTCAAC  180
AGCGGTAAGA TCCTTGAGAG TTTTCGCCCC GAAGAACGTT TTCCAATGAT GAGCACTTTT  240
                                  TEM-A
AAAGTTCTGC TATGTGGCGC GGTATTATCC CGTATTGACG CCGGGCAAGA GCAACTCGGT  300
CGCCGCATAC ACTATTCTCA GAATGACTTG GTTGAGTACT CACCAGTCAC AGAAAAGCAT  360
CTTACGGATG GCATGACAGT AAGAGAATTA TGCAGTGCTG CCATAACCAT GAGTGATAAC  420
ACTGCGGCCA ACTTACTTCT GACAACGATC GGAGGACCGA AGGAGCTAAC CGCTTTTTTG  480
CACAACATGG GGGATCATGT AACTCGCCTT GATCGTTGGG AACCGGAGCT GAATGAAGCC  540
ATACCAAACG ACGAGCGTGA CACCACGATG CCTGTAGCAA TGGCAACAAC GTTGCGCAAA  600
CTATTAACTG GCGAACTACT TACTCTAGCT TCCCGGCAAC AATTAATAGA CTGGATGGAG  660
GCGGATAAAG TTGCAGGACC ACTTCTGCGC TCGGCCCTTC CGGCTGGCTG GTTTATTGCT  720
                                                      TEM-B
GATAAATCTG GAGCCGGTGA GCGTGGGTCT CGCGGTATCA TTGCAGCACT GGGGCCAGAT  760
GGTAAGCCCT CCCGTATCGT AGTTATCTAC ACGACGGGGA GTCAGGCAAC TATGGATGAA  820
CGAAATAGAC AGATCGCTGA GATAGGTGCC TCACTGATTA AGCATTGGTA ACTGTCAGAC  880
                                                  <-

CAAGTTTACT CATATATACT TTAGATTGAT TTAAAACTTC                        921
```

Fig 3.2 DNA sequence of the TEM-1 gene in pUC19 (Yanisch Perron *et al*, 1985) with the intragenic sequence amplified by PCR and used as a probe underlined. The primer sequences are highlighted in italics. TEM-1 coding sequence starts at base 31. Start and stop codons are highlighted with arrowheads.

### *3.2.3.2 Agarose Gel Electrophoresis*

The PCR product was purified by electrophoresis at 90V for 50 - 60 minutes on 1% agarose gels. Negatively charged DNA migrates through agarose towards a positive electrode when a current is applied across it. The rate of migration is proportional to the voltage being applied, the concentration of the agarose and the size of the DNA.

Agarose was dissolved in TAE buffer (Tris-Acetate-EDTA) by heating in a microwave oven, allowed to cool to about 55°C and poured into horizontal moulds. A comb was placed into the molten agarose approximately 1cm from one end of the mould. Once the agarose had set the comb was removed leaving wells into which samples could be loaded.

The gel was submerged in 1xTAE buffer in an electrophoresis apparatus. The samples of DNA were mixed with 2 – 3µl of tracking dye containing bromophenol blue, sucrose and EDTA. The high molecular weight of the sucrose maintains the sample in the well while the dye allows monitoring of the progress of the sample during electrophoresis. After loading the samples a constant voltage (5 - 20 volts/cm gel) was applied until the dye had migrated about 60 - 70% of the length of the gel.

DNA in the gels was visualised using ethidium bromide (EtBr), which intercalates in the DNA molecules and fluoresces under ultraviolet light. Ethidium bromide (10 mg/ml) was usually added to the molten agarose (1µl per 100ml agarose) prior to pouring. However, since it intercalates into the DNA, it affects the electrophoretic mobility of DNA. When accurate size determinations were required, no EtBr was added to the agarose prior to electrophoresis. Instead, the agarose gels were immersed in a solution of TAE containing the same proportions of EtBr (1µl per 100ml of TAE) after electrophoresis. DNA was visualised at 302nm on a Mighty Bright UV transilluminator (Hoefer Scientific, San Francisco) and photographed using a Polaroid Gelcam (Polaroid corporation, Cambridge, Massachusetts).

### *3.2.3.3 DNA Extraction from Agarose Gels*

DNA extraction from agarose gels (Seth, 1984) was carried out by slicing the fragment of agarose containing DNA out of the gel, cutting this gel slice into fragments and placing it in an Eppendorf tube. After weighing these fragments, an equal volume of phenol was added to the agarose to solubilise it. This was incubated at -70°C for 10 minutes and then centrifuged at 14 000 rpm at room temperature for 10 minutes. The aqueous phase was transferred to a clean Eppendorf tube and the DNA was further purified by two phenol/chloroform extractions. To precipitate the DNA, 1/10 volume of 4M LiCl was added followed by 3 volumes of 100% ethanol. One microlitre of glycogen (Boeringer Mannheim, Germany) was added to the mixture to aid in precipitation, which was carried out at -70°C for ten minutes. The DNA was pelleted by centrifugation at 14 000 rpm in a microfuge for ten minutes, washed with 70% ethanol, centrifuged, dried and suspended in TE buffer as before [3.2.1.3].

### *3.2.3.4 Cloning*

#### *i) Preparation of Insert*

Since *Taq* polymerase adds one or more gratuitous 'A' residues to the ends of PCR products, it was necessary to remove these residues before the PCR product could be cloned into an appropriately digested vector. The 'A' residues were removed and the PCR product blunted using a blunting kit supplied by Amersham International (England). In this reaction the ends of the PCR product were blunted by $T_4$ DNA polymerase which has both polymerase and exonuclease activity enabling the enzyme to add bases to a 5' protruding end and remove bases from a 3' protruding end. In the case of PCR products, the 'A' residues are removed by the exonuclease activity. The reaction was carried out according to the manufacturer's instructions and the blunted PCR product was then ligated to prepared vector.

*ii) Preparation of Vector*

Six micrograms of the plasmid vector pUC19 was digested with the restriction enzyme *Sma*I (10 units) (Boehringer Mannheim) at 25°C for one hour. The enzyme was inactivated and removed by carrying out a phenol/chloroform extraction. After adding 3 volumes of 100% ethanol to precipitate the DNA, it was pelleted, washed, dried and suspended in 50µl TE buffer. [3.2.1.3]

*iii) Ligation*

Approximately 100ng of digested vector was ligated to the blunted PCR product (approximately 100ng) using the Amersham ligation kit and following the manufacturer's instructions.

*iv) Transformation*

The ligation mixture (25µl) was introduced into 100µl of competent *E.coli* DH5α cells [3.2.1.2]. All transformation experiments included the following controls. In order to calculate the competency of the cells, 50pg and 500pg of undigested pUC19 were introduced into the cells. Since only circular DNA can be taken up by the competent cells, 100ng of *Sma*I digested vector was transformed to assess the efficiency of the digestion of the vector. During ligation 100ng of digested vector was self-ligated and 25µl of this reaction mix was introduced into the cells to assess the efficiency of the ligation reaction itself. To ensure that the cells were not contaminated by pUC19, competent cells were plated onto selective media containing ampicillin (100µg/ml).

*v) Isolation and Characterisation of Recombinants*

After transformation, the cells were spread onto YT agar containing ampicillin (100µg/ml), X-gal (100µg/ml) and IPTG (100µg/ml). The alpha terminal of the beta galactosidase gene (approximately 150 amino acids) is located in the region of the multiple cloning site of pUC19 while the carboxy terminal of this gene is present in the genome of *E. coli* DH5α. If these two portions of the gene are expressed in the same cell they form

an active enzyme by a process called alpha complementation. IPTG in the agar serves as an inducer of the expression of beta galatosidase, which in turn cleaves the X-gal, thereby imparting a blue colour to colonies of organisms producing the enzyme.

If DNA is ligated into the multiple cloning site, the alpha terminal of the β-galactosidase gene is disrupted, alpha complementation will not occur and recombinants will be white in colour, rather than blue (Sambrook *et al.*, 1989). The entire transformation mixture (1ml) was plated onto selective media in Petri dishes (150mm). With respect to the controls, 200µl of the transformation mix was plated onto the selective media. The tests and controls were incubated at 37°C for 18 hours.

*vi) Plasmid DNA Extraction*

Plasmid DNA was isolated from the putative recombinants using the modified alkaline lysis method of Birnboim and Doly (Sambrook *et al.*, 1989). White colonies were inoculated into YT broth (5-50ml) containing ampicillin (100µg/ml) and incubated at 37°C for four to six hours. The cells were pelleted by centrifugation in a microfuge at 14 000 rpm for 30 seconds. The supernatant was poured off and the bacterial pellet resuspended in 0,2ml of solution 1 (0,025M Tris, 0,05M EDTA, 1% glucose w/v). To lyse the cells and release the DNA twice the volume (0,4ml) of solution 2 (0,2M NaOH, 1% SDS w/v) was added and the solution placed on ice for five minutes. Selective renaturation of the plasmid DNA was achieved by the addition of 0,3ml of solution 3 (3M potassium acetate, 11,5% glacial acetic acid v/v) and the mixture was again kept on ice for five minutes. Cell wall debris was pelleted by centrifugation in a microfuge at 14 000 rpm for ten minutes. Isopropanol (0,6 volumes) was added to the lysate and left at room temperature for at least ten minutes to precipitate the nucleic acids. After centrifugation to pellet the nucleic acids, the DNA was washed, dried and suspended in TE buffer [3.2.1.3].

When large amounts of purified pDNA were required, the plasmids were extracted from 50ml volume cultures using the Nucleobond kit (Machery Nagel, Germany). This is based on the same principle as the alkaline lysis method with some modification of the solutions. Purification was performed through cartridges containing ion exchange silica.

After extraction of DNA, following the manufacturer's instructions, the lysate was filtered through gauze to trap any solid particles that may interfere with the subsequent column purification process. The ion exchange silica was equilibrated with 2ml of buffer N2 (0,1M Tris/$H_3PO_4$, 0,9M KCl, 15% ethanol, pH 6,3) and the lysate was loaded into the columns and allowed to drip through. Plasmid DNA remained bound to the silica. After washing the columns twice with 4 ml each time of buffer N3 (0,1M Tris/$H_3PO_4$, 1,15M KCl, 15% ethanol, pH 6,3), the DNA was eluted with 2 ml of buffer N5 (0,1M Tris/$H_3PO_4$, 1M KCl, 15% ethanol, pH 8,5). Plasmid DNA was then precipitated by adding 0,7 volumes of isopropanol, allowed to stand for ten minutes and then pelleted, dried and suspended in TE buffer [3.2.1.3].

*vii) Restriction Endonuclease Digestion*

The presence of an insert in a plasmid was ascertained by digesting putative recombinant plasmids with a restriction endonuclease. Since the insertion of the blunted PCR product into the vector eliminated the *Sma*I site, an alternative enzyme, *Hind*III, (Boehringer Mannheim) with a site in the multiple cloning site (MCS) was used to digest the recombinanats. The resulting DNA fragments were separated by electrophoresis on 0,8% agarose gels and photographed as before [3.2.3.2]. Recombinants were identified on the basis of size, being approximately 500bp larger than the vector.

### *3.2.3.5 DNA Sequencing*

DNA sequences were determined by Sanger's dideoxy chain termination method (Sambrook *et al.*, 1989) using the $T_7$ Sequencing kit (Pharmacia, USA) and [$^{35}$S]dATP.

Approximately 2-3µg of DNA in 32µl of sterile water was used in the sequencing reaction. DNA was denatured by the addition of 8µl of 2M NaOH followed by incubation at room temperature for 10 minutes. 3M Sodium acetate pH4.8 (7µl) and 4µl of water were then added to neutralise the alkali, and 3 volumes (120µl) of 100% ethanol was added to the mixture and left at -70°C for 15 minutes to precipitate the denatured DNA. The DNA was pelleted by centrifuging at 14 000 rpm for 15 minutes in a microfuge, washed with ice cold 70% ethanol, dried, and resuspended in 10µl of sterile water.

The templates prepared above were annealed to the universal forward and reverse primers as follows. In separate reactions, 2µl of forward and reverse primers (5µM) were added to the denatured DNA template (10µl). After the addition of 2µl of the annealing buffer ($T_7$ Sequenase kit), the primers were annealed to the DNA to be sequenced by incubation at 65°C for 5 minutes, 37°C for 10 minutes and room temperature for 5 minutes.

After annealing of the primers, DNA synthesis was commenced by the addition of 3µl of dNTPs (dCTP, dTTP, dGTP), 1µl of [$^{35}$S]dATP and 2µl of $T_7$ polymerase. DNA synthesis was allowed to continue for five minutes at room temperature. After this incubation, 4,5µl aliquots of the reaction mixture were transferred to each of 4 separate Eppendorf tubes containing dNTPs with either ddATP, ddCTP, ddGTP or ddTTP. When dideoxy nucleotides are incorporated into the extending complementary strand, chain elongation is terminated because the dideoxy nucleotides lack the 3' hydroxyl group necessary for the formation of the next phosphodiester bond. By using limiting amounts of each of the ddNTPs, DNA fragments are generated that terminate at positions occupied by any A, C, G or T in the template strand. The termination reactions were incubated for 5 minutes at 37°C and then 5µl of a stop solution containing formamide, EDTA and a tracking dye was added to each tube.

The sequencing reaction products were separated by electrophoresis on denaturing polyacrylamide gels prepared as follows. Two glass plates separated by spacers (0,25 - 1mm thick) along their long axes and by a strip of filter paper at the bottom end were assembled by clamping them together with bulldog clips. A 6% acrylamide, 8M urea gel was prepared by mixing 6 ml of a 50% acrylamide 2,5% biscrylamide solution with 24g urea and 5ml of 10xNNB. The urea was dissolved by warming the solution prior to adding distilled water to make a volume of 50ml. Polymerisation of the acrylamide was initiated by the addition of 20µl of TEMED (NNN'N' - tetramethylethylene-diamine) and 125µl of 10% ammonium persulphate. The mixture was poured slowly between the two glass plates using a 25ml pipette and running the mixture slowly between the plates while holding them at an angle of about 70° to the vertical. This was done to avoid the formation of bubbles in the acrylamide gel. Once poured, the plates were rested horizontally and a sharks-tooth comb inserted into the open end of the glass plates with the flat side inwards. The top was

then covered with filter paper and soaked with the remaining acrylamide solution. The glass plates were sealed with clingwrap and left to polymerise overnight.

The clingwrap and the comb were removed and the latter replaced with the teeth just touching the flat interface of the polyacrylamide gel created by the flat end of the shark's tooth comb. The plates were placed on vertical supports with the lower end resting in a buffer tank connected to the positive electrode. The upper end was in contact with another buffer tank connected to the negative electrode. The buffer tanks were filled with 1xNNB and a current of 40W passed through the gel for about 30 minutes to preheat the gel to aid in denaturation of the DNA samples. The sequencing reactions were heated to 85°C for 2 minutes to denature the dsDNA and 3µl of the sample loaded into the wells at the top end of the gel in the order 'A', 'C', 'G' and 'T' for each primer/template combination. Electrophoresis was performed at 40W for approximately 150 minutes.

After electrophoresis the glass plates were carefully separated and a sheet of filter paper applied to the gel. This was peeled off with the gel adhering to the filter paper. The gel was wrapped in clingwrap, placed in a drying apparatus (Slab Gel Dryer SE1150, Hoefer Scientific Instruments, San Francisco) and dried under vacuum at 80°C for 2 hours. The dried gel, after removal of the clingwrap, was exposed to X-Ray film (Curix RPI, Agfa) in a light proof X-ray box for 3 days and the film developed.

### 3.2.4 Methods Used in the Preparation of the SHV Probes

Two probes for the detection of SHV type genes were prepared. The first probe consisted of a 34 nucleotide oligoprobe designed to anneal to conserved sequences in SHV-related genes but not to the chromosomal gene (LEN-1) encoding a beta lactamase enzyme in *K. pneumoniae* (Fig 3.3). This probe was synthesised by Whitehead Scientific. The DNA sequence of the probe, 5' CTG ACC AGC CAG CGT CTG AGC GCC CGT TCG CAA C, anneals to nucleotides 570 to 604 of the SHV-1 gene and has 85% homology to the LEN-1 gene.

```
Len-1 CTTTGCTCGC CCTTATCGGC CCTCACTCAA GGAAGTATTG CGGTTATGCG TTATGTTCGC  60
                                                  ***** **** *****
SHV-1                                             ATGCG TTATATTCGC  15

Len-1 CTGTGTGTTA TCTCCCTGTT AGCCACCCTG CCACTGGTGG TATACGCCGG TCCACAGCCG 120
      ****** *** ********** ********** ** **** ** ** ***** *  ** ******
SHV-1 CTGTGTATTA TCTCCCTGTT AGCCACCCTG CCGCTGGCGG TACACGCCAG CCCGCAGCCG  75

Len-1 CTTGAGCAGA TTAAACAAAG CGAAAGCCAG CTGTCGGGCC GCGTGGGGAT GGTGGAAATG 180
      ******** * ****** *** ********** ********** **** ** ** * * ******
SHV-1 CTTGAGCAAA TTAAACTAAG CGAAAGCCAG CTGTCGGGCC GCGTAGGCAT GATAGAAATG 135

Len-1 GATCTGGCCA ACGGCCGCAC GCTGGCCGCC TGGCGCGCCG ATGAACGCTT TCCCATGGTG 240
      **********  ********* **** ***** ********** ********** ******* **
SHV-1 GATCTGGCCA GCGGCCGCAC GCTGACCGCC TGGCGCGCCG ATGAACGCTT TCCCATGATG 195

Len-1 AGCACCTTTA AAGTGCTGCT GTGCGGCGCG GTGCTGGCGC GGGTGGATGC CGGGCTCGAA 300
      ********** ****  ****  ********  ********** ********** ***   ****
SHV-1 AGCACCTTTA AAGTAGTGCT CTGCGGCGCA GTGCTGGCGC GGGTGGATGC CGGTGACGAA 255

Len-1 CAACTGGATC GGCGGATCCA CTACCGCCAG CAGGATCTGG TGGACTACTC CCCGGTCAGC 360
      ** ***** * *   ****** *** ****** ********** **********  *********
SHV-1 CAGCTGGAGC GAAAGATCCA CTATCGCCAG CAGGATCTGG TGGACTACTC GCCGGTCAGC 315

Len-1 GAAAAACACC TTGTCGACGG GATGACGATC GGCGAACTCT GCGCCGCCGC CATCACCCTG 420
      ********** *** *****   ****** ** ********** ********** *** *** **
SHV-1 GAAAAACACC TTGCCGACGC AATGACGGTC GGCGAACTCT GCGCCGCCGC CATTACCATG 375

Len-1 AGCGATAACA GCGCTGGCAA TCTGCTGCTG GCCACCGTCG GCGGCCCCGC GGGATTAACT 480
      ********** **** * *** ****** *** ********** **********  ***** ***
SHV-1 AGCGATAACA GCGCCGCCAA TCTGCTACTG GCCACCGTCG GCGGCCCCGC AGGATTGACT 435

Len-1 GCCTTTCTGC GCCAGATCGG TGACAACGTC ACCCGTCTTG ACCGCTGGGA AACGGCACTG 540
      ****** *** **********  ********* ***** **** ********** ***** ****
SHV-1 GCCTTTTTGC GCCAGATCGG CGACAACGTC ACCCGCCTTG ACCGCTGGGA AACGGAACTG 495

Len-1 AATGAGGCGC TTCCCGGCGA CGCGCGCGAC ACCACCACCC CGGCCAGCAT GGCCGCCACG 600
      ********** ********** *** ****** ***** **** ********** ****** **
SHV-1 AATGAGGCGC TTCCCGGCGA CGCCCGCGAC ACCACTACCC CGGCCAGCAT GGCCGCGACC 555

Len-1 CTGCGCAAAC TA---CTGAC CGCGCAGCAT CTGAGCGCCC GTTCGCAACA GCAACTCCTG 657
      ********   *    ***** *   **** * ********** *********  *** ** ***
SHV-1 CTGCGCAACG TTGGCCTGAC CAGCCAGCGT CTGAGCGCCC GTTCGCAACG GCAGCTGCTG 615

Len-1 CAGTGGATGG TGGACGATCG GGTTGCCGGC CCGCTGATCC GCGCCGTGCT GCCGCCGGGC 717
      ********** ********** *** *****  *** ****** ** ******* **** *****
SHV-1 CAGTGGATGG TGGACGATCG GGTCGCCGGA CCGTTGATCC GCTCCGTGCT GCCGGCGGGC 675

Len-1 TGGTTTATCG CCGACAAAAC CGGGGCTGGC GAACGGGGTG CGCGCGGCAT TGTCGCCCTG 777
      ********** **** ** ** *** ****** ** ******* ******* ** **********
SHV-1 TGGTTTATCG CCGATAAGAC CGGAGCTGGC GAGCGGGGTG CGCGCGGGAT TGTCGCCCTG 735

Len-1 CTCGGCCCGG ACGGCAAACC GGAGCGCATT GTGGTGATCT ATCTGCGGGA TACCCCGGCG 837
      ** ******  *   **** *  ********* ******** * ********** **********
SHV-1 CTTGGCCCGA ATAACAAAGC AGAGCGCATT GTGGTGATTT ATCTGCGGGA TACCCCGGCG 795

Len-1 AGTATGGCCG AGCGTAATCA ACATATCGCC GGGATCGGCC A-GCGCTGAT CGAGCACTGG 896
      ** ******* **** *****  ** ****** *********  * ******   **********
SHV-1 AGCATGGCCG AGCGAAATCA GCAAATCGCC GGGATCGGCA AGGCGCTGTA CGAGCACTGG 855

Len-1 CAACGCTAAC CCGGCGGTAC CGTGCGTTAG CGCGGCCCGC AGCACCTGGC AGGCGTGCCG 956
      *********
SHV-1 CAACGCTAA                                                       864
```

Fig 3.3 – Alignment of the DNA sequences of the LEN-1 (Arakawa *et al.*, 1986) and SHV-1 genes (Mercier & Levesque, 1990), showing the position of the 35-base SHV oligoprobe (underlined in italics). Asterisks represent identical nucleotides in the sequences. Sequence alignment performed with BLAST software (Altschul et al., 1990).

A second probe for the detection of SHV-related genes was prepared by amplifying a portion of the SHV-1 gene in a PCR assay. Primers were designed which annealed to conserved areas of the SHV related genes but not to LEN-1. However, since this probe was 714bp long, and given the fact that there is 89% homology between LEN-1 and the probe over this region, it would be less specific than the oligoprobe for the detection of SHV-related genes.

PCR was carried out in a similar fashion to that described earlier for the TEM probe [3.2.2] using plasmid DNA extracted from the SHV-1 producing control strain as a template. The parameters of the PCR assay were identical to those described for the preparation of the TEM probe. The primers SHV-A (5' CTG GCG GTA CAC GCC AGC) and SHV-B (5' TGC GCT CTG CTT TGT TA) which anneal to positions 48 - 66 and 769 - 743 respectively on the SHV-1 gene (Fig 3.4) yield a product of 714 bp in length.

```
GTTATGCGTT  ATATTCGCCT  GTGTATTATC  TCCCTGTTAG  CCACCCTGCC  GCTGGCGGTA   60
   →                                                          SHV-A
CACGCCAGCC  CGCAGCCGCT  TGAGCAAATT  AAACTAAGCG  AAAGCCAGCT  GTCGGGCCGC   120
GTAGGCATGA  TAGAAATGGA  TCTGGCCAGC  GGCCGCACGC  TGACCGCCTG  GCGCGCCGAT   180
GAACGCTTTC  CCATGATGAG  CACCTTTAAA  GTAGTGCTCT  GCGGCGCAGT  GCTGGCGCGG   240
GTGGATGCCG  GTGACGAACA  GCTGGAGCGA  AAGATCCACT  ATCGCCAGCA  GGATCTGGTG   300
GACTACTCGC  CGGTCAGCGA  AAAACACCTT  GCCGACGGCA  TGACGGTCGG  CGAACTCTGC   360
GCCGCCGCCA  TTACCATGAG  CGATAACAGC  GCCGCCAATC  TGCTACTGGC  CACCGTCGGC   420
GGCCCCGCAG  GATTGACTGC  CTTTTTGCGC  CAGATCGGCG  ACAACGTCAC  CCGCCTTGAC   480
CGCTGGGAAA  CGGAACTGAA  TGAGGCGCTT  CCCGGCGACG  CCCGCGACAC  CACTACCCCG   540
GCCAGCATGG  CCGCGACCCT  GCGCAAGCTG  CTGACCAGCC  AGCGTCTGAG  CGCCCGTTCG   600
CAACGGCAGC  TGCTGCAGTG  GATGGTGGAC  GATCGGGTCG  CCGGACCGTT  GATCCGCTCC   660
GTGCTGCCGG  CGGGCTGGTT  TATCGCCGAT  AAGACCGGAG  CTGGCGAGCG  GGGTGCGCGC   720
GGGATTGTCG  CCCTGCTTGG  CCCGAATAAC  AAAGCAGAGC  GCATTGTGGT  GATTTATCTG   780
                                SHV-B
CGGGATACGC  CGGCGAGCAT  GGCCGAGCGA  AATCAGCAAA  TCGCCGGGAT  CGGCGCGGCG   840
CTGATCGAGC ACTGGCAACG  CTAA                                          864
                        ←
```

Fig 3.4 – DNA sequence of SHV-1 gene (Mercier & Levesque, 1990) with the annealing position of the two primers (SHV-A and SHV-B) illustrated. Coding sequence for SHV-1 starts at base 3. Start and stop codons are highlighted with arrowheads.

After performing PCR, the amplified DNA was purified by agarose gel electrophoresis and extracted from the agarose as described for the TEM probe [3.2.3.1 – 3.2.3.3].

## 3.2.5 Preparation of Probes

### *3.2.5.1 ECL Chemiluminescent Labelling*

Probes were labelled with a non-radioactive chemiluminescent label, horseradish peroxidase, provided in the ECL hybridisation, labelling and detection kit (Amersham, UK). Probe DNA (10µl at 10ng/ml) was denatured by placing it in boiling water for five minutes after which it was kept on ice to prevent re-annealing of the DNA. Equal volumes of the manufacturer's labelling solution and glutaraldehyde were added and the mixture incubated at 37°C for ten minutes. The initial binding of the enzyme to the denatured DNA probe takes place due to charge attraction and subsequently covalent chemical cross-links are formed when glutaraldehyde is added. The labelling reaction was performed immediately prior to hybridisation.

The presence of labelled DNA was detected using the two reagents provided by the manufacturers. The first detection reagent decays to hydrogen peroxide which is the substrate for horseradish peroxidase, and the hydrogen peroxide is degraded to water and oxygen radicals as described in the following equation:

$2H_2O_2$ ----> $2H_2O + O_2^-$

The second reagent contains luminol which when oxidised by the radicals produces a blue light which can be detected by exposing the membrane to an X-Ray film. The production of the blue light is increased in intensity and duration by an enhancer present in the second reagent. The presence of the labelled probe was assessed by exposing the membrane to X-Ray film (Curix) for 15 minutes to 18 hours.

### *3.2.5.2 $P^{32}$ Radioloabelling of Probe*

The oligoprobe for the detection of SHV genes was also labelled with [$\alpha$-$^{32}$P]dCTP using Ready-To-Go™ DNA labelling beads (Pharmacia, USA). Briefly, 50ng of the oligoprobe was dissolved in 45µl TE buffer in the reaction tube containing he DNA labelling beads. After the addition of 5µl of [$\alpha$-$^{32}$P]dCTP, the mixture was incubated at 37°C for 15 minutes, and the labelled probe was then used in a hybridisation reaction.

## 3.2.6 - DNA-DNA Hybridisation Using TEM and SHV Probes

### *3.2.6.1 ECL Labelled Probes*

The probes were hybridised to DNA that had been transferred to nylon membranes. Hybridisation was carried out using the ECL hybridisation and detection kit and were performed in hybridisation buffer provided by the manufacturers. This buffer contains 6M urea which lowers the $T_m$ of the hybridisation and a blocking agent (5% w/v) which prevents the probe binding to areas of the membrane that contain no sample DNA. The recommended concentration of 0,5M NaCl was added to the hybridisation buffer.

The membranes containing the transferred DNA were prehybridised in the hybridisation buffer at 42°C for at least two hours. Freshly labelled probe (100ng) was then added and hybridisation took place at 42°C overnight. The membranes were removed the following day and washed using varying stringency conditions. For the TEM probe, the wash buffer contained 6M urea, 0,4% SDS and 0,2x or 0,1xSSC. Two washes for ten minutes each at 55°C were carried out. The low concentration of SSC and consequently low ionic strength of the wash buffer increases the stringency of the washing procedure. Urea, which acts as a denaturing agent, also increases the stringency of the washing stage. Under these high stringency conditions, only a probe with a high degree of homology (approximately 90% or greater) to the sample DNA will remain annealed to the sample.

When the PCR generated SHV probe was used in hybridisation experiments, the membranes were washed under conditions of high stringency because of the high degree of homology between SHV-1 and the chromosomal LEN-1 of *K. pneumoniae*. These post hybridisation washes consisted of two washes for ten minutes each at 55°C using a buffer containing 6M urea, 0,1xSSC and 0,4% SDS. When the oligoprobe was used, however, the stringency was lowered: the wash buffer contained 0,5xSSC and the washes were performed at 42°C. After the washes, the membranes were sealed in clear plastic and exposed to X-Ray film (Curix RPI, Agfa) for a variable time before being developed. The chemiluminescent signal used in the ECL kit decays after approximately 72 hours, and the probes were thus not stripped from the membranes prior to being used in further hybridisation experiments.

#### *3.2.6.2 [$^{32}$P]Labelled Probe*

The membranes were prehybridised at 68°C in a prehybridisation solution containing 6xSSC, 5x Denhardt's reagent, 0,5% SDS and fragmented salmon sperm DNA (100µg/ml). Denhardt's reagent, salmon sperm DNA and SDS are all used to decrease non-specific binding of the probe to the membrane (Sambrook *et al.*, 1989).

After prehybridisation for an hour at 68°C, the freshly labelled probe was added to the solution, and hybridisation occurred overnight at 68°C. The following day the hybridisation solution was discarded and the membrane was washed in 6xSSC and 0,1% SDS four times at 68°C (ten minutes each wash). Following this, four washes of ten minutes each at 71°C were performed, and a single wash of two minutes at 74°C (Brown, 1991a). The membrane was then exposed to X-ray film (Hyperfilm, Amersham) for 72 hours at –70°C and developed. This hybridisation was only performed once, and the probes were not stripped off the membrane.

## 3.3 RESULTS

### 3.3.1 Preparation of TEM Probe

An internal fragment of the TEM-1 gene was amplified in a PCR assay. After extraction of the PCR product from agarose gel, it was cloned into pUC19 and introduced into competent cells.

Plasmid DNA was extracted from 18 of the putative transformants. Digestion of the DNA with the restriction enzyme *Hind*III confirmed the presence of recombinants in 12 of these 18. A partial DNA sequence was obtained of one of these [3.2.3.5], which showed 100% homology to the TEM-1 gene (Fig 3.5). The PCR product was then used as a probe for the detection of TEM related genes.

```
Clone      ATGAGCACTT TTAAAGTTCT GCTATGTGGC GCGGTATTAT CCCGTATTGA CGCCGGGCAA  60
           ********** ********** ********** ********** ********** **********
TEM-1 229 ATGAGCACTT TTAAAGTTCT GCTATGTGGC GCGGTATTAT CCCGTATTGA CGCCGGGCAA 288
```

Fig 3.5 Alignment of the sequence obtained from the recombinants with that of TEM-1 (numbering of TEM-1 corresponds to that in Fig 3.2). Asterisks indicate identical nucleotides in the sequence. Sequences aligned using BLAST software (Altschul *et al.*, 1990).

## 3.3.2 Results of Hybridisation with the TEM Probe

### *3.3.2.1 Slot Blots*

DNA transferred to a nylon membrane was hybridised with the TEM probe [3.2.6]. A signal was obtained from the DNA extracted from *E. coli* DH5α(pUC19) as well as pUC19 plasmid DNA, while no signal was obtained with the DNA of *E. coli* DH5α (Fig 3.6).

Of the 45 isolates, a strong signal was obtained with the DNA of 11, indicating the presence of a TEM related gene in these 11 isolates (Fig 3.6). A weak signal was obtained with the DNA of one isolate, M43. DNA from this isolate (approximately 2μg) was re-transferred to a nylon membrane along with appropriate controls. Hybridisation with the TEM probe was repeated and the membrane washed under the same stringency conditions and a strong signal was obtained from the isolate (Fig 3.6). The 12 isolates finally shown to contain a TEM related gene or genes consisted of 8 strains of *K. pneumoniae*, 2 *Salmonella* spp., 1 *S. marcescens* and 1 *P. agglomerans* (Table 3.1).

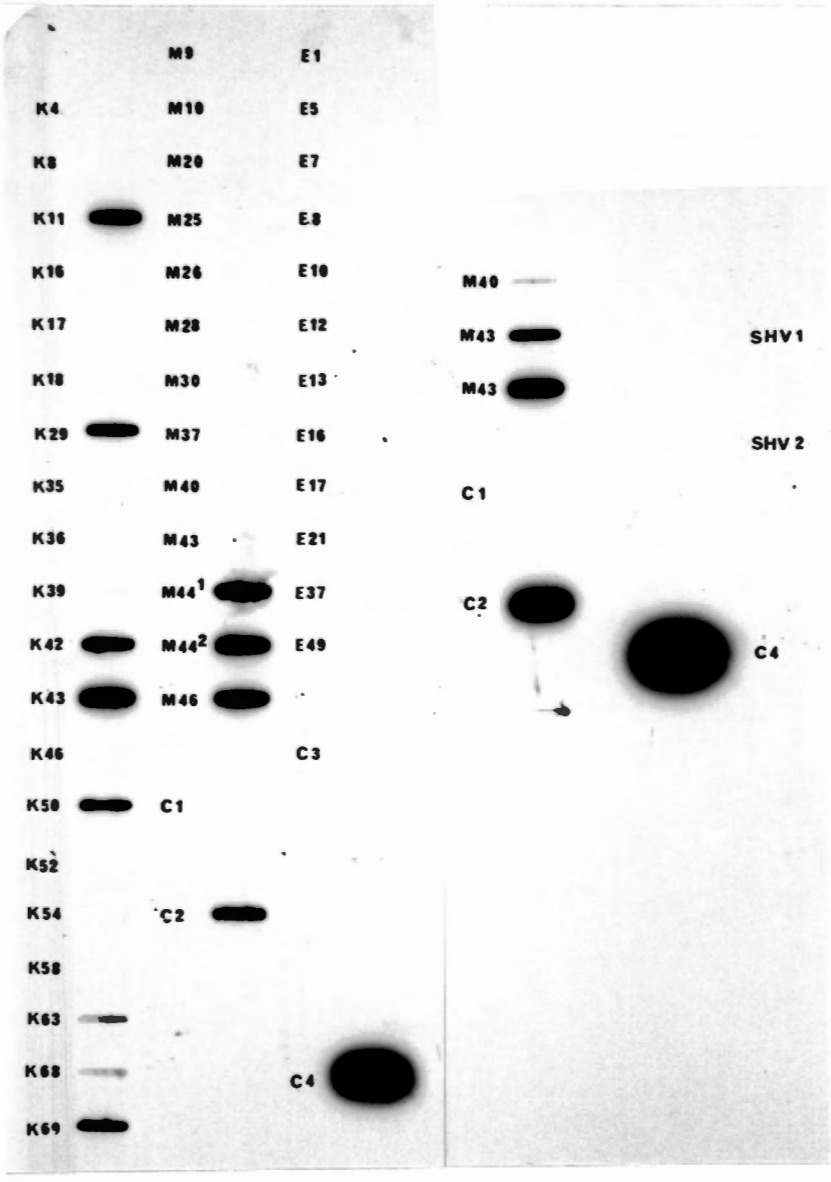



Fig 3.6 Hybridisation of the TEM probe to genomic DNA. The membrane was exposed to X-ray film (Curix RPI, Agfa) for 1 hour.

K4 – K50, *K. pneumoniae*

K52, *K. oxytoca*

K54 – K69, *K. pneumoniae*

M9, *C. freundii*

M10 & M20, *Citrobacter* spp.

M25 – M30, *K. pneumoniae*

M37 & M40, *E. cloacae*

M43, *P. agglomerans*

M44-1, M44-2, *Salmonella* spp

M46, *S. marscecens*

E1 – E7, *E. cloacae*

E8, E10, *Enterobacter* spp.

E12 – E17, *E. cloacae*

E21 & E37, *Enterobacter spp.*

E49, *E. cloacae*

C1, *E. coli* DH5α

C2, *E. coli* DH5α(pUC19)

C3, distilled $H_2O$

C4, pUC19

SHV-1, *E. coli* K12(SHV-1)

SHV-2, *E. coli* K12(SHV-2)

Table 3.1

Summary of results obtained after hybridisation with the TEM probe

| Isolate | Identity | Result | Isolate | Identity | Result | Isolate | Identity | Result |
|---|---|---|---|---|---|---|---|---|
| K4 | *K. pneumoniae* | N | K54 | *K. pneumoniae* | N | M44-1 | *Salmonella* sp. | P |
| K8 | *K. pneumoniae* | N | K58 | *K. pneumoniae* | N | M44-2 | *Salmonella* sp. | P |
| K11 | *K. pneumoniae* | P | K63 | *K. pneumoniae* | P | M46 | *S. marscecens* | P |
| K16 | *K. pneumoniae* | N | K68 | *K. pneumoniae* | P | E1 | *E. cloacae* | N |
| K17 | *K. pneumoniae* | N | K69 | *K. pneumoniae* | P | E5 | *E. cloacae* | N |
| K18 | *K. pneumoniae* | N | M9 | *C. freundii* | N | E7 | *E. cloacae* | N |
| K29 | *K. pneumoniae* | P | M10 | *C.* sp. | N | E8 | *E.* sp. | N |
| K35 | *K. pneumoniae* | N | M20 | *C.* sp. | N | E10 | *E.* sp. | N |
| K36 | *K. pneumoniae* | N | M25 | *K. pneumoniae* | N | E12 | *E. cloacae* | N |
| K39 | *K. pneumoniae* | N | M26 | *K. pneumoniae* | N | E13 | *E. cloacae* | N |
| K42 | *K. pneumoniae* | P | M28 | *K. pneuminiae* | N | E16 | *E. cloacae* | N |
| K43 | *K. pneumoniae* | P | M30 | *K. pneumoniae* | N | E17 | *E. cloacae* | N |
| K46 | *K. pneumoniae* | N | M37 | *E. cloacae* | N | E21 | *E.* sp. | N |
| K50 | *K. pneumoniae* | P | M40 | *E. cloacae* | N | E37 | *E.* sp. | N |
| K52 | *K. oxytoca* | N | M43 | *P. agglomerans* | P | E49 | *E. cloacae* | N |
| | | | *E. coli* DH5α(pUC19) | | P | *E. coli* DH5α | | N |

P = Positive

N= Negative

### *3.3.2.2 Colony Blots*

The TEM probe was hybridised to two colony blots and the membranes were washed under conditions of high stringency [3.2.6]. A strong signal was obtained with the DNA from the positive control, *E. coli* DH5α(pUC19), but signals, although of less intensity, were also obtained from the DNA of the negative control, *E. coli* DH5α.

With respect to the blot prepared by lysing cells which were initially cultured on agar [3.2.2.2], a signal was obtained from 21 of the isolates, including those with a weak signal (Fig 3.7) Of these 21, only one was a *K. pneumoniae* isolate. The other 20 consisted of 14 *Enterobacter* spp., 3 *Citrobacter* spp., 2 *Salmonellae* and 1 *S. marcescens.* As can be seen from the blot (Fig 3.7), the intensity of the signal obtained from the DNA of the positive controls is not consistent and a signal was also obtained from the DNA of the negative

control, albeit weaker than the signal from the positive controls. The signal obtained from the DNA of 9 of the 21 isolates is of a similar intensity to the signal obtained from the negative control.

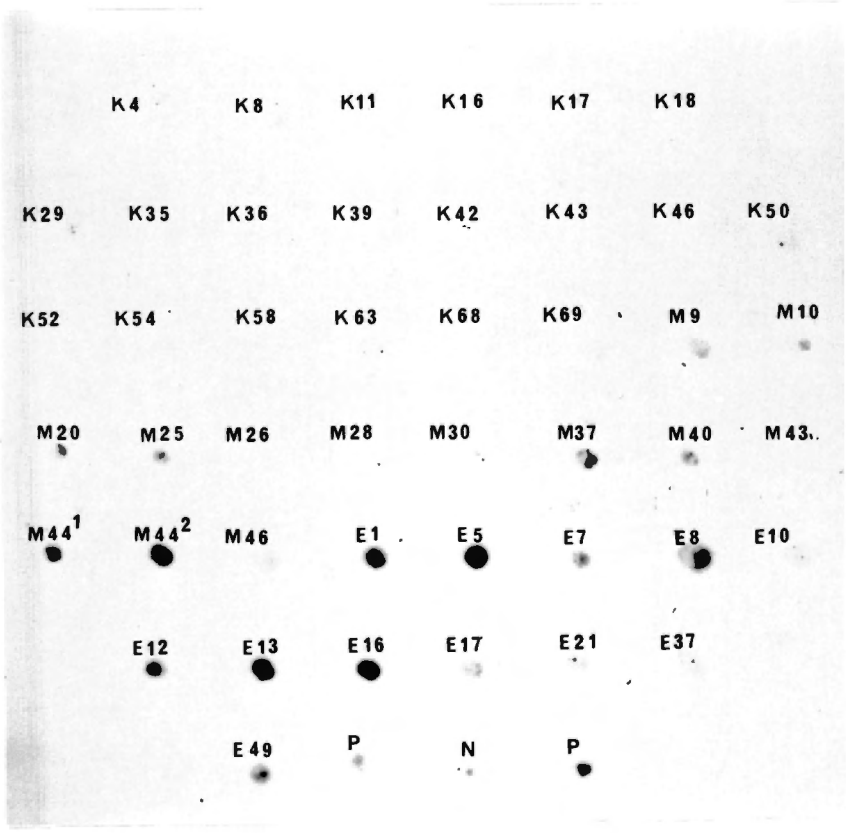



Fig 3.7 Hybridisation of TEM probe to colony blot prepared by culturing colonies of the test isolates on agar, transferring the colonies to a nylon membrane and lysing the cells with NaOH. Exposed to X-ray film (Curix RPI, Agfa) for 30 minutes.

K4 – K50 = *K. pneumoniae*

K52 = *K. oxytoca*

K54 – K69 = *K. pneumoniae*

M9 = *C. freundii*

M10 & M20 = *Citrobacter* spp.

M25 – M30 = *K. pneumoniae*

M37 & M40 = *E. cloacae*

M43 = *P. agglomerans*

M44-1, M44-2 = *Salmonella* spp

M46 = *S. marscecens*

E1 – E7 = *E. cloacae*

E8, E10 = *Enterobacter* spp.

E12 – E17 = *E. cloacae*

E21 & E37 = *Enterobacter* spp.

E49 = *E. cloacae*

P = *E. coli* DH5α(pUC19)

N = *E. coli* DH5α

With respect to the blot prepared by inoculating the cells directly onto the nylon membrane, a signal was obtained from the DNA of 18 of the 45 isolates. Of the 18 positive signals, 13 were from *Enterobacter* spp., 2 from *Citrobacter* spp., 2 from *Salmonella* spp. and one from *S. marcescens.* In this blot, however, the difference between the intensity of the signals obtained from the positive and negative controls was less distinct than in the first blot. Three negative controls were included in this blot and a signal obtained from all of them. The signals obtained from some of the negative controls were stronger than the signal obtained from the positive control. Once again, the differentiation of positive signals from negative signals produced by the isolates was difficult, with the signals obtained from 10 of the isolates being of a similar intensity to the signal generated by the negative control.

Comparison of the results of the colony blot with the results of the slot blots shows no significant correlation. For example, only one isolate of *K. pneumoniae* was shown to contain a TEM-related gene in these hybridisations, while 8 *K. pneumoniae* isolates were shown to contain a TEM-related gene by hybridisation of the same probe to genomic DNA. Given the inconsistent nature of the signals obtained from both the controls and the test isolates as well as the discrepancies with the slot blot hybridisations, meaningful analysis of the results of these colony blots is not feasible.

## 3.3.3 Results of Hybridisation with the SHV Probes

### *3.3.3.1 Slot Blots*

The oligoprobe (labelled with the chemiluminescent label) was hybridised to genomic DNA of all the isolates and washed under conditions of low stringency. The same membranes were used as were used for the hybridisation with the TEM probe and the SHV positive controls are on a separate membrane. These two membranes were hybridised and washed at the same time under the same stringency conditions, and for clarity will be referred to jointly as one membrane. As mentioned in section 3.2.6.1, low stringency conditions were used with the oligoprobe, since no signal could be obtained under conditions of higher stringency.

After hybridisation of the oligoprobe to the membrane, positive signals were obtained from the DNA of the positive controls, *E. coli* K-12 (SHV-1) and *E. coli* K12 (SHV-2). No signal was obtained from the negative controls. A signal was obtained with DNA of 24 of the 45 isolates. Of these 24, 20 were *K. pneumoniae* isolates, 2 were *E. cloacae* and 2 were *Salmonella* spp. A faint signal was obtained from the DNA of isolate K63 (*K. pneumoniae*) (Fig 3.8-i).

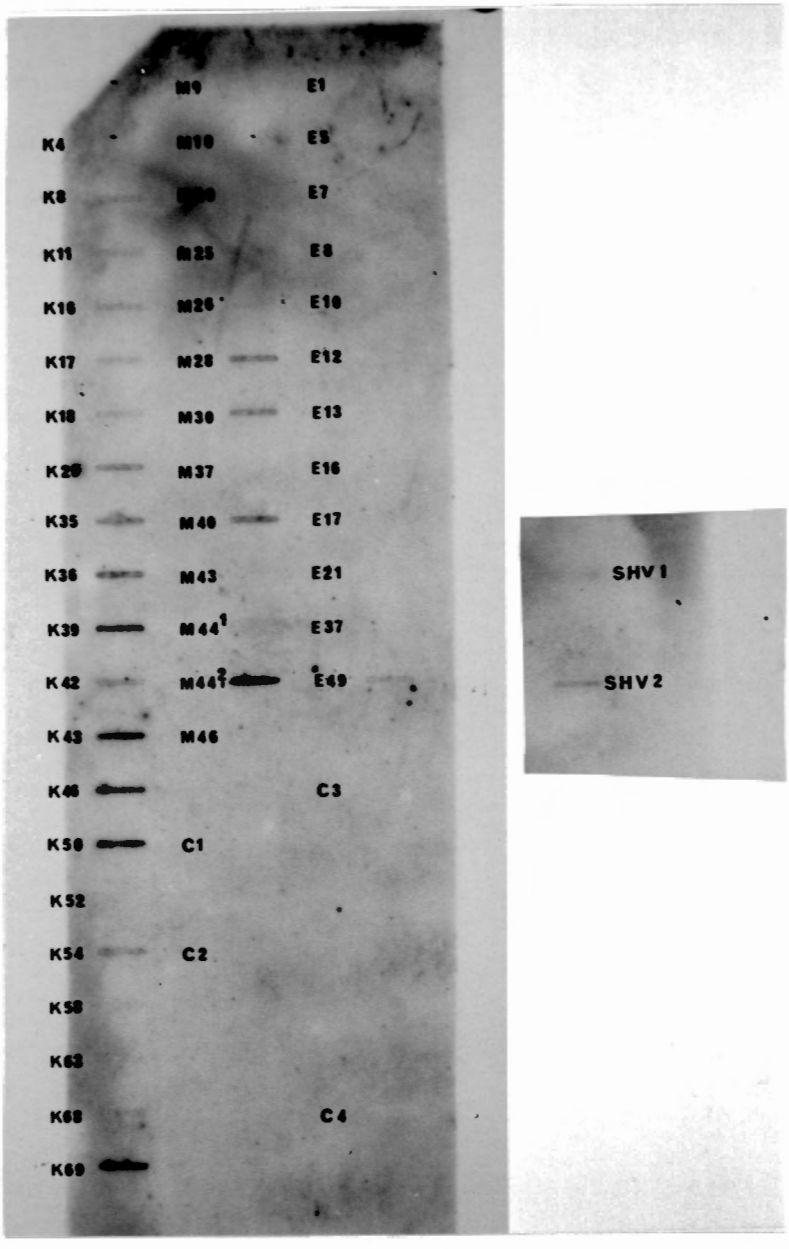



Fig 3.8 - i Hybridisation of the SHV oligoprobe (ECL chemiluminescent label) to genomic DNA. The membrane was washed under low stringency conditions and exposed overnight to X-ray film (Curix RPI, Agfa).

K4 – K50, *K. pneumoniae*

K52 , *K. oxytoca*

K54 – K69, *K. pneumoniae*

M9, *C. freundii*

M10 & M20, *Citrobacter* spp.

M25 – M30, *K. pneumoniae*

M37 & M40, *E. cloacae*

M43, *P. agglomerans*

M44-1, M44-2, *Salmonella* spp

M46, *S. marscecens*

E1 – E7, *E. cloacae*

E8, E10, *Enterobacter* spp

E12 – E17, *E. cloacae*

E21 & E37, *Enterobacter spp.*

E49, *E. cloacae*

C1, *E. coli* DH5α

C2, *E. coli* DH5α(pUC19)

C3, distilled $H_2O$

C4, pUC19

SHV-1, *E. coli* K12(SHV-1)

SHV-2, *E. coli* K12(SHV-2)

In order to increase the sensitivity and specificity of the hybridisation with the oligoprobe, the oligoprobe was labelled with [α-$^{32}$P]dCTP and washed under conditions of higher stringency [3.2.6.2]. Under these conditions the probe hybridised to the DNA from the positive control *E. coli* K12(SHV-1) but not to DNA from *E. coli* DH5α or to the plasmid pUC19 (containing the TEM-1 gene). A signal was obtained with the DNA of 27 of the isolates. These isolates consisted of 23 *K. pneumoniae*, 2 *E. cloacae* and 2 *Salmonella* spp. The signal obtained from 7 (6 *K. pneumoniae* and 1 *E. cloacae)* of these 27 isolates was weaker than the signal from the positive control (Fig 3.8-ii). Despite these signals being weak, they were assessed as being positive results in light of the absence of any signal from the negative controls. The strains to which the radiolabelled probe hybridised and the ECL-labelled probe did not were K4, K63 and M25, all isolates of *K. pneumoniae*.

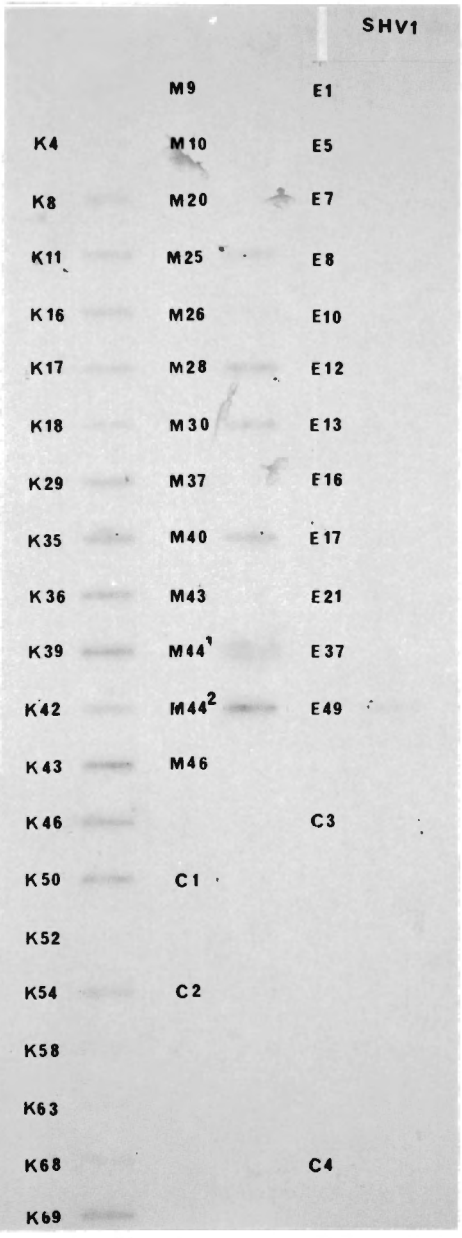



Fig 3.8 - ii Hybridisation of SHV oligoprobe ([$^{32}$P] labelled) to genomic DNA of isolates. Overnight exposure to X-ray film (Hyperfilm, Amersham). The signals obtained from hybridisation to DNA of isolates K4, K63 and M26 are not readily visible in the photograph, but are discernible (albeit weakly) in the original autoradiograph.

K4 – K50, *K. pneumoniae*

K52 , *K. oxytoca*

K54 – K69, *K. pneumoniae*

M9, *C. freundii*

M10 & M20, *Citrobacter* spp.

M25 – M30, *K. pneumoniae*

M37 & M40, *E. cloacae*

M43, *P. agglomerans*

M44-1, M44-2, *Salmonella* spp

M46, *S. marscecens*

E1 – E7, *E. cloacae*

E8, E10, *Enterobacter* spp

E12 – E17, *E. cloacae*

E21 & E37, *Enterobacter spp.*

E49, *E. cloacae*

C1, *E. coli* DH5α

C2, *E. coli* DH5α(pUC19)

C3, distilled $H_2O$

C4, pUC19

SHV-1, *E. coli* K12(SHV-1)

The second SHV probe was designed to assess whether any differences would arise in the results of hybridisation with an oligoprobe or a longer probe. This probe was obtained using primers specific for the SHV genes in a PCR assay and purifying the product. Hybridisation with this probe was carried out under highly stringent conditions given the high degree of homology between SHV-1 and the chromosomal gene of *K. pneumoniae* (90%). The washes were performed at 55°C with the wash buffer containing 0,1xSSC. The same membranes were used as were used for the hybridisation with the chemiluminescent labelled SHV oligoprobe.

Under high stringency conditions a signal was obtained from the DNA of the positive controls. No signal was obtained from genomic DNA extracted from *E. coli* DH5α(pUC19) on the larger membrane. A faint signal was seen from the area in which pUC19 had been transferred to the membrane (position C4 in Fig 3.8-iii), but a similar signal was obtained from a blank area in the smaller membrane (between SHV- and SHV-2, Fig 3.8-iii). This is interpreted as possibly representing some form of binding of the probe to the membrane itself. The difference between these weaker signals and the positive controls is easily distinguishable, and amongst the test isolates no signals of a similarly low intensity were obtained. All 23 of the *K. pneumoniae* isolates yielded a positive signal, but the single *K. oxytoca* did not show any signal. Of the other isolates, 2 *E. cloacae* and 2 *Salmonella* spp. also showed a positive signal (Fig 3.8-iii). The results of the hybridisation with the longer probe thus correlate perfectly with the results obtained using the $^{32}P$ labelled oligoprobe.

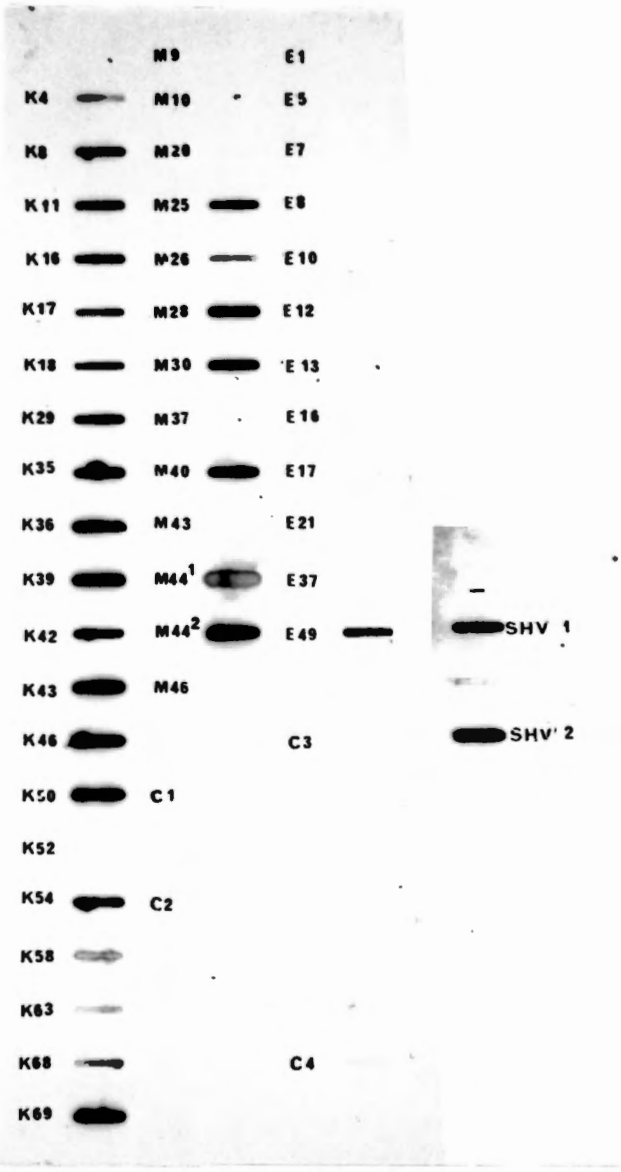



Fig 3.8 - iii Hybridisation of PCR generated SHV probe (ECL chemiluminescent label) to genomic DNA. One hour exposure to X-ray film (Curix RPI, Agfa).

K4 – K50, *K. pneumoniae*

K52 , *K. oxytoca*

K54 – K69, *K. pneumoniae*

M9, *C. freundii*

M10 & M20, *Citrobacter* spp.

M25 – M30, *K. pneumoniae*

M37 & M40, *E. cloacae*

M43, *P. agglomerans*

M44-1 & M44-2, *Salmonella* spp

M46, *S. marscecens*

E1 – E7, *E. cloacae*

E8 & E10, *Enterobacter* spp

E12 – E17, *E. cloacae*

E21 & E37, *Enterobacter spp.*

E49, *E. cloacae*

C1, *E. coli* DH5α

C2, *E. coli* DH5α(pUC19)

C3, distilled $H_2O$

C4, pUC19

SHV-1, *E. coli* K12(SHV-1)

SHV-2, *E. coli* K12(SHV-2)

The results of the three hybridisations are shown in Table 3.2. The results that do not correlate among all three hybridisations are highlighted.

Table 3.2

Results of the hybridisations using the various SHV probes

| Isolate | Identity | 1 | 2 | 3 | Isolate | Identity | 1 | 2 | 3 |
|---|---|---|---|---|---|---|---|---|---|
| K4 | *K. pneumoniae* | N | P | P | M25 | *K. pneumoniae* | N | P | P |
| K8 | *K. pneumoniae* | P | P | P | M26 | *K. pneumoniae* | P | P | P |
| K11 | *K. pneumoniae* | P | P | P | M28 | *K. pneumoniae* | P | P | P |
| K16 | *K. pneumoniae* | P | P | P | M30 | *K. pneumoniae* | P | P | P |
| K17 | *K. pneumoniae* | P | P | P | M37 | *E. cloacae* | N | N | N |
| K18 | *K. pneumoniae* | P | P | P | M40 | *E. cloacae* | P | P | P |
| K29 | *K. pneumoniae* | P | P | P | M43 | *P. agglomerans* | N | N | N |
| K35 | *K. pneumoniae* | P | P | P | M44-1 | *Salmonella* sp. | P | P | P |
| K36 | *K. pneumoniae* | P | P | P | M44-2 | *Salmonella* sp. | P | P | P |
| K39 | *K. pneumoniae* | P | P | P | M46 | *S. marscecens* | N | N | N |
| K42 | *K. pneumoniae* | P | P | P | E1 | *E. cloacae* | N | N | N |
| K43 | *K. pneumoniae* | P | P | P | E5 | *E. cloacae* | N | N | N |
| K46 | *K. pneumoniae* | P | P | P | E7 | *E. cloacae* | N | N | N |
| K50 | *K. pneumoniae* | P | P | P | E8 | *E.* sp. | N | N | N |
| K52 | *K. oxytoca* | N | N | N | E10 | *E.* sp. | N | N | N |
| K54 | *K. pneumoniae* | P | P | P | E12 | *E. cloacae* | N | N | N |
| K58 | *K. pneumoniae* | P | P | P | E13 | *E. cloacae* | N | N | N |
| K63 | *K. pneumoniae* | N | P | P | E16 | *E. cloacae* | N | N | N |
| K68 | *K. pneumoniae* | P | P | P | E17 | *E. cloacae* | N | N | N |
| K69 | *K. pneumoniae* | P | P | P | E21 | *E.* sp. | N | N | N |
| M9 | *C. freundii* | N | N | N | E37 | *E.* sp. | N | N | N |
| M10 | *C.* sp. | N | N | N | E49 | *E. cloacae* | P | P | P |
| M20 | *C.* sp. | N | N | N | | | | | |
| | *E. coli* K12 (SHV-1) | P | | | | *E. coli* DH5α | N | | |

1 - SHV oligoprobe, ECL labelled, low stringency

2 – SHV oligoprobe, radiolabelled, high stringency

3 - SHV PCR Product, high stringency

P = Positive

N= Negative

### *3.3.3.2 Colony Blot*

The SHV probe obtained by PCR was hybridised to a blot where the isolates had been initially inoculated onto agar, incubated and then a nylon membrane placed on the agar and

peeled off. The hybridisation was done under high stringency conditions at 55°C with 0,1xSSC in the wash buffer.

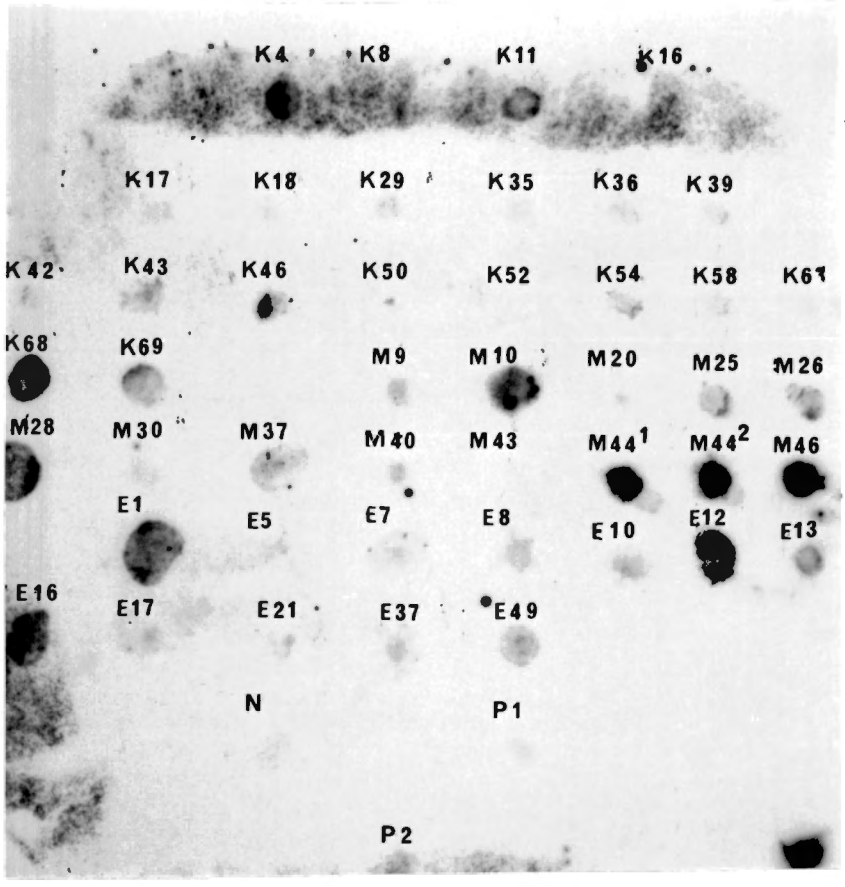



Fig 3.9 Hybridisation of PCR generated SHV probe to colony blot prepared by culturing colonies of the test isolates on agar, transferring the colonies to a nylon membrane and lysing the cells with NaOH. The membrane was exposed to X-ray film (Curix RPI, Agfa) for 1 hour.

K4 – K50, *K. pneumoniae*

K52 , *K. oxytoca*

K54 – K69, *K. pneumoniae*

M9, *C. freundii*

M10 & M20, *Citrobacter* spp.

M25 – M30, *K. pneumoniae*

M37 & M40, *E. cloacae*

M43, *P. agglomerans*

M44-1, M44-2, *Salmonella* spp

M46, *S. marscecens*

E1 – E7, *E. cloacae*

E8, E10, *Enterobacter* spp

E12 – E17, *E. cloacae*

E21 & E37, *Enterobacter spp.*

E49, *E. cloacae*

N, *E. coli* DH5α

P1, *E. coli* K12(SHV-1)

P2, *E. coli* K12(SHV-2)

A weak signal was obtained from the positive controls, and a similarly weak signal obtained from the negative control *E. coli* DH5α, making differentiation of positive from negative in the isolates difficult. Looking at the blot (Fig 3.9), a weak signal can be seen with all the isolates. However, 18 of the isolates, comprising 9 *K. pneumoniae*, 5 *Enterobacter* spp., 2 *Salmonella* spp., 1 *Citrobacter* spp. and 1 *S. marcescens*, show a strongly positive signal. Of these 18 isolates, 11 also gave positive results in the three slot blots. However, 5 of the isolates that produced "positive" results on the colony blot were negative on all three slot blots, while 13 of the isolates with "negative" results from this colony blot were positive on all three slot blots. Given the considerable inconsistencies between the results of the colony blots and the slot blots, the results of these colony blots (as with the TEM hybridised colony blots) were considered to be unreliable and were disregarded.

## 3.4 DISCUSSION

TEM-related genes were detected in 12 of the 45 isolates studied. Of these, 8 were *K. pneumoniae*, 2 *Salmonella* spp., 1 *P. agglomerans* and 1 *S. marcescens*. Thus, of a total of 23 *K. pneumoniae* isolates in the study, 8 were shown to contain TEM related genes. Studies from around the world have found differing proportions of TEM related genes in isolates of *K. pneumoniae*. In 1988, Jarlier *et al.* demonstrated the presence of TEM related genes in 5 of 16 *K. pneumoniae* isolates from Paris with a decreased susceptibility to cefotaxime. These 5 comprised 2 TEM-1 and 3 CTX-1/TEM-3 beta lactamases. It is interesting to note that the isolates in this study that produced TEM-1 beta lactamases also produced SHV-2 beta lactamase.

In another study, 5 out of 11 *K. pneumoniae* isolates from Poland were shown to contain TEM-related ESBLs and 3 of the isolates contained SHV-related ESBLs (Gniadkowski *et al.*, 1998). In South Africa, a study of faecal isolates of *E. coli* and *K. pneumoniae* showed TEM-1 and TEM-2 to be present in over 95% of the isolates. Unfortunately, this study did not discriminate between the incidence in *E. coli* and *K. pneumoniae* (Shanahan *et al.*, 1995). While TEM-1 is by far the most common beta lactamase produced by the *Enterobacteriaceae*, comprising about 80% of all plasmid encoded beta lactamases in

these organisms (Du Bois *et al.*, 1995), TEM-related ESBLs are not necessarily more common than their SHV related counterparts. This is also interesting in view of the fact that there are a larger number of different TEM-related enzymes than SHV-related enzymes. Our finding of TEM-related genes in 8 out of 24 *K. pneumoniae* isolates is in keeping with findings in other areas of the world.

The finding of TEM-related genes in other members of the *Enterobacteriaceae*, such as species of *Serratia* and *Salmonella*, has also been described. (Gutmann *et al.*, 1988; Kariuki *et al.*, 1996; Morosini *et al.*, 1996; Perilli *et al.*, 1997; Luzarro *et al.*, 1998; Shanahan *et al.*, 1998). Similar information on *Pantoea agglomerans* is lacking, although this may be due to *Enterobacter agglomerans* having been reclassified as *P. agglomerans* (Holt *et al.*, 1994). What is interesting, is that both *Serratia* and *Pantoea* species are known to harbour a chromosomal beta lactamase (AmpC) (Livermore, 1995), which makes it unlikely that the acquisition of another beta lactamase encoding gene would confer any survival advantage. However, plasmids conferring antibiotic resistance often contain more than one resistance gene, and the presence of the other resistance genes may confer the survival advantage that allows the strain with the plasmid to predominate (Pitout *et al.*, 1998b). The TEM-related gene (whether encoding an ESBL or not) in this case is something of an "innocent bystander".

The initial results using the oligoprobe for detection of SHV-related genes in isolates of *K. pneumoniae* were promising. This probe did not hybridise to some of the isolates, suggesting that hybridisation to non-specific, SHV-like chromosomal beta lactamase genes did not occur. However, in subsequent hybridisation studies using the same oligoprobe with a radioactive label, as well as the longer SHV probe, signals were obtained with DNA from all the *K. pneumoniae* isolates. As it is unlikely that all the *K. pneumoniae* isolates contain a plasmid mediated SHV related gene, these results were disregarded.

While some studies have found a predominance of SHV-related ESBLs in *K. pneumoniae* isolates, no studies have demonstrated SHV-related ESBLs in all the *K. pneumoniae* isolates studied. In 1996, Jacoby and Han characterised the beta lactamases produced by 117 transconjugants from crosses involving clinical isolates of *E.coli* and *K. pneumoniae* by iso-electric focussing. Their findings indicated that SHV-related ESBLs (especially

SHV-4 and SHV-5) were the commonest ESBLs produced by the transconjugants, although TEM-related enzymes were also found. Unfortunately, the study did not specify which organisms contained which beta lactamases.

Jarlier *et al.* (1988) conducted a similar study, and found that all the *K. pneumoniae* donor strains produced an enzyme with an isoelectric point of 7.7, identified as SHV-1, and presumed it to be chromosomal. Unfortunately this gene was not sequenced and any similarity with LEN-1 is unknown. However, since LEN-1 has a pI of 7,0 – 7,1 (Hæggman *et al.*, 1997) it is probable that the chromosomal beta lactamase gene with a pI of 7.7 differs in some respects from LEN-1. Hæggman *et al.* (1997) also found that the majority of chromosomal beta lactamases from isolates of *K. pneumoniae* had a pI of either 7.6 (SHV-1 like) or 7.1 (LEN-1 like). Similarly, Leung *et al.* (1997) found that non-transferable beta lactamases from 77 isolates of *K. pneumoniae* had pIs ranging from 6,7-8,0 with the majority (~80%) ranging from 7,1 – 7,6. It therefore appears that while LEN-1 is the only chromosomal beta lactamase from *K. pneumoniae* that has been sequenced to date, there are probably other SHV-like chromosomal genes in *K. pneumoniae* with even greater homology to the plasmid mediated SHV-1.

Since both probes hybridised to DNA from all of the *K. pneumoniae* isolates, it is assumed that both probes hybridised to a chromosomal beta lactamase gene similar to SHV-1 or LEN-1, as well as to any plasmid mediated SHV-related gene/s in the organisms. Workers who have demonstrated plasmid mediated SHV-related genes in *K. pneumoniae* isolates have relied on analysis of *E. coli* transconjugants following conjugation experiments. A similar approach was adopted in this study and conjugation experiments were carried out [chapter 5].

Hybridisations using the SHV probes proved useful for detecting SHV-related genes in genera other than *Klebsiella.* Since any chromosomal beta lactamase genes in these organisms should not cross hybridise with a SHV probe, interpretation of the results is possible. Of the 22 non-*K. pneumoniae* isolates (including members of the genera *Citrobacter, Enterobacter, Pantoea, Serratia* and *Salmonella*), a positive signal was obtained from 2 isolates of *E. cloacae* and 2 isolates of *Salmonella* sp.

The finding of SHV-related genes in *Enterobacter* species is unusual, although it has been described (Pitout *et al.*, 1997; Pitout *et al.*, 1998a,b). What is possibly more interesting is the demonstration of SHV genes in the *Salmonella* isolates in addition to the TEM genes [3.3.2.1]. Although *Salmonella* species are usually sensitive to extended spectrum cephalosporins, instances of *Salmonella* species harbouring either SHV or TEM related beta lactamases have been reported (Barguellil *et al.*, 1995). Although there is no reason why both types of beta lactamase may not be present in a single isolate, it is unusual. It may be that one of the enzymes has extended spectrum activity, conferring a survival advantage, which is absent in the other enzyme. It should be mentioned here that the *Salmonella* isolates examined in this study were obtained from the same patient, albeit 4 or 5 days apart and are probably the same strain.

Some other considerations should also be mentioned pertaining to the use of hybridisation for detection of beta lactamases. Whether an oligoprobe or longer probe is used, it should be designed to anneal to the intragenic portion of a beta lactamase gene rather than upstream or downstream regions, since the latter can lead to false positive results (Payne & Thomson, 1998). With respect to the use of hybridisations in a routine diagnostic laboratory: it is too labour intensive and technically demanding, and is not appropriate in a clinical laboratory.

## Part II

# CHAPTER 4

# DETECTION OF TEM AND SHV RELATED GENES USING THE POLYMERASE CHAIN REACTION (PCR)

## 4.1 INTRODUCTION

One of the many applications of the polymerase chain reaction (PCR) is the detection of resistance genes, including the detection of TEM and SHV related genes in Gram negative bacilli (Leung *et al*, 1997; Payne & Thomson, 1998; Van Belkum *et al*, 1998). This chapter will describe PCR assays performed to detect TEM- and SHV-related genes in the 45 clinical isolates selected in chapter 2, and the use of PCR will be evaluated and compared to the hybridisation techniques described in chapter 3.

## 4.2 MATERIALS AND METHODS

A variety of methods were used to prepare DNA for use as a template in the PCR assays, particularly during optimisation. This was done to ascertain a method of DNA preparation that would both yield a suitable template and be feasible in a clinical laboratory. The different methods used to isolate DNA are described below, followed by the details of the PCR assays. All PCR assays were performed in a Perkin Elmer GeneAmp 2400 PCR system.

## 4.2.1 Isolation of DNA

### *4.2.1.1 Plasmid DNA*

Plasmid DNA was extracted using the alkaline lysis method and purified further either by centrifugation in a CsCl density gradient or by using a commercial kit (Nucleobond, Machery Nagel, Germany) according to the manufacturer's instructions [3.2.3.4.vi].

Plasmid DNA was purified by centrifugation in a CsCl gradient (Sambrook *et al.*, 1989) by adding of 1g CsCl per ml of solution to the cleared lysate. Ethidium bromide (EtBr) was then added at 0,1 ml per ml of solution. The refractive index of the solution was adjusted by adding either CsCl or sterile water until the refractive index was 1,391, equivalent to a density of 1,55g/ml. The solution was then transferred to Beckman quickseal tubes and centrifuged overnight at 50 000 rpm (Beckman L70 Ultracentrifuge with V-ti 65-2 rotor). After centrifugation plasmid DNA was recovered in an Eppendorf tube.

Ethidium bromide was removed from the solution by repeated extractions with salt saturated isopropanol until the solution was colourless (usually 4 – 5 extractions). Two volumes of TE buffer were added, followed by isopropanol equal to the new volume. After standing at room temperature for ten minutes the solution was centrifuged in a microfuge at 12 000 rpm for ten minutes to pellet the DNA which was then washed, dried and resuspended in TE buffer [3.2.1.3].

### *4.2.1.2 Genomic DNA from Broth Cultures*

Genomic DNA was extracted [3.2.1.3] and resuspended in TE buffer. Aliquots of the DNA solution were subjected to agarose gel electrophoresis alongside known standards in order to estimate the concentration of DNA.

#### *4.2.1.3 Genomic DNA from Colonies*

Three methods were used to extract DNA from colonies of bacteria cultured on YT agar.

a) Two or three bacterial colonies were transferred from YT agar plates into 50µl of sterile water in an Eppendorf tube. These were then placed in a bath of boiling water for five to fifteen minutes to lyse the cells and release the DNA. The samples were then centrifuged in a microfuge at 14 000rpm at room temperature for 2 minutes to pellet bacterial cell wall debris and the tubes were placed on ice. A 5µl aliquot of the supernatant fluid was used as the template in a PCR reaction.

b) Two or three colonies were emulsified in 50µl of sterile water, placed in a bath of boiling water for five to fifteen minutes and then placed on ice. These samples were not centrifuged and 5µl of the mixture was used in the PCR reaction.

c) Two or three colonies were emulsified in 50µl of sterile water and 5µl used as the template without prior boiling or centrifugation.

#### *4.2.1.4 Control Strains*

The positive control strains were those used in the hybridisation experiments [3.2.1]. Negative controls consisted of DNA extracted from broth cultures or colonies of *E. coli* LK111 or DH5α. Sterile water was also used as a negative control.

## 4.2.2 PCR Assay for the Detection of TEM Related Genes

### *4.2.2.1 Primers*

The same primers used to amplify an intragenic portion of the pUC19 TEM-1 gene [3.2.3.1] were used in the PCR assays. The primers anneal to conserved sequences in the intragenic portion of TEM-related genes and when used in PCR assays an amplification product 517bp long is obtained. The primers (TEM-A 5' -CCC CGA AGA ACG TTT TC and TEM-B 5' -ATC AGC AAT AAA CCA GC), which anneal to bases 177-194 and 677-693 of the TEM-1 gene, respectively, were synthesised in the Department of Medical Biochemistry, University of Cape Town [3.2.3.1 – Fig 3.2].

### *4.2.2.2 PCR Assay: Parameters*

#### *i) Primer concentration*

Aliquots of concentrated primer solutions were stored at –20°C and diluted to working stocks of 10µM which were used in the PCR assays at concentrations ranging from 0,2µM to 0,6µM.

#### *ii) dNTPs*

dNTPs were used at a final concentration of 200µM of each dNTP.

#### *iii) Annealing Temperature and Time*

The melting temperature of the primers was determined by applying the following equation.

$T_M = 22 + 1{,}46\ (2x\ N^O\ G\&C + N^O\ A\&T)$ (Wu *et al.*, 1991)

*$N^O$ G&C and $N^O$ A&T represents the number of each of these bases in the primer sequence.*

Using this formula the melting temperatures for TEM-A and TEM-B were 60°C and 57°C, respectively. The recommended annealing temperature is approximately 5°C below the melting temperature (Innis & Gelfand, 1990). The annealing temperatures used in the

PCR assays ranged from 55°C – 62 °C and the duration of annealing was from 30 to 50 seconds.

*iv) Denaturation and Extension*

An initial denaturation step at 94°C for five minutes was performed. This was followed by cycles of denaturation, annealing and extension. Denaturation was carried out at 94°C and extension at 72°C. The duration of each stage varied slightly and denaturation time was increased from an initial 20 seconds to 30 seconds. Extension took place for 60 seconds with one exception, where the time was decreased to 30 seconds. A final extension step at 72°C for seven minutes occurred once the cycles had finished.

*v) Number of Amplification Cycles*

To increase the number of amplicons produced, the number of cycles was increased from 25 to 35 and then to 40.

#### *4.2.2.3 Predigestion with AvaII*

Contamination of PCR mixtures resulting in false positives is a well recognised hazard of this technique (Vaneechoutte & Van Eldere, 1997). To minimise this problem, digestion of the reaction mix with the restriction enzyme *AvaII* prior to the addition of template was carried out. *Ava*II recognises the restriction site, G/GWCC (W = A/T). This site occurs twice between the two primer sites in the TEM-1 gene but is not present in either of the primers. By pre-digesting the PCR mixture with *Ava*II, any contaminating DNA containing a TEM related gene would be digested into fragments too small to serve as a template.

## 4.2.3 PCR Assay for the Detection of SHV Related Genes

### *4.2.3.1 Primers*

A set of primers was designed to anneal to conserved sequences of all the SHV-related genes but not to the chromosomal LEN-1 gene of *K. pneumoniae.* The primers were designed to render the 3' ends incompatible with the corresponding regions of the LEN-1 gene, while still being compatible with all SHV related genes. The primers, SHV-A (5' CTG GCG GTA CAC GCC AGC) and SHV-B (5' TGC GCT CTG CTT TGT TA) anneal to bases 154-171 and 852-868, respectively, of the SHV-1 gene. SHV-A contains 4 mismatches with LEN-1, of which 2 (C/T and A/G) are in the last 3 nucleotides at the 3' end. Similarly, SHV-B contains 5 mismatches: 3 at the 3' end, in addition to two mismatches which are 8 and 10 base pairs from the 3' end. The mismatches are highlighted in Fig 4.1. The primers were synthesised by an oligonucleotide synthesiser at the University of Cape Town Department of Medical Biochemistry.

```
LEN-1 CCACTGGTGGTATACGCCGGTCCA      LEN-1 TGCCGTTTGGCCTCGCGTAA
      ** **** **** ***** * **             *   **** * *********
SHV-1 CCGCTGGCGGTACACGCCAGCCCG      SHV-1 TATTGTTTCGTCTCGCGTAA
            SHV-A       3'                  3'     SHV-B
```

Fig 4.1 – Annealing positions of the primers SHV-A and SHV-B, showing the mismatches with LEN-1

A second set of SHV primers, SHV-C and SHV-D, described by Nuesch-Inderbinen *et al* (1996), were also synthesised. Products amplified by these primers contain a site recognised by the restriction enzyme *Nhe*I. This site is present only in SHV related ESBL genes and not in the SHV-1 or LEN-1 genes. The presence of an SHV related ESBL gene can thus be detected by digesting the amplicon with the enzyme *Nhe*I. The primers, SHV-C and SHV-D (5' GCC CGG GTT ATT CTT ATT TGT CGC and 5' TCT TTC CGA TGC CGC CGC CAG TCA respectively), which anneal to sequences flanking the structural SHV-related genes (Nuesch-Inderbinen *et al.*, 1996) (Fig 4.2), were obtained from Boehringer Mannheim, Germany.

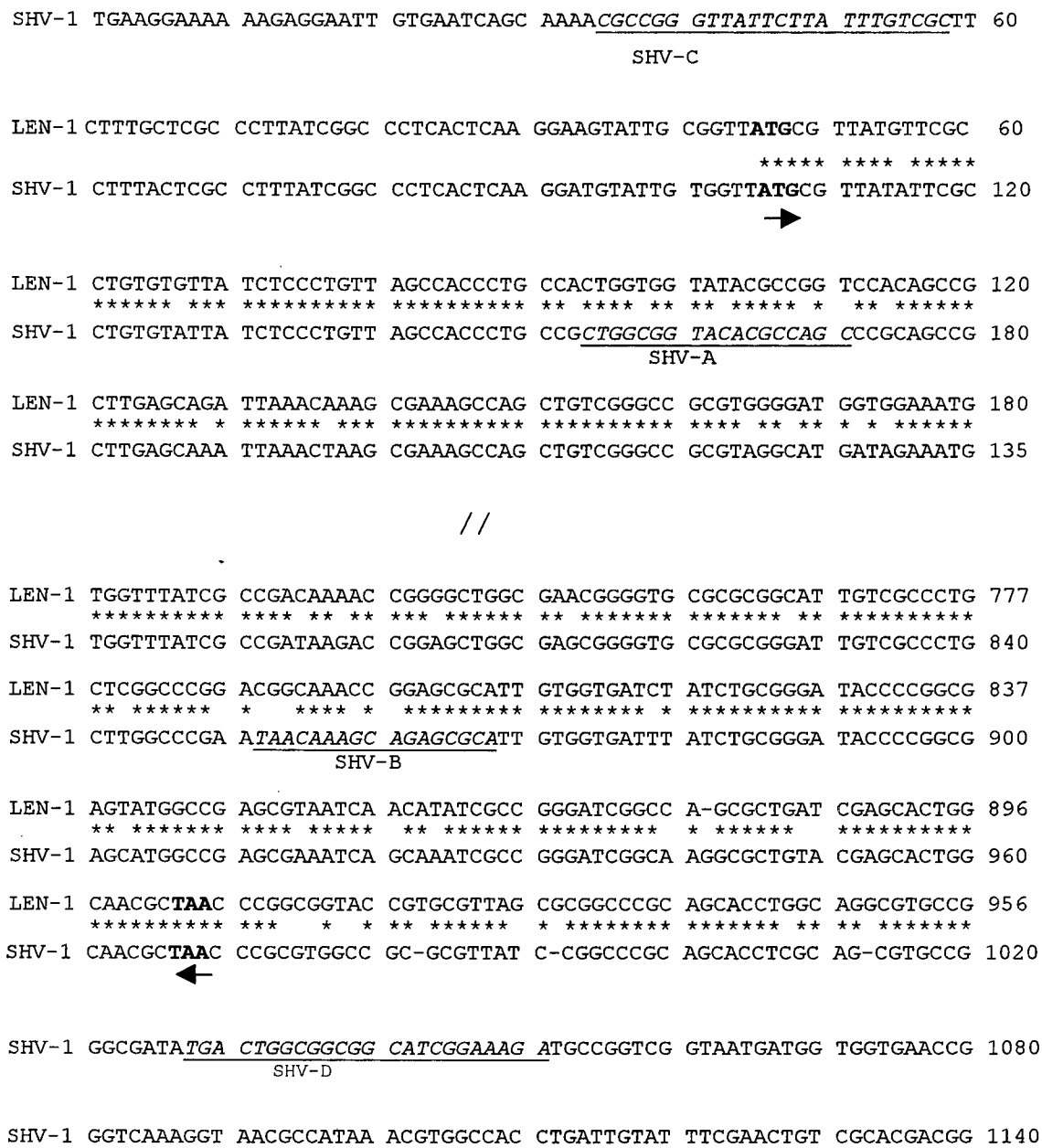



Fig 4.2 Sequence of the SHV-1 gene and flanking areas showing the sites of the primers used in PCR assays for detection of SHV–related genes. The start and stop codons are highlighted with arrowheads. Asterisks indicate identical nucleotides in the sequences of SHV-1 (Mercier & Levesque, 1990) and LEN-1 (Arakawa *et al.*, 1986). Sequences aligned using BLAST software (Altschul *et al.*, 1990). It should be noted that the first two 5' nucleotides of the SHV-C primer [4.2.3.1] do not correspond to the published sequence of the SHV-1 gene. The SHV-C sequence detailed in 4.2.3.1 is as described by Nuesch-Inderbinen *et al.* (1996).

#### *4.2.3.2 PCR Assays: Parameters*

The same parameters as those investigated for the TEM-PCR assays were used to optimise the SHV-PCR assays, and the calculations for annealing temperature of the primers were done in the same manner. The ranges of the various parameters altered while optimising the assays using the two sets of primers are represented in Table 4.1.

Table 4.1

| PARAMETER | PRIMERS | |
|---|---|---|
| | **SHV A and B** | **SHV C and D** |
| Primer concentration | 0,2 – 0,6μM | 0,2 – 0,6μM |
| dNTP concentration | 100 – 400μM | 100 – 400μM |
| Melting temperature (calculated) | SHV-A 67°C | SHV-C - 74°C |
| | SHV-B 59°C | SHV-D - 77°C |
| Denaturation temp and time | 94°C or 95°C for 30s | 94°C or 95°C for 30s |
| Annealing temp and time | 48°C - 60°C for 30 – 50s | 45°C - 60°C for 30 – 50s |
| Extension temp and time | 72°C for 30 – 50s | 72°C for 50s |
| Number of cycles | 35 - 40 | 40 |

# 4.3 RESULTS AND DISCUSSION

## 4.3.1 Results of Optimisation of PCR Assay to Detect TEM-Related Genes

Purified pUC19 or genomic DNA extracted from either broth cultures [3.2.1.3] or colonies [4.2.1.3] of *E. coli* DH5α(pUC19) were used as templates in optimisation of the PCR assays with the primers specific for TEM-related genes. Negative controls were as described earlier [4.2.1.4].

### *4.3.1.1 Parameters*

A summary of the results of optimisation experiments showing the variations in primer concentration, template and thermal cycle is presented in Table 4.2.

Table 4.2

| Template | Primer concentration | Denaturation | Annealing | Extension | Number of Cycles | Amplicon |
|---|---|---|---|---|---|---|
| pUC19 | 0,2μM | 94°C 20 s | 55°C 30 s | 72°C 60 s | 25 | No |
| | 0,4μM | 94°C 20 s | 55°C 30 s | 72°C 60 s | 25 | Yes |
| | 0,6μM | 94°C 20 s | 55°C 30 s | 72°C 60 s | 25 | Yes |
| | 0,4μM | 94°C 20 s | 60°C 30 s | 72°C 60 s | 25, 35, 40 | Yes (with all 3 cycles) |
| | 0,4μM | 94°C 20 s | 62°C 30 s | 72°C 60 s | 25 | Yes |
| Genomic DNA of *E.coli* DH5α(pUC19) | 0,4μM | 94°C 20 s | 55°C 30 s | 72°C 60 s | 25 | Yes (faint) |
| | 0,4μM | 94°C 30 s | 60°C 30 s | 72°C 30 s | 25 | Yes (faint) |
| Boiled and spun *E.coli* DH5α(pUC19) | 0,4μM | 94°C 20 s | 55°C 30 s | 72°C 60 s | 25, 35 | Yes (with both cycles) |
| | 0,4μM | 94°C 30 s | 60°C 50 s | 72°C 60 s | 25,35, 40 | Yes (with all 3 cycles) |
| | 0,4μM | 94°C 30 s | 62°C 30 s | 72°C 30 s | 25 | Yes |

*An initial denaturing step at 94°C for 5 minutes and a final extension at 72°C for seven minutes was always performed. dNTPs always at 200μM each. PCR carried out in the same machine on all occasions.*

*i) Primer Concentration*

The appropriate sized amplicon was produced using primer concentrations of both 0,4μM and 0,6μM. No product was obtained using a primer concentration of 0,2μM. All subsequent reactions were carried out using a primer concentration of 0,4uM.

*ii) Annealing Temperature*

Using an initial annealing temperature of 55°C, an amplification product of the correct size was obtained from the positive controls. The same size product however, was also obtained from the DNA of the negative controls using the same annealing temperature. To eliminate what was thought to be non-specific annealing of the primers, the annealing temperature was raised to 60°C and then to 62°C. At these annealing temperatures the false positive reactions were initially eliminated, but reappeared sporadically in subsequent experiments. It was at this stage that contamination of one of the stock solutions of primer, buffer or dNTP was suspected, with the primer solution thought to be the most likely source of contaminating DNA. Predigestion of the PCR mixtures with *Ava*II was initiated, and using an annealing temperature of 60°C no further false positive results were encountered. The results of assays performed on both test isolates and controls before digestion with *Ava*II was initiated are shown in Fig 4.3. The faint bands of DNA resulting from contamination of the PCR mixture, clearly distinguishable from the intense bands representing true positive results, can be seen (E7, E8, E12, E13 in Fig 4.3). The results of subsequent assays on the test isolates, after digestion with *Ava*II, will be shown later.

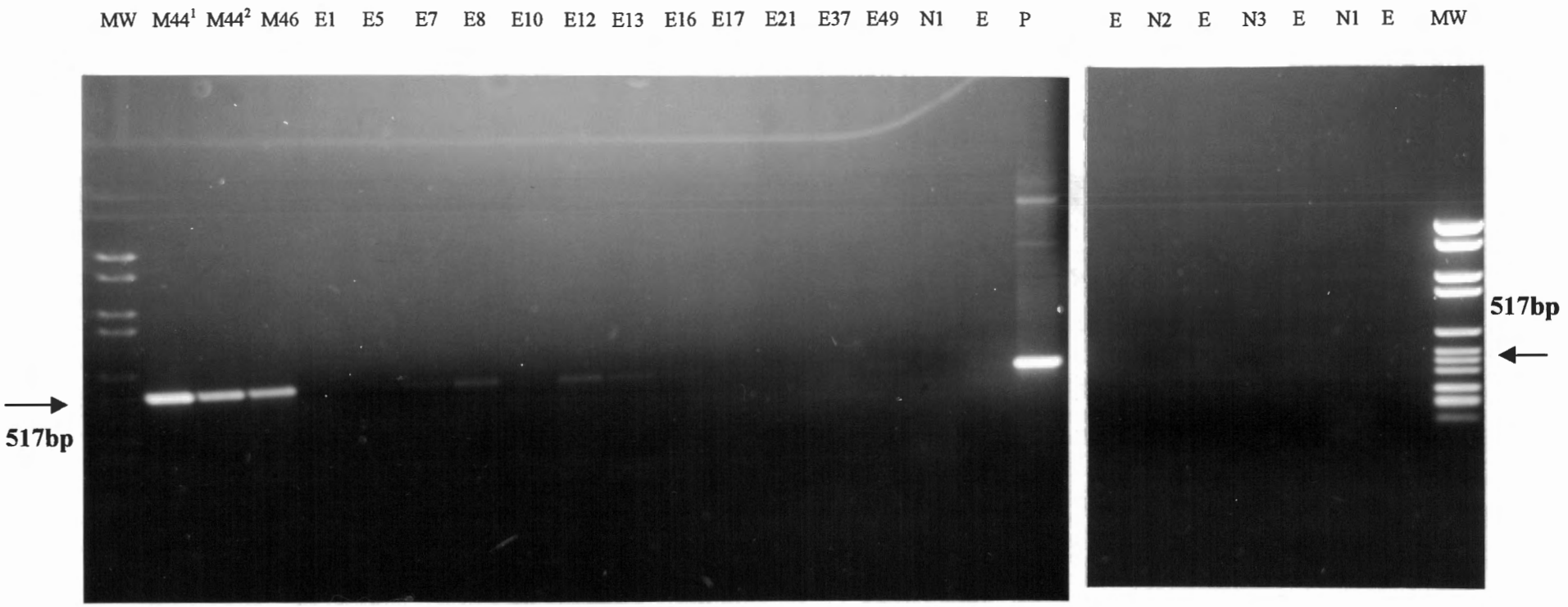



Fig 4.3 PCR assay performed on test isolates and controls prior to predigestion with *AvaI*II, showing faint bands of DNA amplified from some of the test isolates. Agarose gel elctriophoresis (AGE) performed on 1,2% gel at 10V/cm for 90 minutes.

**M44-1**, *Salmonella* sp.
**M44-2**, *Salmonella* sp
**M46**, *S. marcescens*
**E1**, *E. cloacae*
**E5**, *E. cloacae*
**E7**, *E. cloacae*
**E8**, *E.* sp
**E10**, *E* sp.
**E12**, *E. cloacae*
**E13**, *E. cloacae*
**E16**, *E. cloacae*
**E17**, *E. cloacae*
**E21**, *E.* sp
**E37**, *E.* sp
**E49**, *E. cloacae*
**N1**, *E. coli* DH5α
**P**, *E coli* DH5α(pUC19)
**N2**, Distilled H2O
**N3**, No template
**E**, Empty lanes
**MW**, Molecular weight marker VI, with the size of one of the fragments highlighted

*iii) Denaturation and Extension*

None of the changes in the duration of any of the cycles could be seen to make a difference to the amount of PCR product obtained. A final time cycle of 30 seconds, 50 seconds and 60 seconds for the denaturation, annealing and extension steps, respectively, was chosen.

*iv) Number of Cycles*

No visually discernible difference could be found in the intensity of the band of PCR product on the agarose gel using different numbers of cycles. The final assays were performed with 40 cycles of the thermal profile.

#### *4.3.1.2 Templates*

*i) Plasmid DNA*

Of the various templates used, purified pUC19 was used in the majority of the optimisation assays and produced consistent results (lane 14 in Fig 4.4).

*ii) Genomic DNA*

The results using genomic DNA were disappointing, with inconsistent results, and often fainter bands of DNA, obtained from the positive controls (lane 12 in Fig 4.4). For this reason as well as the laborious nature of the DNA extraction, little work was done using genomic DNA as a template.

*iii) Colonies*

Using colonies after boiling and centrifugation provided good results. The correct sized amplicon was consistently obtained from the positive control (*E. coli* DH5α(pUC19)). The PCR product was readily discernible on agarose gels (lane 9 in Fig 4.4), and the ease

of sample preparation made it suitable for use in a diagnostic laboratory, in marked contrast to the previous two templates.

To conclude optimisation, it was shown that an amplicon of the correct size was produced from DNA extracted directly from colonies (Fig 4.4). The conditions consisted of an initial denaturing step of 94°C for five minutes followed by 40 cycles of 94°C for 30 seconds (denaturation), 60°C for 50 seconds (annealing) and 72°C for 60 seconds (extension). A final extension step of 72°C for 7 minutes was performed. Primers were used at a concentration of 0,4µM and dNTPs at 200µM, and all PCR mixtures were digested with the restriction enzyme *Ava*II prior to addition of the template.

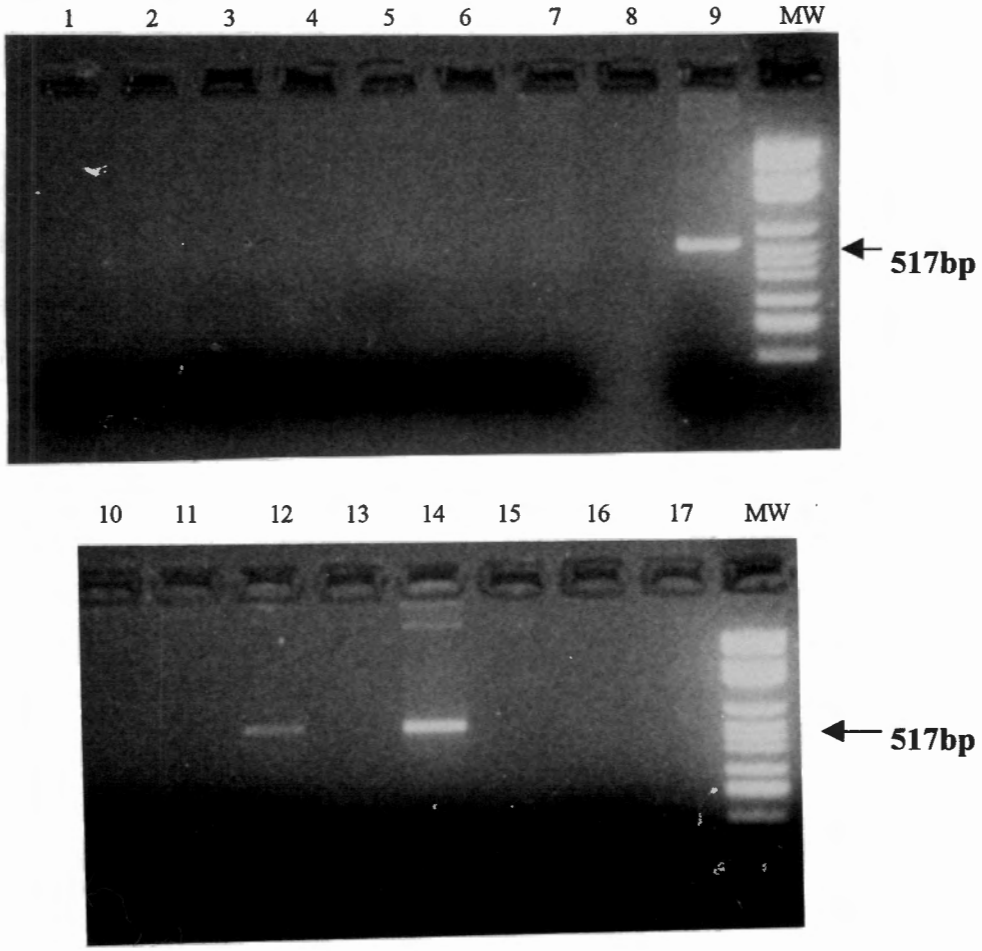



Fig 4.4 – PCR assay under the conditions described in the text, showing the nature of amplification product obtained from the 3 different template preparations.

***Lanes 1 – 6,*** *Miscellaneous test isolates*

**Lane 7,** *E. coli* DH5a

**Lane 8,** Empty

**Lane 9,** *E. coli* DH5α(pUC19)

**Lane 10,** Distilled H2O

**Lane 11,** Empty

**Lane 12,** Genomic DNA of *E. coli* DH5α(pUC19)

**Lane 13,** Empty

**Lane 14,** pUC19

**Lane 15,** Empty

**Lane 16,** No template

**MW,** Molecular weight marker VI

## 4.3.2 Detection of TEM – Related Genes in the Clinical Isolates

PCR assays to detect TEM-related genes were carried out on all 45 clinical isolates described in chapter 2 using the conditions described above. The PCR products were stored at 4°C prior to detection by electrophoresis. DNA was extracted from all the isolates by emulsifying colonies in water, boiling for five minutes followed by

centrifugation at 14 000 rpm in a microfuge for 20 – 30 seconds. The supernatant (5µl) was used as the template.

The assays were carried out in triplicate and the results are shown in Table 4.3 and Figs 4.5-i to 4.5-iii. An amplicon was obtained from colonies of *E.coli* DH5α(pUC19) in all the assays. No product was detected using the DNA from colonies of *E.coli* DH5α. Similarly, amplicons were not detected when distilled water was used as a template nor were they detected when no template was added to the PCR mixture. Amplicons were obtained from 11 of the 45 isolates in all three assays, indicating the presence of a TEM related gene in these isolates. In two isolates (K35 and K39), an amplicon was detected in only one of the assays (Fig 4.6-i). When the assay was repeated on these isolates no amplicons were detected (Fig 4.6-ii), and these two isolates were assumed not to contain a TEM-related gene. Amplicons were detected in 2 out of 3 assays containing template from isolate M46, one of which is shown in Fig 4.5-iii. A negative result was obtained in a repeat assay (Fig 4.7). Nevertheless, this isolate was assumed to contain a TEM-related gene as a positive signal was obtained with the TEM probe in the hybridisation experiments [3.3.2.1].

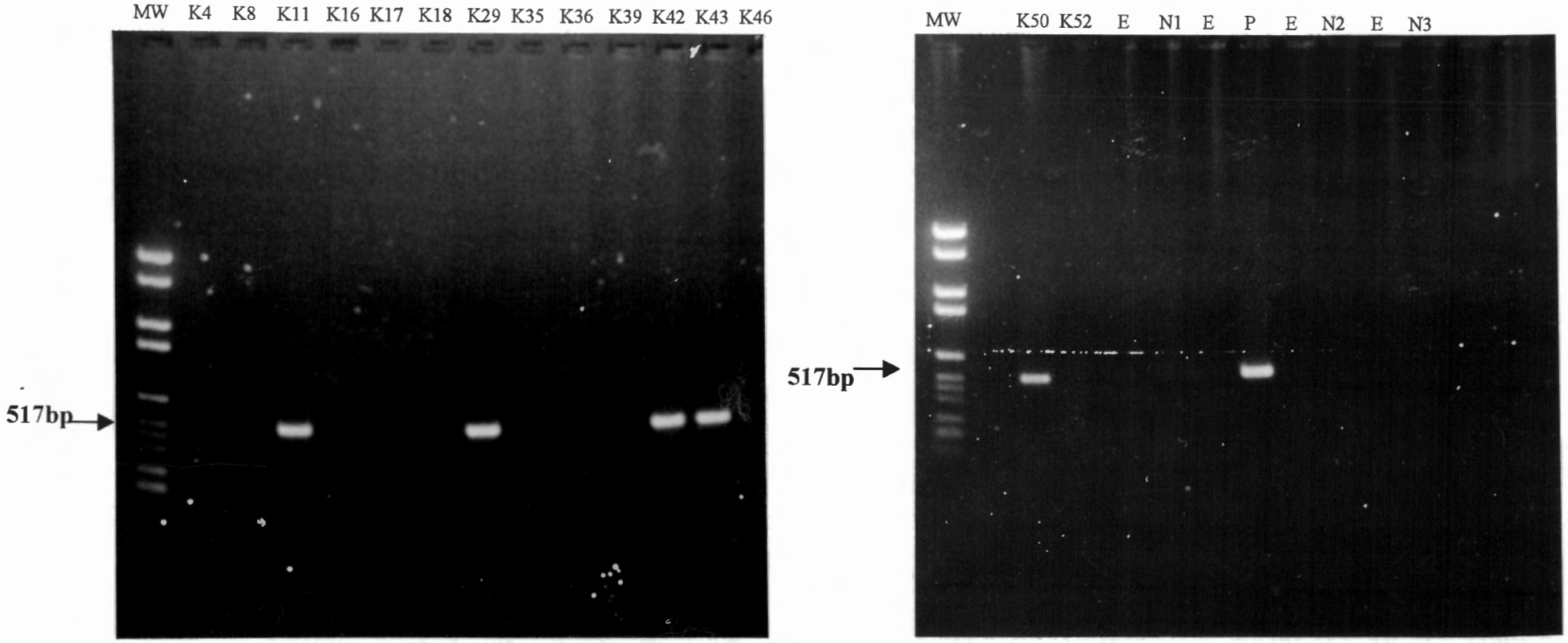



Fig 4.5-i PCR assays performed on test isolates and controls. AGE performed at 10V/cm for 90 minutes.

**K4**, *K. pneumoniae*
**K8**, *K. pneumoniae*
**K11**, *K. pneumoniae*
**K16**, *K. pneumoniae*
**K17**, *K. pneumoniae*
**K18**, *K. pneumoniae*
**K29**, *K. pneumoniae*
**K35**, *K. pneumoniae*
**K36**, *K. pneumoniae*
**K39**, *K. pneumoniae*
**K42**, *K. pneumoniae*
**K43**, *K. pneumoniae*
**K46**, *K. pneumoniae*
**K50**, *K. pneumoniae*
**K52**, *K. oxytoca*
**N1**, *E. coli* DH5α
**P**, *E coli* DH5α(pUC19)
**N2**, Distilled H2O
**N3**, No template
**E**, Empty lanes
**MW**, Molecular weight marker VI

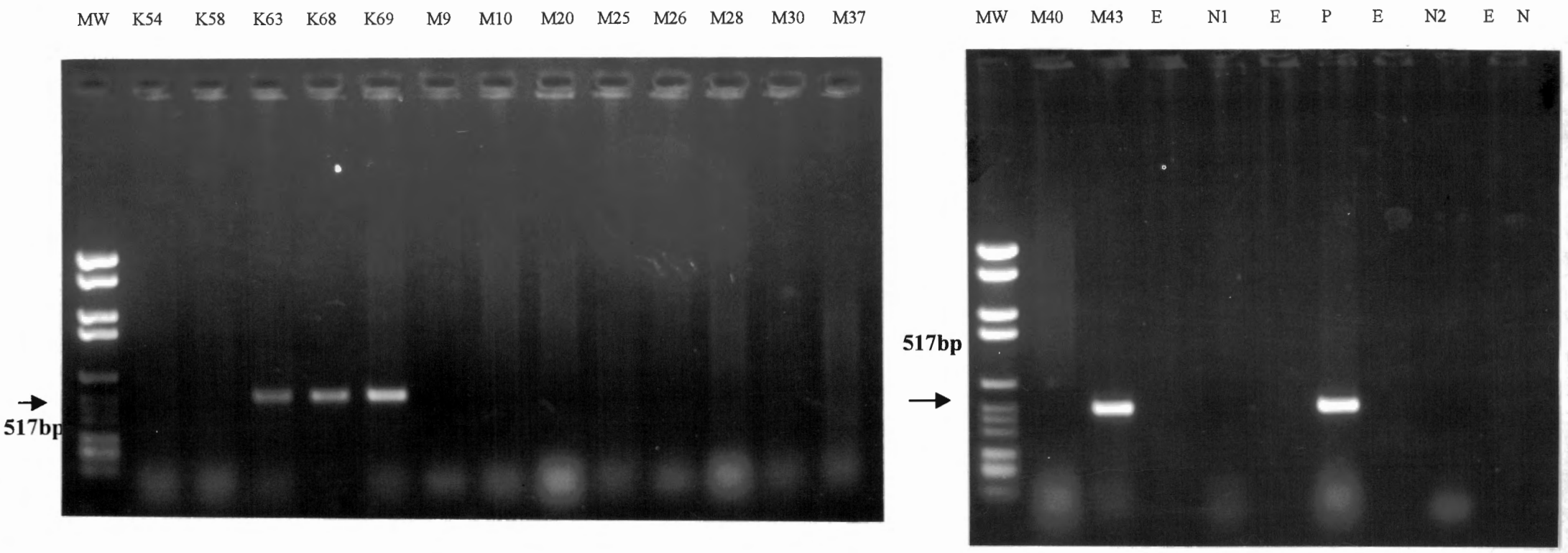



Fig 4.5-ii PCR assay performed on test isolates and controls. AGE performed at 10V/cm for 90 minutes.

**K54**, *K. pneumoniae*
**K58**, *K. pneumoniae*
**K63**, *K. pneumoniae*
**K68**, *K. pneumoniae*
**K69**, *K. pneumoniae*
**M9**, *C. freundii*
**M10**, *C.* sp.
**M20**, *C.* sp.
**M25**, *K. pneumoniae*
**M26**, *K. pneumoniae*
**M28**, *K. pneumoniae*
**M30**, *K. pneumoniae*
**M37**, *E. cloacae*
**M40**, *E. cloacae*
**M43**, *P. agglomerans*
**N1**, *E. coli* DH5α
**P**, *E coli* DH5α(pUC19)
**N2**, Distilled H2O
**N3**, No template
**E**, Empty lanes
**MW**, Molecular weight marker VI

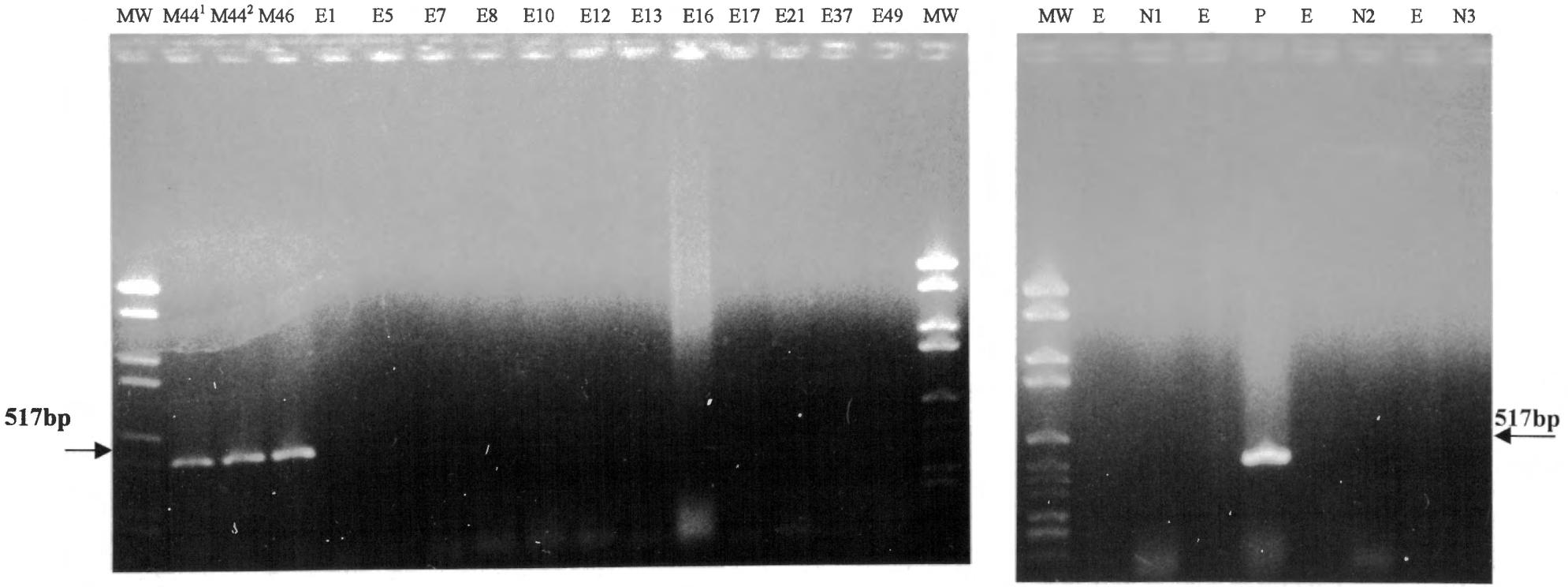



Fig 4.5-iii PCR assay performed on test isolates and controls. AGE performed at 10V/cm for 90 minutes.

**M44-1**, *Salmonella* sp.
**M44-2**, *Salmonella* sp
**M46**, *S. marcescens*
**E1**, *E. cloacae*
**E5**, *E. cloacae*
**E7**, *E. cloacae*
**E8**, *E.* sp
**E10**, *E* sp.
**E12**, *E. cloacae*
**E13**, *E. cloacae*
**E16**, *E. cloacae*
**E17**, *E. cloacae*
**E21**, *E.* sp
**E37**, *E.* sp
**E49**, *E. cloacae*
**N1**, *E. coli* DH5α
**P**, *E coli* DH5α(pUC19)
**N2**, distilled H2O
**N3**, no template
**E**, Empty lanes
**MW**, Molecular weight marker V

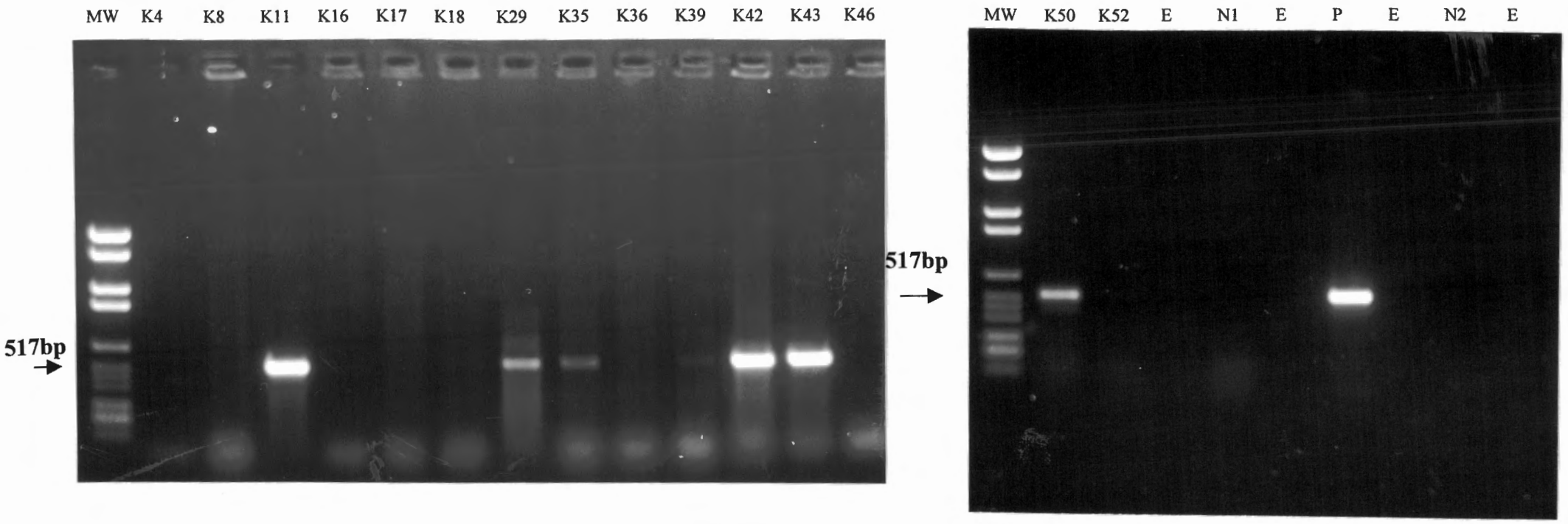



Fig 4.6-i PCR assay performed on test isolates K4 – K52 and controls, showing the presence of amplification product from the templates of isolates K35 and K39.

**K4**, *K. pneumoniae*
**K8**, *K. pneumoniae*
**K11**, *K. pneumoniae*
**K16**, *K. pneumoniae*
**K17**, *K. pneumoniae*
**K18**, *K. pneumoniae*
**K29**, *K. pneumoniae*
**K35**, *K. pneumoniae*
**K36**, *K. pneumoniae*
**K39**, *K. pneumoniae*
**K42**, *K. pneumoniae*
**K43**, *K. pneumoniae*
**K46**, *K. pneumoniae*
**K50**, *K. pneumoniae*
**K52**, *K. oxytoca*
**N1**, *E. coli* DH5α
**P**, *E coli* DH5α(pUC19)
**N2**, Distilled H2O
**E**, Empty lanes
**MW**, Molecular weight marker VI

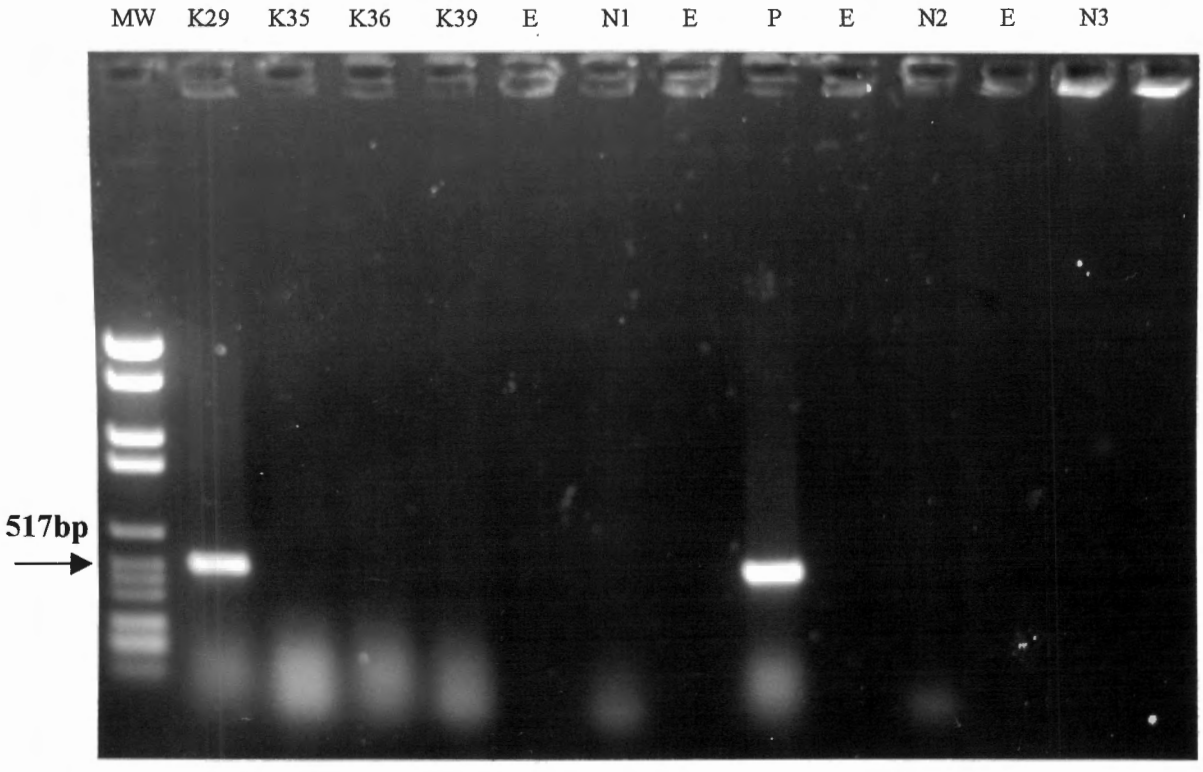



Fig 4.6-ii Repeat PCR assay performed on isolates K29, K35, K36 and K39 after the results seen in Fig 4.6-i

**K29**, *K. pneumoniae*
**K35**, *K. pneumoniae*
**K36**, *K. pneumoniae*
**K39**, *K. pneumoniae*
**N1**, *E. coli* DH5α
**P**, *E. coli* DH5α(pUC19)
**N2**, Distilled H2O
**N3**, No template
**E**, Empty
**MW**, Molecular weight marker VI

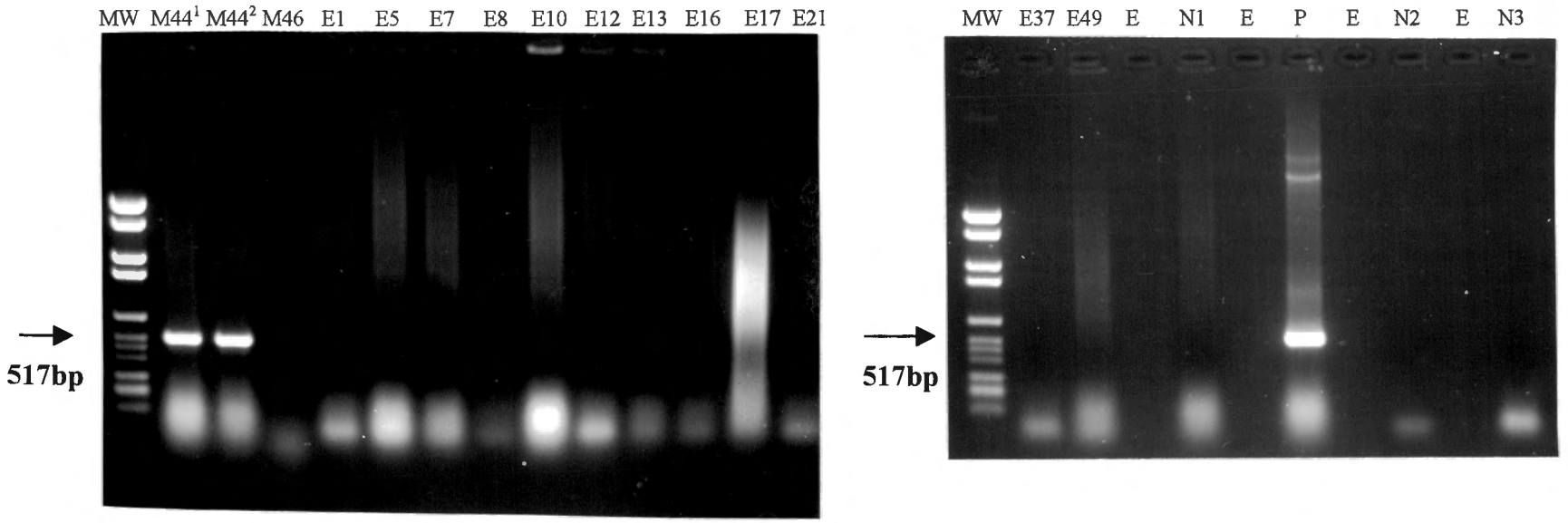



Fig 4.7 PCR assay performed on test isolates M44-1 to E49, showing the absence of an amplicon from isolate M46, compared to the amplicon detected previously (Fig 4.5-iii).

**M44-1**, *Salmonella* sp.
**M44-2**, *Salmonella* sp
**M46**, *S. marcescens*
**E1**, *E. cloacae*
**E5**, *E. cloacae*
**E7**, *E. cloacae*
**E8**, *E.* sp
**E10**, *E* sp.
**E12**, *E. cloacae*
**E13**, *E. cloacae*
**E16**, *E. cloacae*
**E17**, *E. cloacae*
**E21**, *E.* sp
**E37**, *E.* sp
**E49**, *E. cloacae*
**N1**, *E. coli* DH5a
**P**, *E coli* DH5a(pUC19)
**N2**, distilled H2O
**N3**, no template
**E**, Empty lanes
**MW**, Molecular weight marker VI

Table 4.3

Summary of results of PCR assays for detection of TEM-related genes

| Isolate | Identity | Assay 1 | Assay 2 | Assay 3 | Isolate | Identity | Assay 1 | Assay 2 | Assay 3 |
|---|---|---|---|---|---|---|---|---|---|
| K4 | *K. pneumoniae* | N | N | N | M25 | *K. pneumoniae* | N | N | N |
| K8 | *K. pneumoniae* | N | N | N | M26 | *K. pneumoniae* | N | N | N |
| K11 | *K. pneumoniae* | P | P | P | M28 | *K. pneumoniae* | N | N | N |
| K16 | *K. pneumoniae* | N | N | N | M30 | *K. pneumoniae* | N | N | N |
| K17 | *K. pneumoniae* | N | N | N | M37 | *E. cloacae* | N | N | N |
| K18 | *K. pneumoniae* | N | N | N | M40 | *E. cloacae* | N | N | N |
| K29 | *K. pneumoniae* | P | P | P | M43 | *P. agglomerans* | P | P | P |
| K35 | *K. pneumoniae* | **N** | **N** | **P ⇒ N** | M44-1 | *Salmonella* sp. | P | P | P |
| K36 | *K. pneumoniae* | N | N | N | M44-2 | *Salmonella* sp. | P | P | P |
| K39 | *K. pneumoniae* | **N** | **N** | **P ⇒ N** | M46 | *S. marcescens* | **P** | **P** | **N ⇒ N** |
| K42 | *K. pneumoniae* | P | P | P | E1 | *E. cloacae* | N | N | N |
| K43 | *K. pneumoniae* | P | P | P | E5 | *E. cloacae* | N | N | N |
| K46 | *K. pneumoniae* | N | N | N | E7 | *E. cloacae* | N | N | N |
| K50 | *K. pneumoniae* | P | P | P | E8 | *E.* sp. | N | N | N |
| K52 | *K. oxytoca* | N | N | N | E10 | *E.* sp. | N | N | N |
| K54 | *K. pneumoniae* | N | N | N | E12 | *E. cloacae* | N | N | N |
| K58 | *K. pneumoniae* | N | N | N | E13 | *E. cloacae* | N | N | N |
| K63 | *K. pneumoniae* | P | P | P | E16 | *E. cloacae* | N | N | N |
| K68 | *K. pneumoniae* | P | P | P | E17 | *E. cloacae* | N | N | N |
| K69 | *K. pneumoniae* | P | P | P | E21 | *E.* sp. | N | N | N |
| M9 | *C. freundii* | N | N | N | E37 | *E.* sp. | N | N | N |
| M10 | *C.* sp. | N | N | N | E49 | *E. cloacae* | N | N | N |
| M20 | *C.* sp. | N | N | N | | | | | |
| *E.coli* DH5α (pUC19) | | P | P | P | *E. coli* DH5α | | N | N | N |

Those results in boldface are those for which a discrepancy between the first two and third sets of PCR results was noted (see text).

N = No amplicon detected P = amplicon detected

Assay 1,2 and 3 are the 1st, 2nd and 3rd sets of PCR assays performed on the isolates.

TEM related genes were detected in 11 of the 45 isolates using the PCR assay – K11, K29, K42, K43, K50, K63, K68, K69, M43, M44-1 and M44-2. These results correlate almost perfectly with the results of the slot-blot hybridisation experiments using the TEM probe. The only discrepancy arises when the PCR and hybridisation results on isolate M46 are compared. A TEM-related gene was detected by hybridisation in this isolate, but PCR assays detected the gene inconsistently. The reason for the failure of the PCR assays to consistently detect the gene is unknown.

Two of the isolates (M43 and M46) that were shown to contain TEM-related genes were not shown to be ESBL producers by either the double disc diffusion test or the Etest ESBL screen. [2.3]. This suggests that the TEM-related genes in these isolates are TEM-1 or TEM-2. However, it is noteworthy that the ceftazidime and ceftazidime/clavulanate MICs of M43 (*P. agglomerans*) were >32μg/ml and >8μg/ml respectively. This is analogous to the MIC results of isolate K8 [2.4] where the presence of an ESBL cannot be excluded since the MICs of both antibiotics for that isolate are higher than the upper limit that can be determined by the Estrip, and a ratio cannot be calculated. In the case of isolate M46 (*S. marscecens*) the MICs of the two agents were 12μg/ml and >8μg/ml respectively, which is certainly not indicative of the presence of an ESBL.

The ease of sample preparation makes the PCR assay eminently suitable for use in a clinical laboratory. The cost of the equipment is not expensive, and the total time involved in carrying out the assay, from sample preparation to detection, is approximately 3,5 to 4 hours. It could quite easily be used to determine the number of TEM and non-TEM related enzymes in a hospital setting.

During the course of this study, contamination was a problem, and this is probably the largest drawback to the use of PCR. The primers in this study were thought to have been contaminated by pUC19 (or a similar TEM-1 containing plasmid vector). These plasmids are widely used in molecular biology laboratories and it is easy to envisage the accidental introduction of such a plasmid into the primer stock solution. The problem was overcome by pre-digestion of the PCR mixture with *Ava*II, and underscores the need for adequate controls and dedicated areas and equipment for the

setting up and performing of PCR. Nevertheless, the PCR assays as described detected TEM-related genes in clinical isolates with a high degree of reliability, and a minimum of difficulty.

## 4.3.3 Results of Optimisation of PCR to Detect SHV Related Genes First Set of Primers (SHV-A and SHV-B)

As with the optimisation of the PCR assays using the TEM specific primes, optimisation with both sets of SHV specific primers was carried out using templates consisting of purified plasmid DNA extracted from *E. coli* K12 (SHV-1), *E. coli* K12 (SHV-2) and DNA extracted from colonies of the same organisms by boiling and centrifuging the colonies. Negative controls were included in all assays and these were similar to those used in the TEM-related PCR assays, although the TEM producing control (*E. coli* DH5α(puc19)) was used as a negative control. The results of the optimisation assays with both sets of primers are discussed below.

### *4.3.3.1 Parameters Using SHV-A and SHV-B*

Conditions for a PCR assay with the primers SHV-A and -B using DNA from the control strains were determined relatively quickly, and the results of these optimisation assays are presented below in Table 4.4.

Table 4.4

| Primer (μM) | Denaturing Temperature (°C) | Annealing Temperature (°C) | dNTPs (μM each) | Product from Plasmid | Product from Colonies |
|---|---|---|---|---|---|
| 0,2 | 94 | 60 | 200 | + | |
| 0,4 | 94 | 60 | 200 | + (2) | - (2) |
| 0,6 | 94 | 60 | 200 | + (2) | |
| 0,8 | 94 | 60 | 200 | + | |
| 0,4 | 95 | 55 | 200 | | + |
| 0,4 | 95 | 60 | 200 | | + |

Numbers in parentheses indicate the number of occasions on which the assay was performed

+ = presence of an amplicon

- = no amplicon detected

*i) Primer Concentration*

The correct sized amplicon was obtained from plasmid DNA template when primer concentrations of 0,4; 0,6 and 0,8μM were used, and significantly less (lane 1, Fig 4.8) obtained using a primer concentration of 0,2uM. Using the same parameters (Table 4.4) and a primer concentration of 0,4μM no product was obtained with DNA prepared from colonies of *E. coli* K12 (SHV-1), on two occasions. However, when the denaturing temperature was raised to 95°C, amplicons were obtained from colony preparations using a primer concentration of 0,4μM (Fig 4.9).

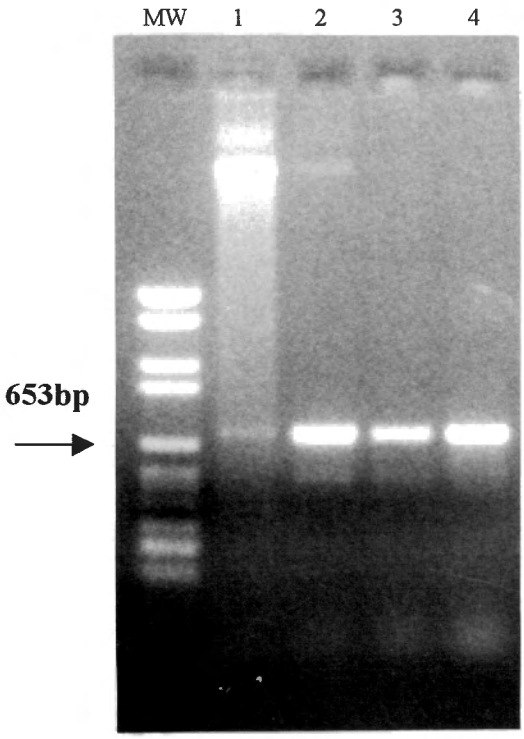



Fig 4.8 – PCR assay using plasmid DNA prepared from *E. coli* K12(SHV-1) as a template. The thermal profile consisted of 40 cycles of denaturation at 94°C, annealing at 60°C and extension at 72°C. dNTPs were used at 200μM each and primer concentrations varied.

**MW**, Molecular weight marker VI, with the position of one of the fragments illustrated.

**Lane 1**, Primer concentration 0,2uM

**Lane 2**, Primer concentration 0,4uM

**Lane 3**, Primer concentration 0,6uM

**Lane 4**, Primer concentration 0,8uM

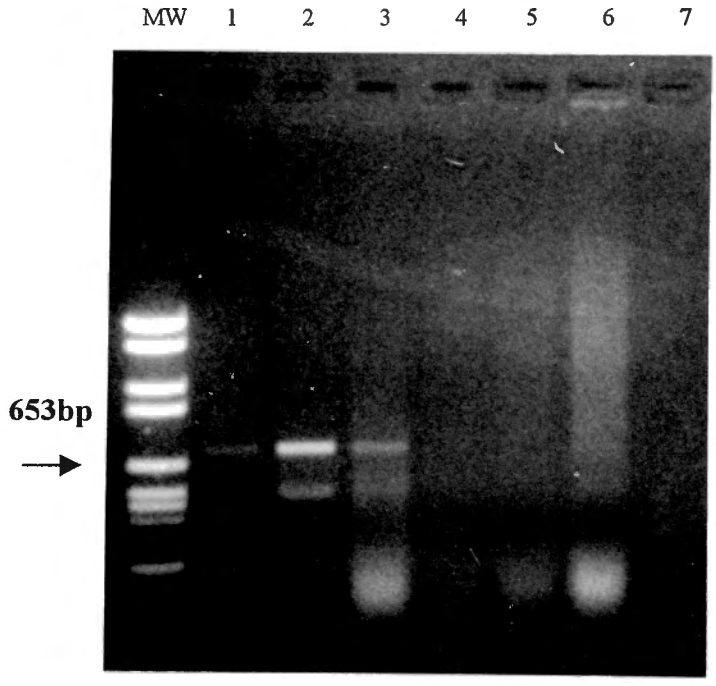



Fig 4.9 – PCR assay using template prepared by boiling SHV-containing control strains, and increasing the denaturation temperature to 95°C, with annealing at 55°C and extension at 72°C. Primer concentration 0,4µm, dNTP concentration 200µM.

**MW**, Molecular weight marker VI
**Lane 1**, E. coli R1010-6(SHV-1)
**Lane 2**, E. coli K12(SHV-1)
**Lane 3**, E.coli K12(SHV-2)
**Lane 4**, E. coli DH5α
**Lane 5**, E. coli DH5α(pUC19)
**Lane 6**, Distilled water
**Lane 7**, No template

*ii) Annealing Temperature*

Amplicons were produced at an annealing temperature of 60°C using a variety of primer concentrations, different denaturing temperatures and different template preparations.

*iii) Denaturing Temperature*

As mentioned previously, raising the annealing temperature from 94°C to 95°C resulted in successful amplification of DNA from templates prepared from colonies

(Figs 4.8 and 4.9). It is interesting that the denaturing temperature of 94°C resulted in amplification using plasmid DNA as the template.

In summary, the following conditions were initially found to result in the production of the correct sized amplicon from templates prepared by boiling and centrifugation of colonies (Fig 4.10) and using primer and dNTP concentrations of 0,4µM and 200µM, respectively. An initial denaturing step of 5 minutes at 95°C was followed by 40 cycles of 95°C (30s), 60°C (45s) and 72°C (50s) (denaturing, annealing and extension respectively) followed by final extension at 72°C for 7 minutes. Storage and detection were carried out as before [4.3.2].

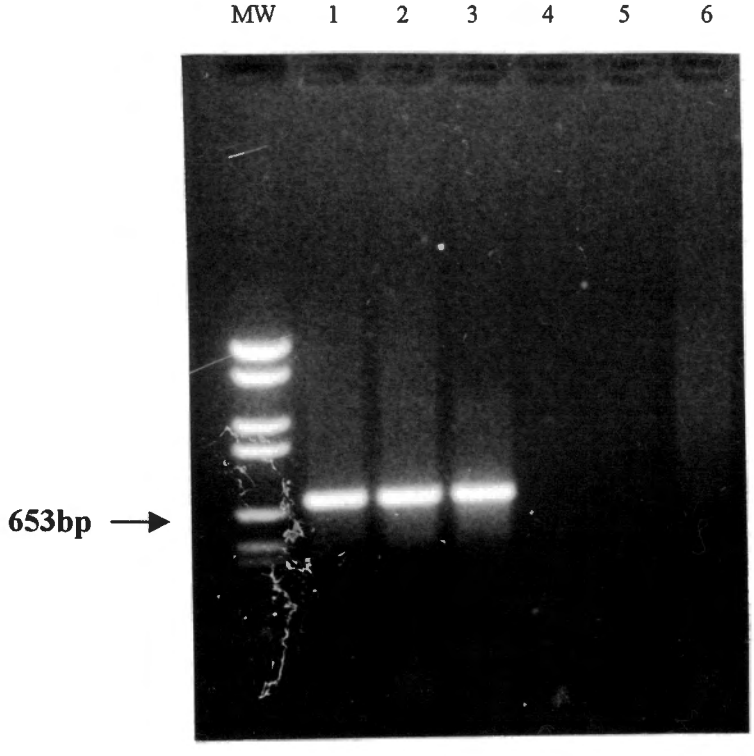



Fig 4.10 – PCR assay using SHV primers. Denaturation performed at 95C for 5 minutes, followed by 40 cycles of denaturation (95°C for 30s), annealing (60°C for 45s) and extension (72°C for 50s), and a final extension at 72°C for 7 minutes. Primers used at 0,2µM each and dNTPs at 200µM each.

**MW**, Molecular weight marker VI
**Lane 1**, *E. coli* R1010-6(SHV-1)
**Lane 2**, *E. coli* K12(SHV-1)
**Lane 3**, *E. coli* K12(SHV-2)
**Lane 4**, *E. coli* DH5a(pUC19)
**Lane 5**, Distilled water
**Lane 6**, No template

The assay as described above was performed on the 45 test isolates. The appropriate sized amplicons were obtained from colonies of one of the positive controls and no amplicons were detected from the negative controls. However, the amount of amplicon produced, judged by the intensity of the band on the agarose gel, varied from assay to assay (Fig 4.11-i to 4.11-iii). Similarly, the results of the assays on the 45 isolates were not consistent from one assay to the next (Fig 4.11-i to 4.11-iii; Table 4.5).

Table 4.5

Summary of results of PCR assays for detection of SHV-related Genes

| Isolate | Identity | Assay 1 | Assay 2 | Assay 3 | Isolate | Identity | Assay 1 | Assay 2 | Assay 3 |
|---|---|---|---|---|---|---|---|---|---|
| K4 | *K. pneumoniae* | P | P | N | M25 | *K. pneumoniae* | P | N | NR |
| K8 | *K. pneumoniae* | P | P | Weak | M26 | *K. pneumoniae* | P | Weak | NR |
| K11 | *K. pneumoniae* | P | P | P | M28 | *K. pneumoniae* | P | Weak | NR |
| K16 | *K. pneumoniae* | N | P | P | M30 | *K. pneumoniae* | P | P | NR |
| K17 | *K. pneumoniae* | N | P | P | M37 | *E. cloacae* | N | N | NR |
| K18 | *K. pneumoniae* | N | P | P | M40 | *E. cloacae* | P | P | NR |
| K29 | *K. pneumoniae* | P | P | P | M43 | *P. agglomerans* | N | N | NR |
| K35 | *K. pneumoniae* | N | P | P | M44-1 | *Salmonella* sp. | P | P | NR |
| K36 | *K. pneumoniae* | P | N | N | M44-2 | *Salmonella* sp. | P | P | NR |
| K39 | *K. pneumoniae* | P | N | N | M46 | *S. marcescens* | N | N | NR |
| K42 | *K. pneumoniae* | P | N | N | E1 | *E. cloacae* | N | N | NR |
| K43 | *K. pneumoniae* | N | P | N | E5 | *E. cloacae* | N | N | NR |
| K46 | *K. pneumoniae* | P | P | P | E7 | *E. cloacae* | N | N | NR |
| K50 | *K. pneumoniae* | P | P | N | E8 | *E.* sp. | N | N | NR |
| K52 | *K. oxytoca* | N | N | N | E10 | *E.* sp. | N | N | NR |
| K54 | *K. pneumoniae* | P | P | NR | E12 | *E. cloacae* | N | N | NR |
| K58 | *K. pneumoniae* | P | P | NR | E13 | *E. cloacae* | N | N | NR |
| K63 | *K. pneumoniae* | P | P | NR | E16 | *E. cloacae* | N | N | NR |
| K68 | *K. pneumoniae* | P | P | NR | E17 | *E. cloacae* | N | N | NR |
| K69 | *K. pneumoniae* | P | P | NR | E21 | *E.* sp. | N | N | NR |
| M9 | *C. freundii* | N | N | NR | E37 | *E.* sp. | N | N | NR |
| M10 | *C.* sp. | N | N | NR | E49 | *E. cloacae* | P | N | NR |
| M20 | *C.* sp. | N | N | NR | | | | | |
| Positive Control | | P | P | P*/NR | Positive Control | | P | P | NR |
| Negative Control | | N | N | N | Negative Control | | N | N | NR |

P = Amplicon detected

Assays 1,2 & 3 – 1st, 2nd and 3rd set of PCR assays

N = No amplicon detected

P*/NR = Amplicons detected from positive control of assays involving isolates K4 – K52, and no amplicons detected from positive controls of assays involving isolates K54 – E49.

NR = No amplicon obtained from controls or test isolates

Weak = A faint band of DNA (of the correct size) was visible on the EtBr stained gel.

Positive control = *E. coli* K12 (SHV-1) or *E. coli* K12(SHV2)

Negative control = *E. coli* DH5α (pUC19)

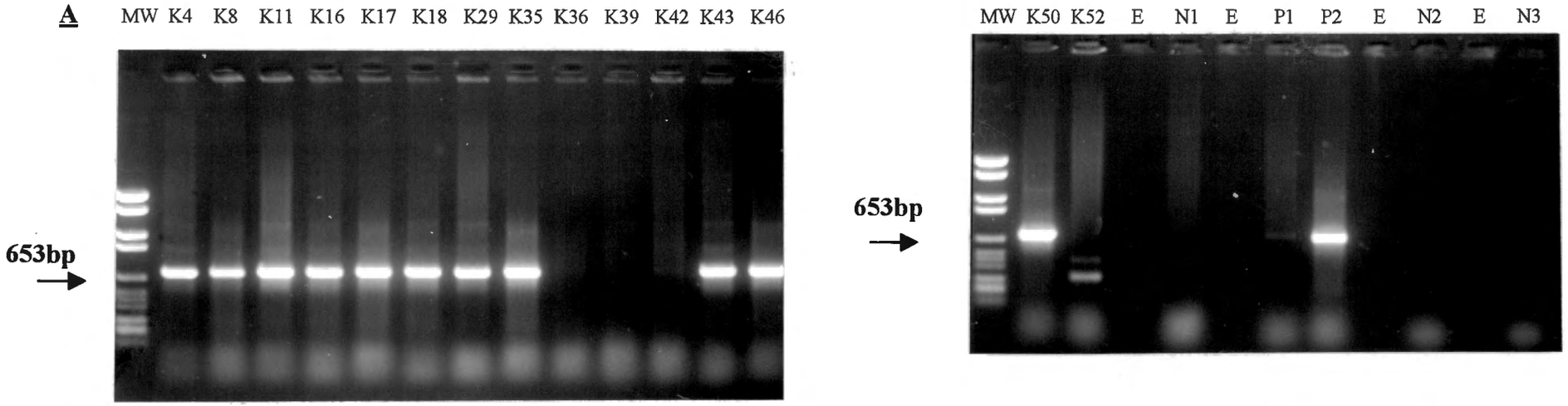



Fig 4.11-i 2 sets of PCR assays (A this page and B overleaf) performed on isolates K4 – K52. Note the different intensities of the DNA bands in the two sets of results, as well as the differences in which isolates produced an amplicon. AGE performed at 10V/cm for 90 minutes.

**K4**, *K. pneumoniae*
**K8**, *K. pneumoniae*
**K11**, *K. pneumoniae*
**K16**, *K. pneumoniae*
**K17**, *K. pneumoniae*
**K18**, *K. pneumoniae*
**K29**, *K. pneumoniae*
**K35**, *K. pneumoniae*
**K36**, *K. pneumoniae*
**K39**, *K. pneumoniae*
**K42**, *K. pneumoniae*
**K43**, *K. pneumoniae*
**K46**, *K. pneumoniae*
**K50**, *K. pneumoniae*
**K52**, *K. oxytoca*
**N1**, *E coli* DH5a(pUC19)
**P1**, *E coli* K12(SHV-1)
**P2**, *E. coli* K12(SHV-2)
**N2**, Distilled H2O
**N3**, No template
**E**, Empty lanes
**MW**, Molecular weight marker VI

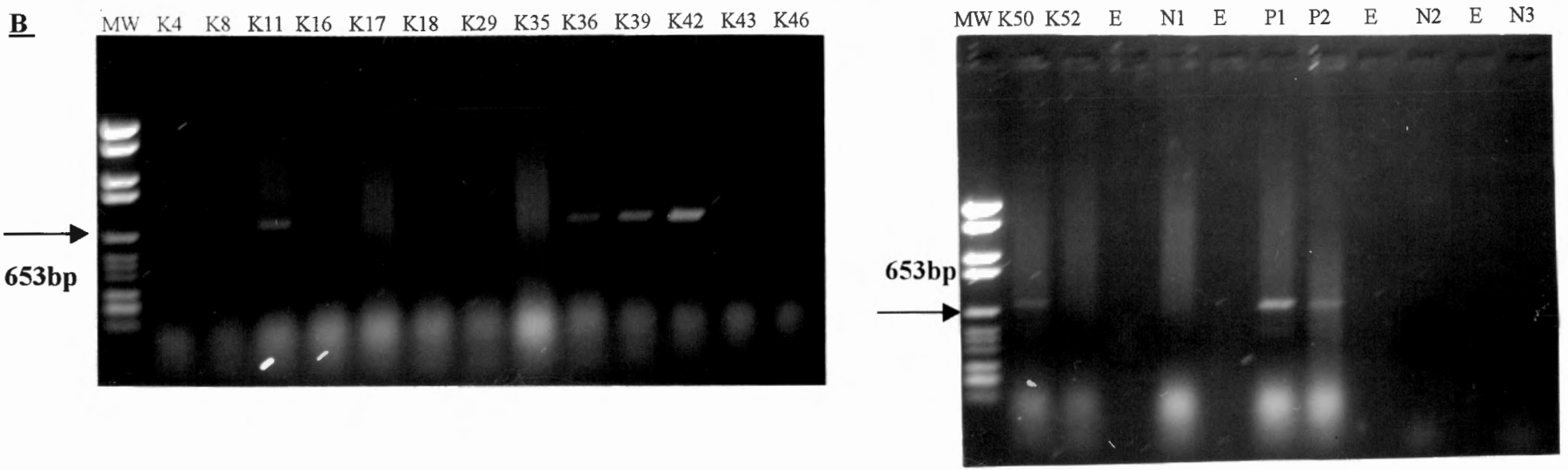



Fig 4.11-i 2nd set of PCR assays (B) performed on isolates K4 – K52.

**K4**, *K. pneumoniae*
**K8**, *K. pneumoniae*
**K11**, *K. pneumoniae*
**K16**, *K. pneumoniae*
**K17**, *K. pneumoniae*
**K18**, *K. pneumoniae*
**K29**, *K. pneumoniae*
**K35**, *K. pneumoniae*
**K36**, *K. pneumoniae*
**K39**, *K. pneumoniae*
**K42**, *K. pneumoniae*
**K43**, *K. pneumoniae*
**K46**, *K. pneumoniae*
**K50**, *K. pneumoniae*
**K52**, *K. oxytoca*
**N1**, *E coli* DH5a(pUC19)
**P1**, *E coli* K12(SHV-1)
**P2**, *E. coli* K12(SHV-2)
**N2**, Distilled H2O
**N3**, No template
**E**, Empty lanes
**MW**, Molecular weight marker VI

**A**

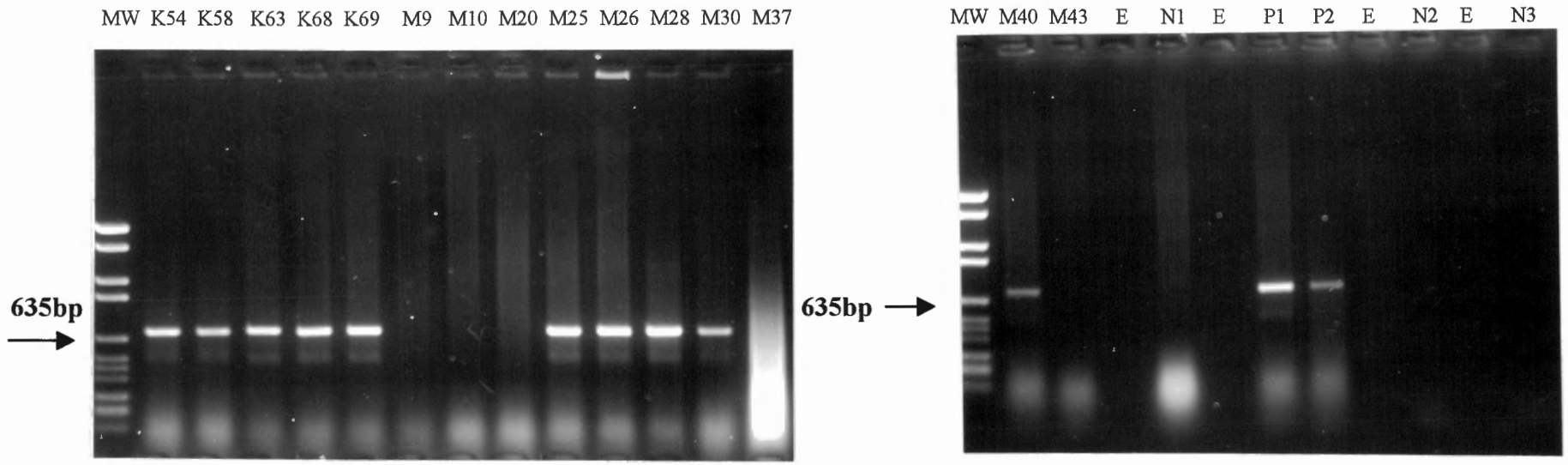



Fig 4.11-ii 2 sets of PCR assays (A this page and and B overleaf) performed on isolates K54 – M43. Note the different intensities of the DNA bands in the two sets of results, as well as the differences in which isolates produced an amplicon. AGE performed at 10V/cm for 90 minutes

**K54**, *K. pneumoniae*
**K58**, *K. pneumoniae*
**K63**, *K. pneumoniae*
**K68**, *K. pneumoniae*
**K69**, *K. pneumoniae*
**M9**, *C. freundii*
**M10**, *C.* sp.
**M20**, *C.* sp.
**M25**, *K. pneumoniae*
**M26**, *K. pneumoniae*
**M28**, *K. pneumoniae*
**M30**, *K. pneumoniae*
**M37**, *E. cloacae*
**M40**, *E. cloacae*
**M43**, *P. agglomerans*
**N1**, *E coli* DH5α(pUC19)
**P1**, *E coli* K12(SHV-1)
**P2**, *E. coli* K12(SHV-2)
**N2**, Distilled H2O
**N3**, No template
**E**, Empty lanes
**MW**, Molecular weight marker VI

**B**

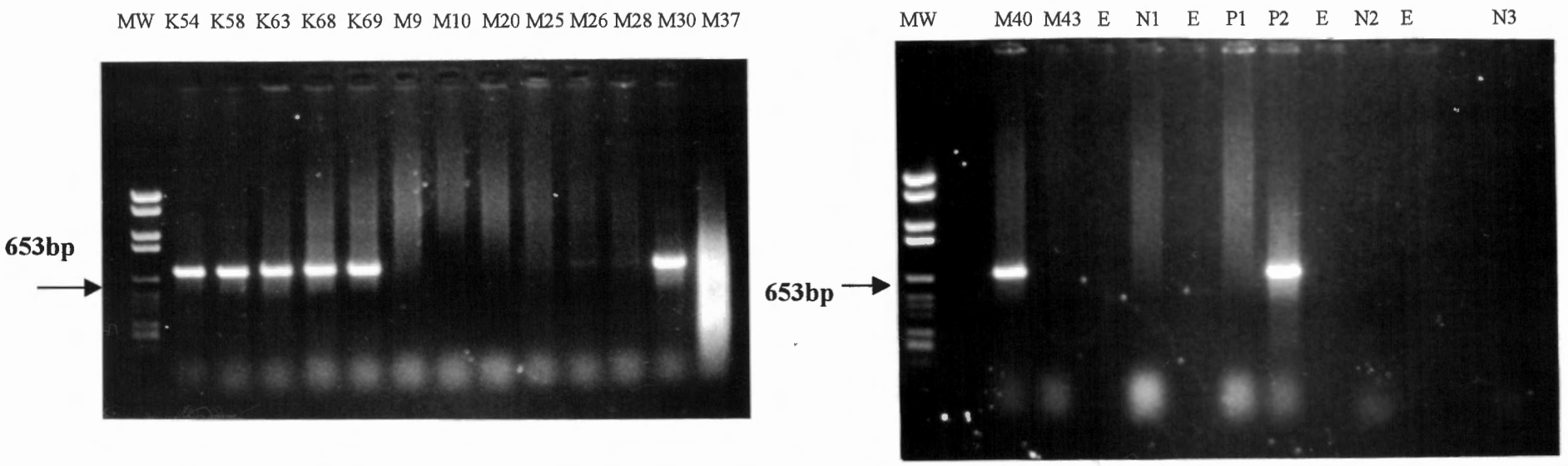



Fig 4.11-ii 2ND set of PCR assays (B) performed on isolates K54 – M43.

**K54**, *K. pneumoniae*
**K58**, *K. pneumoniae*
**K63**, *K. pneumoniae*
**K68**, *K. pneumoniae*
**K69**, *K. pneumoniae*
**M9**, *C. freundii*
**M10**, *C.* sp.
**M20**, *C.* sp.
**M25**, *K. pneumoniae*
**M26**, *K. pneumoniae*
**M28**, *K. pneumoniae*
**M30**, *K. pneumoniae*
**M37**, *E. cloacae*
**M40**, *E. cloacae*
**M43**, *P. agglomerans*
**N1**, *E coli* DH5α(pUC19)
**P1**, *E coli* K12(SHV-1)
**P2**, *E. coli* K12(SHV-2)
**N2**, Distilled H2O
**N3**, No template
**E**, Empty lanes
**MW**, Molecular weight marker VI

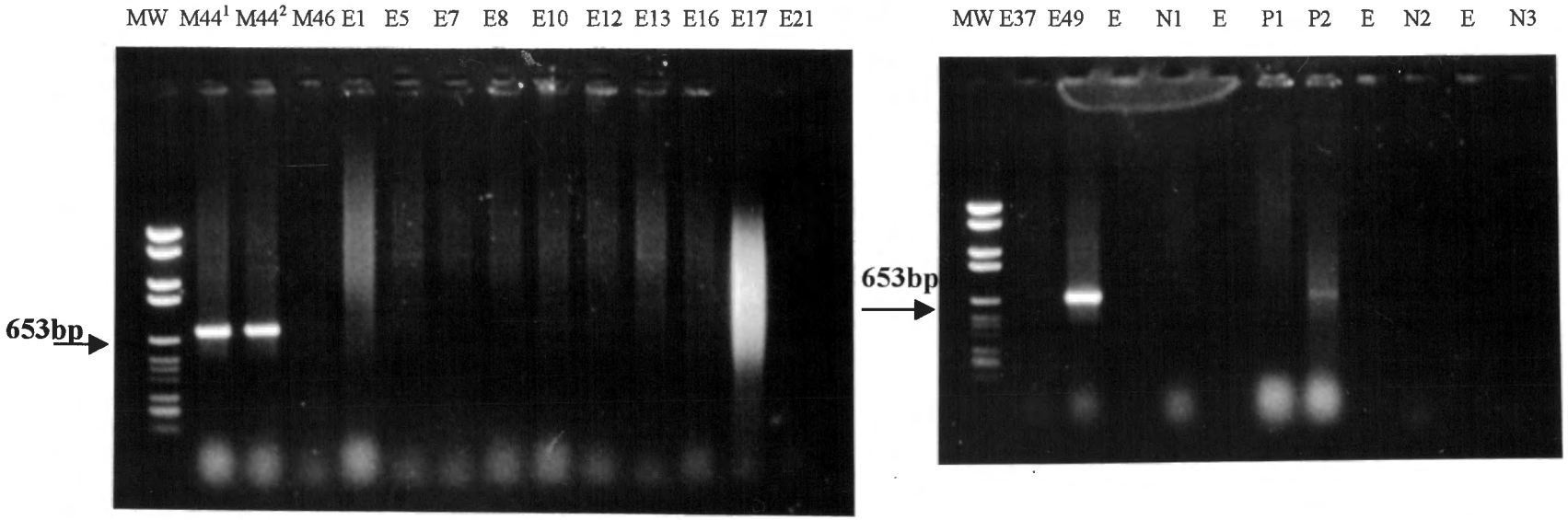



Fig 4.11-iii PCR assay performed on test isolates M44-1 – E49. AGE performed at 10V/cm for 90 minutes.

**M44-1**, *Salmonella* sp.
**M44-2**, *Salmonella* sp
**M46**, *S. marcescens*
**E1**, *E. cloacae*
**E5**, *E. cloacae*
**E7**, *E. cloacae*
**E8**, *E.* sp
**E10**, *E* sp.
**E12**, *E. cloacae*
**E13**, *E. cloacae*
**E16**, *E. cloacae*
**E17**, *E. cloacae*
**E21**, *E.* sp
**E37**, *E.* sp
**E49**, *E. cloacae*
**N1**, *E. coli* DH5α(Puc19)
**P1**, *E coli* K12(SHV-1)
**P2**, *E. coli* K12(SHV-2)
**N2**, distilled H2O
**N3**, no template
**E**, Empty lanes
**MW**, Molecular weight marker VI

On repeating some of the assays for the third time, no amplicon was obtained from either the positive controls or the test isolates. These assays were repeated and once again no amplicon was obtained. Given the inconsistency of the results, a further round of more extensive optimisation assays was carried out. The results of the further optimisation assays are shown in Table 4.6 followed by a discussion of the results of the changes in the various parameters. The table shows the effects of variation in primer and dNTP concentration as well as annealing and denaturing temperatures. The results of the initial optimisation assays (Table 4.4) have been included in this table to allow for better comparison of various conditions.

Table 4.6

| Primers (µM) | Denaturing temperature (°C) | Annealing temperature (°C) | dNTPs (µM each) | Product obtained from | | | |
|---|---|---|---|---|---|---|---|
| | | | | Plasmid DNA | | Colonies | |
| 0,2 | 94 | 50 | 100 | Yes | | Yes | |
| | | | 200 | No | | No | |
| | | | 300 | No | | No | |
| | | | 400 | No | | No | |
| | | 57 | 100 | Faint | | No | |
| | | | 200 | No | | | |
| | | | 400 | No | | | |
| | | 60 | 100 | No | | | |
| | | | 200 | No | | | |
| | | | 200 | Faint | | Faint | |
| | | | 300 | No | | | |
| | 95 | 55 | 200 | | | Yes | |
| | | 60 | 200 | | | Yes | |
| 0,4 | 94 | 48 | 200 | | | Yes | |
| | | 50 | 100 | Yes | | Yes | |
| | | | 200 | No | | No | |
| | | | 300 | No | | No | |
| | | | 400 | No | | No | |
| | | 57 | 100 | Yes | | | |
| | | | 200 | No | | | |
| | | | 400 | No | | | |
| | | 60 | 100 | No | (3) | No | (1) |
| | | | | | | Yes | (1) |
| | | | 200 | No | (7) | No | (4) |
| | | | | Yes | (2) | Yes | (1) |
| | | | 300 | No | (2) | | |
| | | | 400 | No | (3) | No | (2) |
| | 95 | 55 | 200 | Faint | | Yes | (2) |
| | | 55 | 400 | | | No | |
| | | 55,5 | 200 | Yes | | Faint | |
| | | 56 | 200 | No | | No | |
| | | 57 | 200 | No | | | |
| | | 60 | 200 | No | (4) | No | (2) |
| | | | | | | Yes | (3) |
| 0,6 | 94 | 50 | 100 | Yes | | Yes | |
| | | | 200 | No | | No | |
| | | | 300 | No | | No | |
| | | | 400 | No | | No | |
| | | 57 | 100 | Faint | | | |
| | | | 200 | Yes | | | |
| | | | 400 | No | | | |
| | | 60 | 100 | No | (2) | No | (3) |
| | | | | Yes | (3) | Yes | (1) |
| | | | 200 | No | (3) | No | (5) |
| | | | | Faint | (4) | Yes | (2) |
| | | | 300 | No | (2) | No | (2) |
| | | | 400 | No | (3) | No | (3) |

Numbers in parentheses indicate the number of assays performed that gave a particular result

Faint = A faint band of the correct sized DNA was visualised on EtBr stained agarose gels.

*iv) Primers, Annealing Temperature and dNTP Concentration*

The use of primers at a concentration of 0,2μM resulted in amplification of the expected fragment of DNA at annealing temperatures of 55°C and 60°C and a denaturing temperature of 95°C. However, these results were not reproducible. Despite utilising a range of annealing temperatures (50°C – 60°C) and dNTP concentrations (100 – 400μM), a reliable amplification could not be achieved with a primer concentration of 0,2μM.

Increasing the primer concentration to 0,4μM resulted in the expected amplicon at an annealing temperature of 57°C and a dNTP concentration of 100μM. Increasing the annealing temperature to 60°C also resulted in amplification of DNA from both plasmid DNA and colonies, this time with a dNTP concentration of 200μM. Unfortunately this too was not reproducible, the amplification product being produced in only two out of nine assays using plasmid DNA and one out of five assays using DNA extracted from colonies (Table 4.6).

The denaturing temperature was again increased to 95°C, with primer concentrations of 0,4μM. Assays using annealing temperatures from 55°C – 57°C, with dNTPs at 200μM and 400μM were carried out, but these conditions resulted in either additional non-specific amplicons ($T_A$ 55°C), only small amounts of amplification products or no product at all. When the annealing temperature was increased to 60°C (dNTP 200μM), no product was obtained from purified pDNA. However, amplicons of the expected size were obtained from boiled and centrifuged colonies in three out of five assays. In the assays using purified plasmid DNA a fresh stock of *Taq* polymerase and dNTPs was used after the initial negative assays. This did not result in successful amplification of the expected fragment.

No amplification of the expected fragment was achieved using primer concentrations of 0,6μM at an annealing temperature of 50°C (dNTPs ranging from 100 – 400μM). Raising the annealing temperature to 57°C resulted in a variable amount of product with dNTPs at 100μM or 200μM, but with the formation of additional products at both dNTP concentrations. At an annealing temperature of 60°C, inconsistent results

were attained with dNTP concentrations of 100μM and 200μM, and no amplification achieved with either 300μM or 400μM. At 100μM dNTP, three out of five assays using plasmid DNA and one out of four assays using DNA from colonies yielded amplicons. In a similar vein, four of seven assays using plasmid DNA and two of seven assays using boiled colonies were successful with a dNTP concentration of 200μM.

The duration of the annealing step was initially 30 seconds, but was subsequently increased to 45 and then 50 seconds. Although it is difficult to comment on the value of increasing the annealing time given the inconsistencies of the results, the changes did not appear to have any long-lasting effects on the reliability of the assay.

*v) Denaturation and Extension*

Raising the denaturating temperature to 95°C did appear to improve the consistency of the yield of PCR product, particularly at a higher annealing temperature (60°C). However these results were again not reliable in the long term. Denaturation was carried out for 30 seconds at both 94°C and 95°C. Extension was initially performed for 50 seconds, and was increased to 60 seconds. The theory behind this change is based on the fact that the shorter time may not have allowed extension to continue to completion, with the formation of only partially synthesised amplicons. However, this alteration had no noticeable effect on the assay.

*vi) Number of Cycles*

In the initial experiments the number of cycles ranged from 30 to 35 with no noticeable effect. Subsequent experiments were performed using 40 amplification cycles in an attempt to increase the yield of product, with little success.

#### *4.3.3.2 Template*

Both purified plasmid DNA extracted from the positive controls and DNA extracted from colonies of the positive controls were used in some of the optimisation experiments. This was done since the chance of impurities affecting the PCR reaction would be less with plasmid DNA, while the colony DNA extractions are easier to prepare and it was hoped that the assays on the clinical isolates would be carried out using this form of template.

The initial results of PCR assays using both plasmid DNA and colonies as the template were promising, with good yield obtained from both. (Figs 4.8 – 4.10). As described in section 4.3.3.1(iv) the further results using plasmid DNA were not consistent. The use of plasmid DNA as the template did yield positive results when the colony preparations were negative but this situation was reversed on occasion. This lack of consistency proved to be the most troublesome factor in these experiments.

Although it is difficult to ascribe the inconsistencies using plasmid DNA to the template alone, there was a possibility that templates prepared from the colonies may have been insufficient and contain inhibitory substances. For this reason, assays using colony suspensions boiled for fifteen minutes were attempted, on the basis that boiling for longer may have released more DNA and also eliminated any potential inhibitory substances. Plasmid and genomic DNA from the controls were used in these assays, which were carried out with primer concentrations of 0,4μM, dNTP concentrations of 200μM and denaturing and annealing temperatures of 95°C and 60°C, respectively.

The results showed the production of the expected amplicon using the colony preparations, although less product was amplified from both plasmid and genomic DNA templates. This result using genomic DNA was not entirely surprising given the similar lack of success with this template in the TEM series of experiments. The lack of results with the plasmid DNA was unexpected and no explanation could be found for this. Although boiling colonies for 15 minutes initially seemed to be a useful strategy, further assays using this method once again proved unreliable.

Despite extensive optimisation experiments, conditions that would result in reliable amplification of SHV-related genes using primers SHV-A and SHV-B could not be determined.

## 4.3.4 Results of Optimisation of PCR to Detect SHV Related Genes Second Set of Primers (SHV-C and SHV-D)

The same controls were used in these optimisation assays as were used with the first set of SHV primers [4.3.3], with templates consisting of both plasmid DNA and colonies prepared by boiling and centrifugation.

### *4.3.4.1 Parameters Using the Second Set of Primers*

The results of the optimisation assays using the primers SHV-C and SHV-D are presented in Table 4.7. No product of the correct size was obtained at any of the parameters using primers SHV-C and SHV-D.

Table 4.7

Optimisation of PCR assay using SHV-C and SHV-D

| Template | Primer µM | dNTP µM | Denaturing | Annealing | Amplicon |
|---|---|---|---|---|---|
| Plasmid[a] | 0,2 | 100 | 95°C | 50°C | Nil |
| | | 200 | | | |
| | | 300 | | | |
| Plasmid[a] | 0,4 | 100 | 95°C | 50°C | Nil |
| | | 200 | | | |
| | | 300 | | | |
| Plasmid[a] | 0,6 | 100 | 95°C | 50°C | Nil |
| | | 200 | | | |
| | | 300 | | | |
| Plasmid | 0,4 | 200 | 94°C | 55°C | Nil |
| Colonies | 0,4 | 200 | 94°C | 45°C | Wrong size |
| Colonies | 0,2 | 100 | 94°C | 50°C | Nil |
| | | 200 | | | |
| | | 300 | | | |
| Colonies | 0,4 | 100 | 94°C | 50°C | Nil |
| | | 200 | | | |
| | | 300 | | | |
| Colonies | 0,6 | 100 | 94°C | 50°C | Nil |
| | | 200 | | | |
| | | 300 | | | |
| Colonies | 0,4 | 200 | 94°C | 53°C | Wrong size |
| Colonies | 0,2 | 100 | 94°C | 55°C | Nil |
| | | 200 | | | |
| | | 300 | | | |
| Colonies[a] | 0,4 | 100 | 94°C | 55°C | Nil |
| | | 200 | | | |
| | | 300 | | | |
| Colonies | 0,6 | 100 | 94°C | 55°C | Nil |
| | | 200 | | | |
| | | 300 | | | |
| Colonies | 0,4 | 100 | 95°C | 55°C | Nil |
| | | 200 | | | |
| | | 300 | | | |
| Colonies | 0,4 | 200 | 94°C | 60°C | Wrong size |

*a – These assays were repeated with negative results*

### 4.3.5 Detection of SHV – Related Genes in the Clinical Isolates

The results of the PCR assays for the detection of SHV related genes were disappointing. The first set of primers were designed to anneal to SHV related genes in areas where there was as little homology as possible with LEN-1, and the 3' ends of both primers contained mismatches with the LEN-1 gene. Certain primer/template mismatches (C-C, G-A, A-G) have been shown to reduce the product yield from the primer-template pairing to less than 1%, while an A-A mismatch reduces the product approximately 20-fold (Kwok *et al.*, 1990). SHV-A and SHV-B each contain one A-G mismatched nucleotide pair at the 3' end. While there are also two T-C mismatches at the 3' end of SHV-B, these mismatches are not thought to be as critical in preventing annealing and extension in PCR assays.

Despite the mismatches, the data suggest that the primers annealed to the LEN-1 gene as well as SHV-related genes. The results of the initial assay (Table 4.5) showed that an amplicon was produced from all of the *K. pneumoniae* isolates on at least one occasion, a situation similar to that seen with the results of hybridisation using the SHV probes [3.3.3.1]. One method of proving that the primers were also annealing to LEN-1 would have been to extract plasmid DNA from the clinical isolates and compare PCR assays on the plasmid DNA to assays on genomic DNA extracted from the colonies. Since the assay using purified plasmid DNA from the controls yielded equivocal results, this experiment was not carried out.

Importantly, the results obtained from the isolates other than *K. pneumoniae* do correlate with the hybridisation results on the same isolates. Specifically, amplicons were obtained from the 2 *Salmonella* isolates (M44-1 & M44-2) and 2 strains of *E. cloacae* (M40 & E49). It is noteworthy that an amplicon was obtained from isolate E49 (*E. cloacae*) in only one of the two assays performed on this isolate, which indicates, yet again, the inconsistent nature of the PCR assay in this study.

No amplification product was obtained using the primers (SHV-C and SHV-D) described by Nuesch-Inderbinen *et al.* (1996). This is in contrast to the work done by

these workers where the assay was successfully used both to detect SHV-related genes and to distinguish genes coding non-ESBL enzymes from those coding for ESBLs. The fact that the primers anneal to sequences flanking the structural SHV gene and not the gene itself may, at least to some extent, be part of the explanation.

Taken together, these data suggest that PCR could be useful for the detection of SHV-related genes in *Enterobacteriaceae* other than *K. pneumoniae*. The technique is more "user friendly" than DNA-DNA hybridisation and would be suitable for use in a diagnostic laboratory with the *caveat*: PCR may be a quick and easy technique but it can also be unreliable if not adequately optimised, and optimisation itself is not always quick and easy.

# CHAPTER 5

# CONJUGATION STUDIES

## 5.1 INTRODUCTION

Transfer of genetic information from one bacterial species to another can occur by transformation, transduction or conjugation. Transformation is the uptake of free DNA from the environment by naturally competent cells (Davis, 1980).

Transduction is a process whereby bacteria acquire DNA through the action of a bacteriophage. These viruses specifically infect bacterial cells and utilise the organism's own cellular "machinery" to synthesise new viral protein coats as well as viral nucleic acid. When viral particles are reassembled, fragments of bacterial DNA may be accidentally incorporated into the viral genome and can subsequently be introduced into other bacteria (Boyd & Marr, 1980b).

Conjugation relies on the presence of transmissible plasmids within organisms to transfer DNA from one cell to another. Following cell to cell contact, plasmid DNA is transferred through a pilus from one organism to another. It is important to remember that the transfer is unidirectional *i.e.* one organism acts as a donor and the other as a recipient. Self-transmissible plasmids, which contain the genes required for conjugation, including synthesis of a pilus, also often contain antibiotic resistance determinants and are a major cause of the spread of resistance, especially in the hospital environment. Conjugation is the commonest mechanism of gene transfer among members of the *Enterobacteriaceae* (Boyd & Marr, 1980b; Davis, 1980).

Resistance to beta lactams mediated by plasmid mediated TEM- and SHV-related ESBLs has frequently been shown to be transferable. (Jarlier *et al.*, 1988; Nicolas *et al.*, 1989; Petit *et al.*, 1990; Barguellil *et al.*, 1995; Sirot, 1995). Conjugation experiments were carried out in order to ascertain whether the TEM- and SHV-related

genes identified in this study are carried on self-transmissible plasmids. Since equivocal results were obtained when looking for SHV-related genes in isolates of *K. pneumoniae*, all these isolates were included.

# 5.2 MATERIALS AND METHODS

## 5.2.1 Conjugation

### *5.2.1.1 Bacterial strains*

*i) Recipient*

*E. coli* J53 ($Nal^R$) was used as the recipient organism in the conjugation experiments.

*ii) Donors*

All the isolates containing either TEM- or SHV-related genes (demonstrated by PCR or hybridisation) were used as donors in the conjugation experiments. As mentioned, all the isolates of *K. pneumoniae* were included in the experiments. A total of 29 isolates were used as donor strains. Sensitivity of the donors to nalidixic acid was confirmed by inoculating all the donors onto YT agar containing nalidixic acid at 50μg/ml and 100μg/ml and assessing growth after 18 hours incubation at 37°C.

### *5.2.1.2 Bacterial Conjugation*

Conjugation studies were carried out to determine whether resistance to ampicillin could be transferred from the donors to the ampicilllin-sensitive *E. coli* recipient. An overnight culture of *E. coli* J53 was diluted 1:100 in YT broth and incubated at 37°C for 4 hours with agitation. Overnight cultures of the donors were diluted 1:10 in YT broth and incubated for 2 hours at 37°C without agitation. Four drops of each donor and 5 drops of the recipient were combined on MH agar and left undisturbed for two hours at room temperature to allow conjugation to take place. This was followed by incubation for 18 hours at 37°C

The cells were washed off the agar with physiological saline (10ml), harvested by centrifugation at 3000rpm for 15 minutes (Beckman GS-6 centrifuge with bucket rotor) and resuspended in 0,5ml physiological saline. Aliquots (100µl) of this suspension were inoculated onto selective media containing ampicillin (200µg/ml) and nalidixic acid (100µg/ml) and incubated overnight at 37°C.

The transconjugants were identified on the basis of the distinctive colonial morphology of *E. coli* on MacConkey agar and single colonies were inoculated into YT broth and incubated at 37°C. When growth was confluent and discrete colonies were not evident, a sweep of organisms was inoculated into YT broth containing ampicillin (100µg/ml) and nalidixic acid (100µg/ml) and incubated at 37°C overnight, followed by inoculation onto MacConkey agar and incubation at 37°C. After 18 hours the MacConkey agar was examined for the presence of single colonies and these cultured as above.

## 5.2.2 Characterisation of Transconjugants by DNA-DNA Hybridisation

### *5.2.2.1 Transfer of DNA to a Stable Matrix*

DNA was transferred from agarose gels to a nylon membrane (Hybond $N^+$, Amersham UK) by capillary transfer (Southern, 1975; Smith & Summers, 1980) as described below. The agarose was soaked twice in 0,25M HCl for 15 minutes to depurinate the DNA and thereby aid the transfer of large fragments of DNA. The DNA in the gel was then denatured by soaking it twice in a solution of 1,5M NaCl 0,5M NaOH and neutralisation was achieved by soaking the gel twice in 1,5M NaCl 0,5M Tris-HCl pH 7,4.

The DNA was transferred to the nylon membrane by means of capillary transfer using 20xSSC as the transfer buffer and filter paper as a wick. A strip of Whatman 3M filter paper was placed on a perspex sheet in a bath of 20xSSC with its free ends submerged in the SSC. The gel was placed on top of this filter paper, with the gel not in contact with the SSC. An appropriately sized piece of nylon membrane (Hybond $N^+$) was

placed on top of the gel and then covered by three pieces of Whatman 3M filter paper soaked in 20xSSC. Folded up paper towel about 5 cm thick was placed on top, and the whole covered by a second piece of perspex and a brick (Fig 5.1). DNA transfer was allowed to occur overnight. The following day the apparatus was dismantled and the DNA cross linked to the nylon membrane by exposure to 254nm UV light [3.2.2.1]. Where necessary, the membranes were stored in sealed plastic at 4°C until such time as they were required.

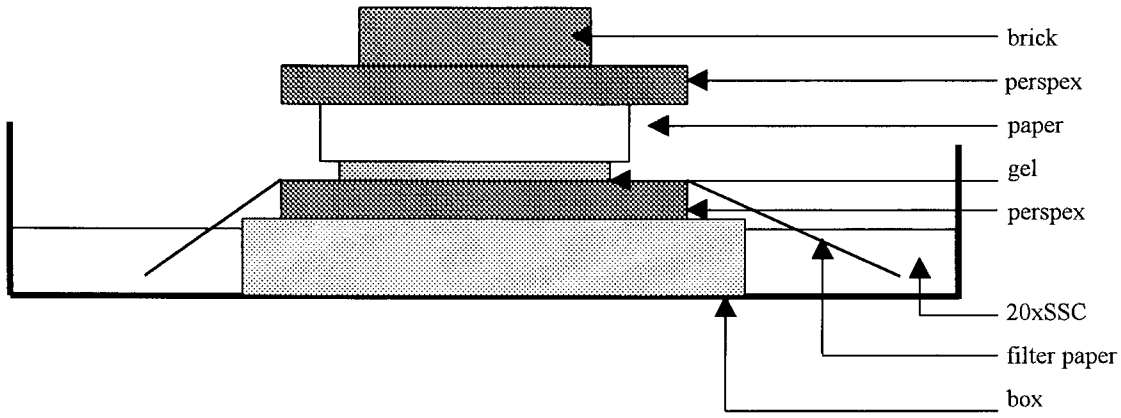



Fig 5.1 Set-up of apparatus used for Southern transfer of DNA. The nylon membrane, covered with 3 pieces of filter paper, lies between the gel and the folded up paper towel.

### *5.2.2.2 DNA-DNA Hybridisation*

The probe specific for TEM-related genes [3.2.3] was hybridised to the DNA transferred to the nylon membranes. Similarly, the PCR generated probe [3.2.4] labelled with the ECL chemiluminescent label [3.2.5.1] was used in hybridisations to detect SHV-related genes.

# 5.3 RESULTS

## 5.3.1 Bacterial Conjugation

As expected, the recipient, *E. coli* J53 $Nal^R$, did not grow on the selective medium containing ampicillin and nalidixic acid. None of the donors grew on agar containing nalidixic acid at 100μg/ml. However, after conjugation, 3 of the 29 donor isolates showed scanty growth (maximum of 11 colonies) on the selective media. The morphology of these isolates was sufficiently distinct from that of *E. coli* J53 to make selection of transconjugants possible.

Following conjugation, putative transconjugants were isolated from each of the 29 crosses. Single colonies were successfully cultured from 20 of the transconjugants, but despite repeated attempts, the remaining 9 transconjugants could not be cultured further in broth suggesting that the plasmids may be unstable in *E. coli*.

## 5.3.2 - Antibiotic Susceptibility of Transconjugants

To determine the beta lactam resistance profile of the transconjugants, ceftazidime, cefotaxime and ceftazidime/clavulanic acid MICs were determined using E-strips [2.2] as shown in Table 5.1.

Table 5.1

| *ISOLATE* | | *CEFOTAXIME* $R \geq 64$ $S \leq 8\mu g/ml$ | | *CEFTAZIDIME* $R \geq 32$ $S \leq 8\mu g/ml$ | | *CEFTAZ/CLAV* | | *ESBL PRESENCE* | |
|---|---|---|---|---|---|---|---|---|---|
| | | *donor* | *tconj* | *donor* | *tconj* | *donor* | *tconj* | *donor* | *tconj* |
| K4 | *K. pneumoniae* | 1,5 | 0,75 | 8 | 0,75 | 0,75 | 0,75 | Yes | No |
| K8 | *K. pneumoniae* | >256 **R** | 0,38 | >32 **R** | 1,5 | >8 | 1,5 | No [A] | No |
| K11 | *K. pneumoniae* | 3,0 | 0,25 | 12 **I** | 1,5 | 0,75 | 1 | Yes | No |
| K16 | *K. pneumoniae* | 12 **I** | 0,25 | 12 **I** | 1 | 0,75 | 1 | Yes | No |
| K29 | *K. pneumoniae* | 2,0 | 2,0 | >32 **R** | >32 **R** | 0,38 | 1,5 | Yes | Yes |
| K42 | *K. pneumoniae* | 0,5 | 1,5 | >32 **R** | >32 **R** | 0,5 | 4 | Yes | Yes |
| K43 | *K. pneumoniae* | 12 **I** | 6 | 8 | 8 | 0,125 | 0,75 | Yes | Yes |
| K46 | *K. pneumoniae* | 16-24 **I** | 4 | >32 **R** | >32 **R** | 0,75 | 1,5 | Yes | Yes |
| K50 | *K. pneumoniae* | 4 | 2 | >32 **R** | 6 | 1-1,5 | 1,5 | Yes | B |
| K54 | *K. pneumoniae* | >256 **R** | 0,25 | >32 **R** | 1,5 | 0,75 | 1,5 | Yes | No |
| K63 | *K. pneumoniae* | 3 | 1,5 | >32 **R** | >32 **R** | 0,5 | 6 | Yes | Yes |
| K68 | *K. pneumoniae* | 2 | 0,25 | 24 **I** | 1,5 | 1,5 | 1,5 | Yes | No |
| K69 | *K. pneumoniae* | 6-8 | 0,5 | >32 **R** | 0,5 | 0,5 | 0,5 | Yes | No |
| M26 | *K. pneumoniae* | 1,0 | 1,5 | 2,0 | 0,75 | 0,38 | 0,5 | Yes | No |
| M30 | *K. pneumoniae* | 8 | 1,5 | 12 **I** | 6 | 0,5 | 0,5 | Yes | Yes |
| M40 | *E. cloacae* | 4 | 1,5 | >32 **R** | >32 **R** | 1,0 | 1,5 | Yes | Yes |
| M44-1 | *Salmonella* sp. | 16 **I** | 0,19 | >32 **R** | 0,5 | 0,75 | 0,38 | Yes | No |
| M44-2 | *Salmonella* sp. | 16 **I** | 0,19 | >32 **R** | 0,5 | 0,75 | 0,5 | Yes | No |
| M46 | *S. marcescens* | 64 **R** | 1,5 | 12 **I** | >32 **R** | >8 | 1,5 | No | Yes |
| E49 | *E. cloacae* | 4 | 1,0 | 8 | 0,75 | 0,25 | 0,5 | Yes | No |
| E coli J53 | | 0,032 | | <0,5 | | 0,125 | | No | |

MICs in µg/ml Unless stated, the MICs represent sensitivity to cefotaxime or ceftazidime. No criteria exist for the ceftazidime/clavulanate combination

I = intermediate susceptibility R = resistant

Tconj = transconjugant

A – Section 2.5 for discussion of the ESBL in isolate K8

B – Ratio of ceftazidime to ceftazidime/clavulanate =4, borderline for presence of ESBL.

The results of the MIC testing show a number of interesting features. Firstly, comparison of the MICs of various beta lactams for the transconjugants and the corresponding donors shows very little correlation. This may reflect the altered expression of the genes in the different hosts, or be due to differences in membrane permeability (Podbielski *et al.*, 1991).

What can be seen from the MIC results is that 6 of the transconjugants are resistant to ceftazidime (MIC>32µg/ml), and the Etest ESBL test shows that these 6

transconjugants contain an ESBL. In addition, another 2 transconjugants that produce an ESBL (based on the Etest ESBL test), have ceftazidime MICs of 8μg/ml and 6μg/ml. Another transconjugant (from the cross with K50), with a ceftazidime MIC of 6μg/ml, has a ratio of ceftazidime MIC to cefaztidime/clavulanate MIC of 4, which is the cut-off value for determination of ESBL activity.

Although ceftazidime MICs of 8μg/ml or less indicate clinical susceptibility of the organism to the antibiotic, this does not exclude the presence of an ESBL. As discussed in chapter 1, an organism may be sensitive to a beta lactam *in vitro*, but still display *in vivo* resistance due to the production of an ESBL. It is precisely this problem that makes detection of ESBLs so difficult and so clinically important.

The 11 transconjugants without detectable ESBL activity have ceftazidime MICs of 1,5μg/ml or less. These MICs are higher than the ceftazidime MIC of the recipient, *E. coli* J53, and this is most probably due to the transfer of a beta lactamase gene encoding an enzyme without extended spectrum activity (*e.g* TEM-1 or SHV-1). The presence of such an enzyme, however, can result in some hydrolysis of ceftazidime, which is reflected in the slightly elevated MIC.

The nine transconjugants with ESBL activity, determined by the Etest ESBL test, (including the transconjugant from the cross with K50) have cefotaxime MICs ranging from 1,5μg/ml to 6μg/ml. These MICs indicate susceptibility of the organisms to cefotaxime, and this again illustrates the problem of correlating the results of *in vitro* testing to *in vivo* effect of an antibiotic. It is interesting that, with one exception (from the cross with M26), the cefotaxime MICs of those transconjugants with no ESBL activity are lower than the cefotaxime MICs of the ESBL producing transconjugants. These MICs are still higher than the cefotaxime MIC of the recipient *E. coli* J53, again reflecting the acquisition of a beta lactamase, which, although not an ESBL, still has some activity against cefotaxime.

One transconjugant, from the cross with isolate M46 (*S. marscecens*), contains an ESBL while the donor strain does not. This is in itself unusual, and suggests that the gene, if present in isolate M46, was not expressed in the donor. Another, and more

likely, explanation hinges on the fact the *S. marscecens* produces a chromosomal beta lactamase (AmpC) which is resistant to the effects of clavulanic acid and may mask the presence of the ESBL in the donor. Since AmpC is chromosomally mediated, it would not be transferable to *E. coli* while a plasmid containing an ESBL gene would be transferable, and the enzyme could then be detected in the transconjugant. This again illustrates the difficulties in detecting ESBL activity, using both the double disc test and the E-test ESBL test.

### 5.3.3 Characterisation of Plasmids from Transconjugants

To study the plasmid content of the transconjugants, plasmid DNA was extracted [3.2.3.4.vi] from 19 of those transconjugants that were successfully cultured. The 19 transconjugants from which plasmids were successfully purified were from conjugations with the donors K4, K8, K16, K29, K42, K43, K46, K50, K54, K63, K68, K69, M26, M30, M40, M44-1, M44-2, M46 and E49. Plasmid DNA was digested with *Bam*HI (Boehringer Mannheim) and the fragments separated by agarose gel electrophoresis (Fig 5.2 (i & ii)).

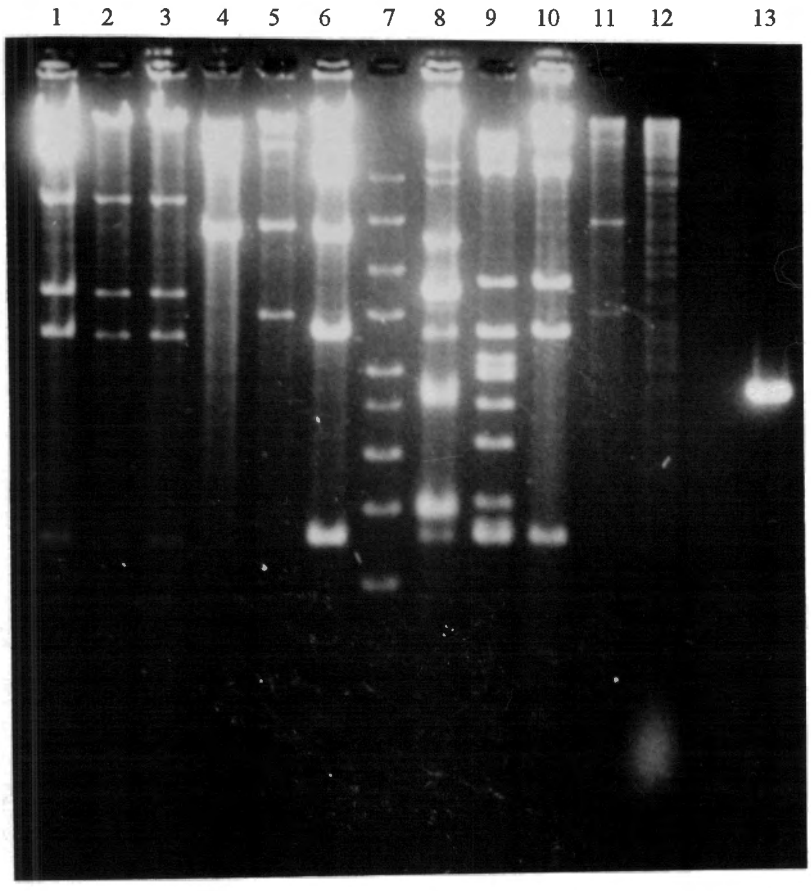



Fig 5.2 Digestion of plasmids extracted from the transconjugants, using *Bam*HI (5 units), at 37°C for 6 hours. Electrophoresis carried out on 0,8% agarose at 2 –3 V/cm for 18 hours and stained after electrophoresis with EtBr (10mg/ml), using 1µl EtBr per 100ml TAE [3.2.3.2].

(i) **lanes 1 – 6**: Plasmid from transconjugants of K4, K8, K16, K29, K42, and K43, respectively.
**lane 7**: Molecular weight marker (10kb BioMarker)
**lanes 8 – 11**: Plasmids from transconjugants of K46, K50, K54 and K63.
**lane 12**: Genomic DNA from *E. coli* DH5α
**lane 13**: pUC19

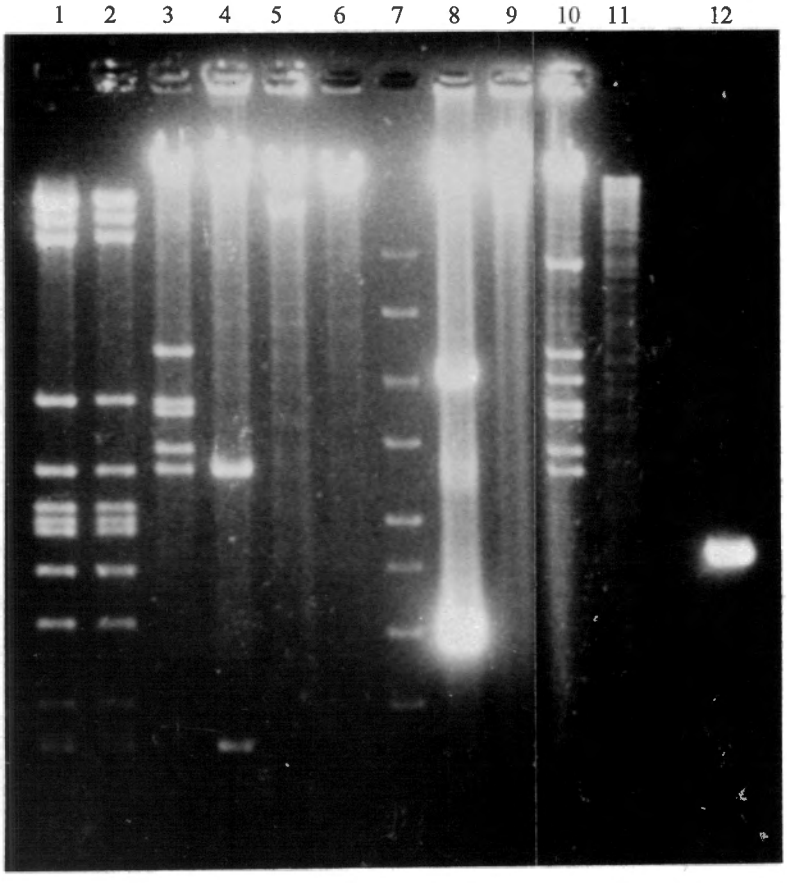



Fig 5.2 (ii) **lanes 1 – 6**: Plasmid from transconjugants of K68, K69, M26, M30, M40 & M44-1 respectively.

**lane 7**: Molecular weight marker (10kb BioMarker)

**lanes 8 – 10**: Plasmids from transconjugants of M44-2, M46 & E49

**lane 11**: Genomic DNA from *E. coli* DH5α

**lane 12**: pUC19

Analysis of the restriction patterns generated (Fig 5.2) shows two predominant restriction profiles, 1 and 2. Profile 1 is present in transconjugants from crosses involving K4, K8, K16 and K54 and profile 2 was obtained from the crosses involving K50, K68 and K69. The sizes of the DNA fragments present in these two profiles are shown in Table 5.2.

Table 5.2

Common *Bam*HI restriction profiles

| Profile 1 | Profile 2 |
|---|---|
| >20kb | >20kb |
| | >15kb |
| | >10kb |
| ~8kb | |
| ~4,5kb | ~4,5kb |
| ~3,5kb | ~3,5kb |
| | ~3,2kb |
| | ~3kb |
| | ~2,8kb |
| | ~2,5kb |
| | ~2,1kb |
| | ~1,5kb |
| | ~1,3kb |
| ~1,2kb | ~1,2kb |

Profile 1 – Present in plasmids from transconjugants of K4, K8, K16, K54

Profile 2 – Present in plasmids from transconjugants of K50, K68, K69

kb = kilobases.

Although the restriction profiles of the other plasmids are unique, a number of fragments are common to some of the profiles. A ~3,5kb fragment, present in profiles 1 and 2, can also be seen in the profiles of plasmids from donors K43, K46, M26, M30 and E49. The plasmid profiles from the transconjugants of M26 and E49, while not identical, share a number of similarities. In addition to the ~3,5kb fragment, *Bam*H1 fragments of ~5,5kb, ~4,6kb, ~4,5kb, ~4kb are present in both plasmids.

It is noteworthy that the plasmids from the transconjugants of the two *Salmonella* strains have slightly different restriction patterns. Specifically, the plasmid from the transconjugant of M44-2 is larger than that from M44-1, with 2 extra fragments of ~5kb and ~2kb. Since M44-2 was isolated 5 days after M44-1, it is possible that the plasmid in M44-1 acquired additional genetic material.

## 5.3.4 DNA-DNA Hybridisations

To characterise the plasmids further, the DNA fragments generated after digestion with *Bam*H1 were transferred to nylon membranes and the TEM and SHV probes were hybridised to these membranes in separate experiments.

### *5.3.4.1 Hybridisation with TEM Probe*

When the membranes were hybridised with the TEM probe, a positive signal was obtained from the control, pUC19 and no signal was obtained from the negative control, *E. coli* DH5α genomic DNA. A positive signal was obtained from 8 of the 20 samples (K42, K43, K54, K63, M40, M44-1, M44-2 and M46) (Fig 5.3 (i & ii)) indicating the presence of a TEM-related gene. The probe hybridised to large (>15kb) fragments in these plasmids, and examination of the plasmid profile suggests that the fragments to which the probe hybridised may be undigested DNA.

Importantly, a TEM related gene was identified in all but two of the donor strains by hybridisation and PCR [chapters 3 & 4]. No evidence of a TEM related gene by either hybridisation or PCR was detected in donors K54 or M40. Curiously, a signal was not obtained from the plasmids isolated from transconjugants involving crosses between K29, K50, K68 and K69, although TEM-related genes had been demonstrated in each of these donors by hybridisation and PCR

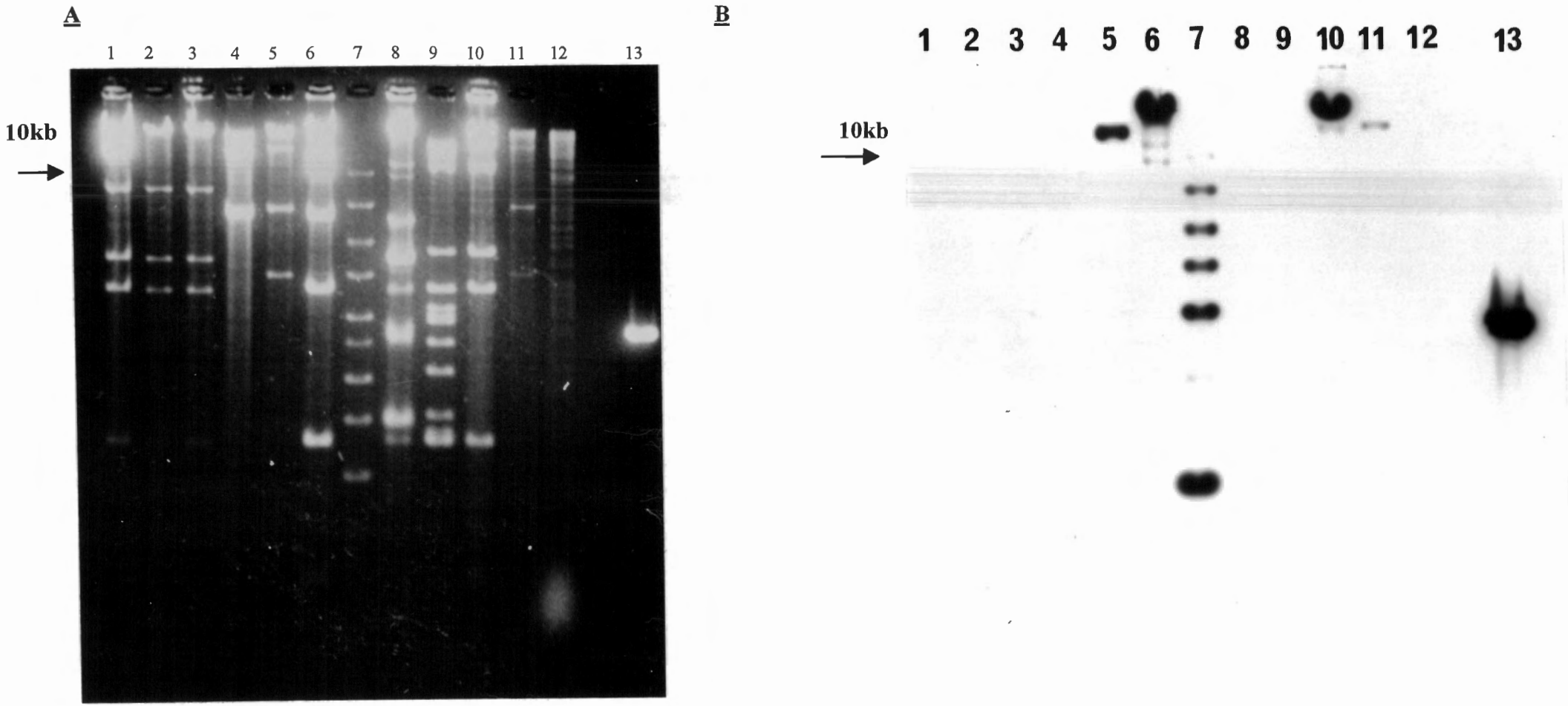



Fig 5.3 (i) Hybridisation of TEM probe to *Bam*HI digested plasmid DNA extracted from the transconjugants.

**A** *Bam*HI Restriction profiles following electrophoresis **B** Autoradiograph of DNA shown in A. Membrane exposed to Curix X-ray film for 2 hours

**lanes 1 – 6**: plasmid from transconjugants of K4, K8, K16, K29, K42, and K43, respectively.

**lane 7**: Molecular weight marker (10kb BioMarker). The position of the 10kb fragment is indicated.

**lanes 8 – 11**: Plasmids from transconjugants of K46, K50, K54 and K63, respectively.

**lane 12**: Genomic DNA from *E. coli* DH5α (negative control)

**lane 13**: pUC19 (TEM positive control)

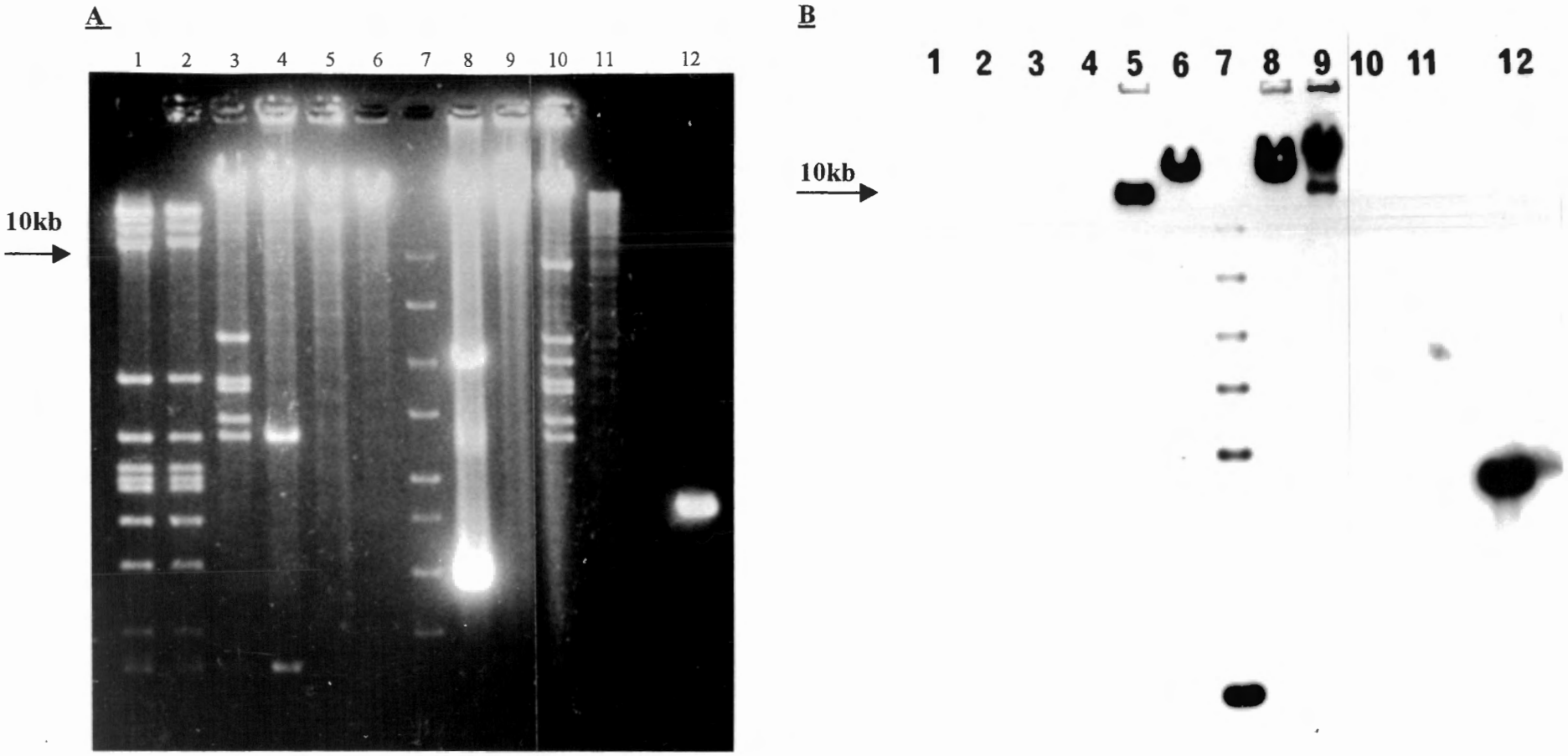



Fig 5.3 (ii) Hybridisation of TEM probe to *Bam*HI digested plasmid DNA extracted from the transconjugants. After hybridisation the membranes were washed under high stringency condidions, and exposed to Curix X-Ray film for 2 hours.

**A** *Bam*HI Restriction profiles following electrophoresis **B** Autoradiograph of DNA shown in A. Membrane exposed to Curix X-ray film for 2 hours

**lanes 1 – 6**: Plasmid from transconjugants of K68, K69, M26, M30, M40 & M44-1 respectively

**lane 7**: Molecular weight marker (10kb BioMarker). The position of the 10kb fragment is indicated.

**lanes 8 – 10**: Plasmids from transconjugants of M44-2, M46 & E49, respectively.

**lane 11**: Genomic DNA from *E. coli* DH5α (negative control)

**lane 12**: pUC19 (TEM positive control)

The hybridisation studies were repeated: plasmids from these transconjugants and genomic DNA from the corresponding donors was digested with *Bam*H1, transferred to a nylon membrane and hybridised with the TEM probe. The original donor M40 could not be cultured from the glycerol stocks in order to extract fresh genomic DNA, and the hybridisation on this donor could thus not be repeated.

As shown (Fig. 5.4) a strong signal was obtained from the positive control (*E. coli* DH5α(pUC19) genomic DNA). Strong signals were obtained from the genomic DNA from the donors K29, K50, K68 and K69, as well as from fragments in the plasmids from crosses involving K29 and K54. Weak signals were obtained from fragments in the plasmids from crosses involving the donors K50, K68 and K69. These weak signals were interpreted as non-specific hybridisation based on the observation that a strong signal was obtained from the corresponding donor's genomic DNA. No signal was obtained from the genomic DNA of isolate K54. In the case of genomic DNA from donor K29 and the corresponding plasmid, the fragment of genomic DNA to which the probe hybridised is larger than the fragment from the plasmid, suggesting incomplete digestion of the genomic DNA.

The discrepancy between the hybridisation to genomic DNA of isolate K54 and hybridisation to the plasmid from the corresponding transconjugant is worrying. No signal was obtained when the TEM probe was hybridised to genomic DNA from the donor, and it appears that the plasmid from this transconjugant did not originate from the purported donor K54. Consequently, all results pertaining to the transconjugant of isolate K54 were ignored. Similarly, the results pertaining to the transconjugant of isolate M40 were ignored, since a second hybridisation could not be performed on the donor to clarify the discrepancy between the results of the hybridisations on the donor and recipient.

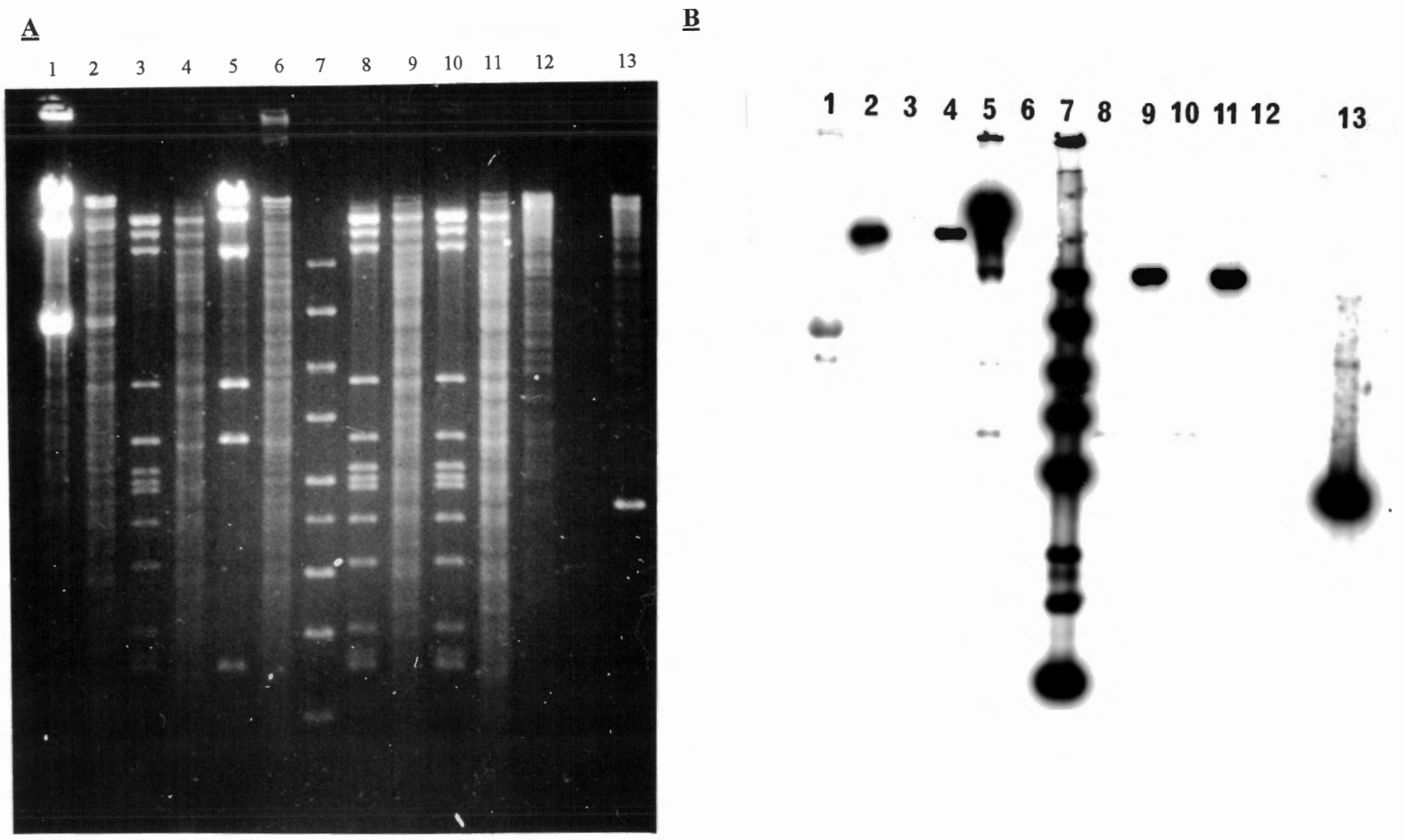



Fig 5.4 Legend overleaf

Fig 5.4 Hybridisation of TEM probe to *Bam*HI digested DNA extracted from transconjugants (plasmid DNA) and respective donors (genomic DNA).

**A** Restriction profiles following electrophoresis. Digestion was carried out with 5 units of enzyme at 37°C for 6 hours. DNA was separated by electrophoresis on a 0,8% agarose gel at 2-3 V/cm

**B** Autoradiograph of DNA shown in A. After hybridisation the membranes were washed under for 18 high stringency conditions, and exposed to Curix X-Ray film for 2 hours.

**Lane 1**: Plasmid DNA from transconjugant of K29

**Lane 2**: Genomic DNA from isolate K29

**Lane 3**: Plasmid DNA from transconjugant of K50

**Lane 4**: Genomic DNA from isolate K50

**Lane 5**: Plasmid DNA from transconjugant of K54

**Lane 6**: Genomic DNA from isolate K54

**Lane 7**: 10kb BioMarker

**Lane 8**: Plasmid DNA from transconjugant of K68

**Lane 9**: Genomic DNA from isolate K68

**Lane 10**: Plasmid DNA from transconjugant of K69

**Lane 11**: Genomic DNA from isolate K69

**Lane 12**: Genomic DNA from *E. coli* DH5α

**Lane 13**: Genomic DNA from *E. coli* DH5α(pUC19)

#### *5.3.4.2 Hybridisation with SHV Probe*

When the SHV probe was hybridised to the membranes, a strong signal was obtained from the positive control (*E. coli* K12 (SHV-1)) and no signal obtained from the negative control (pUC19) using the membrane containing only the controls (Fig 5.5(i)). However, a weak signal was obtained from the same negative control on those membranes containing the plasmid DNA from the transconjugants (Fig 5.5 (ii & iii)). Although this suggests hybridisation of the SHV probe to the TEM-1 gene in pUC19, this is surprising since sequence alignment of SHV-1 and TEM-1 shows that there is 60% DNA homology between the two genes. Under the stringency conditions used in this hybridisation, the SHV probe should not have remained annealed to the TEM-1 gene, and the fact that it may have cannot be easily explained. Alternatively, the probe may have hybridised to a different pUC19-related sequence.

A weak signal, of similar intensity to the signal from the negative control, was obtained from the plasmids of 3 of the transconjugants – those resulting from crosses with M44-1, M44-2 and M46. In view of the fact that these plasmids contain TEM-related genes [5.3.4.1], it would be tempting to assume that the probe had annealed to the TEM genes in these plasmids. The autoradiographs (Figs 5.3 & 5.5), however, show that the SHV and TEM probes hybridised to different *Bam*HI generated fragments. As the signal was of similar intensity to that obtained from the negative control, it was assumed that these transconjugants do not contain an SHV-related gene.

The probe hybridised to *Bam*HI generated fragments of the plasmids extracted from 13 of the transconjugants – those resulting from crosses with isolates K4, K8, K16, K29, K43, K46, K50, K68, K69, M26, M30, M40 and E49. The signals obtained from these fragments were of a similar intensity to the signal obtained from the positive control. The probe hybridised to an approximately 3,5kb fragment in 11 of the 13 plasmids, originating from the donors K4, K8, K16, K43, K46, K50, K68, K69, M26, M30 and E49.

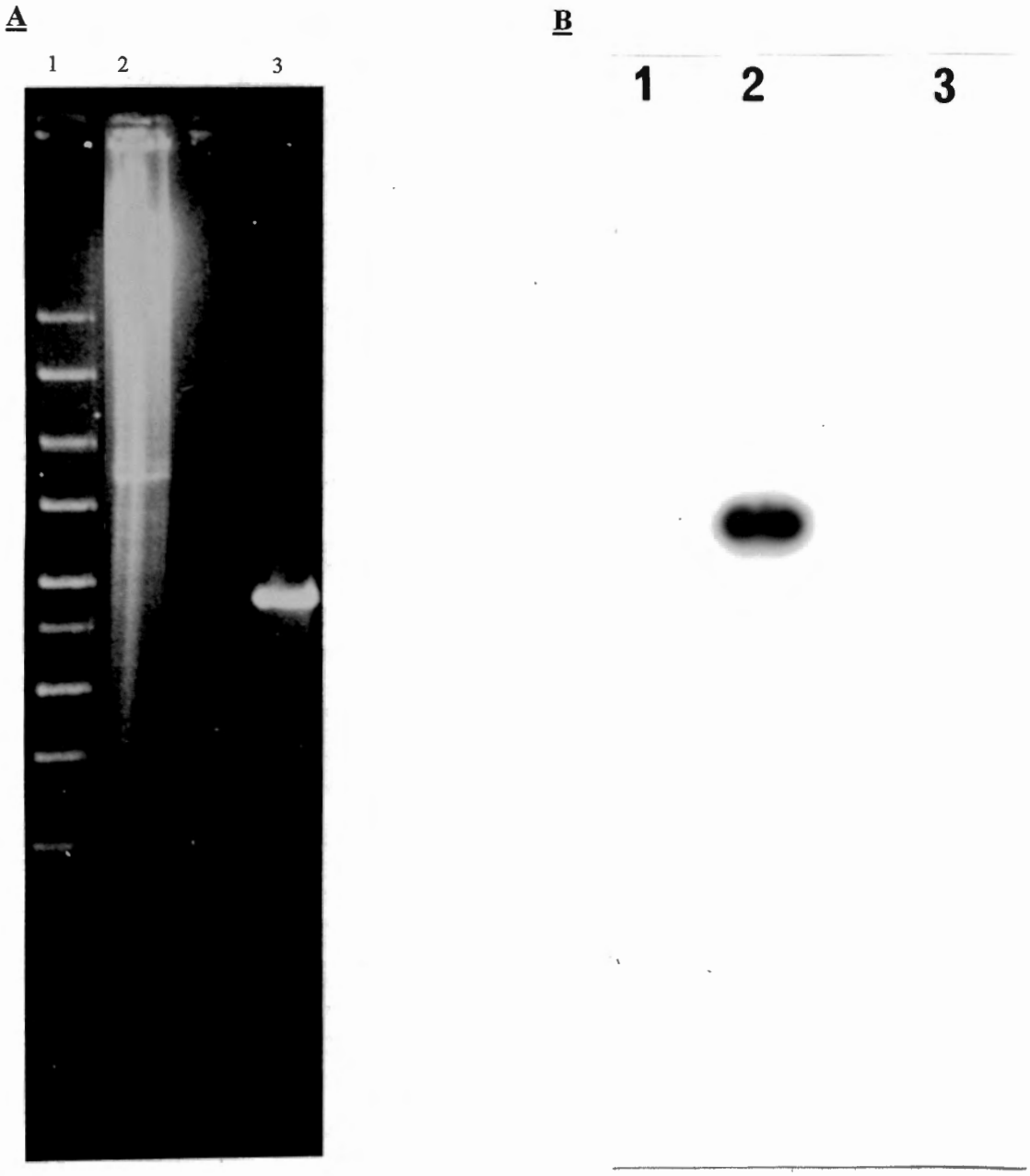



Fig 5.5 (i) Hybridisation of the SHV probe to *Bam*HI digested pUC19 and plasmid DNA extracted from *E. coli* K12 (SHV-1). After hybridisation the membranes were washed under the same stringency conditions applied to the membranes with the plasmid DNA from the transconjugants. The membrane was exposed to Curix X-Ray film for 2 hours.

**A** Restriction profiles following electrophoresis

**lane 1**: Molecular weight marker (10kb BioMarker)

**lane 2**: DNA from K12 (SHV-1)

**lane 3**: pUC19

**B** Autoradiograph of DNA shown in A

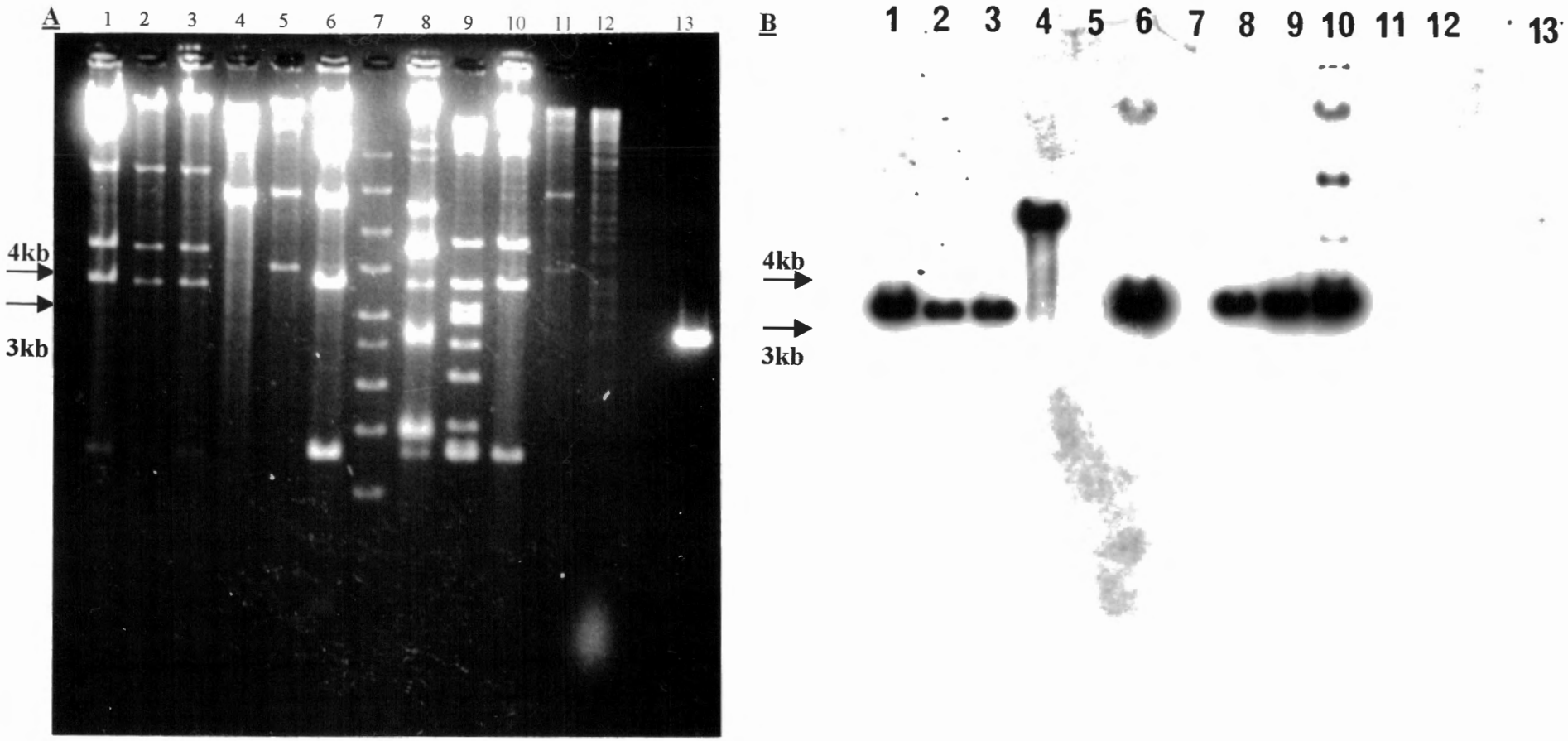



Fig 5.5 (ii) Hybridisation of SHV probe to *Bam*HI digested plasmid DNA extracted from the transconjugants.

**A** *Bam*HI restriction profiles following electrophoresis

**B** Autoradiograph of DNA shown in A After hybridisation the membranes were exposed to Curix X-Ray film for 2 hours.

**lanes 1 – 6**: Plasmid from transconjugants of K4, K8, K16, K29, K42, and K43, respectively.

**lane 7**: Molecular weight marker (10kb BioMarker). The position of 2 of the fragments is illustrated.

**lanes 8 – 11**: Plasmids from transconjugants of K46, K50, K54 and K63, respectively.

**lane 12**: Genomic DNA from *E. coli* DH5α (negative control)

**lane 13**: pUC19 (negative control)

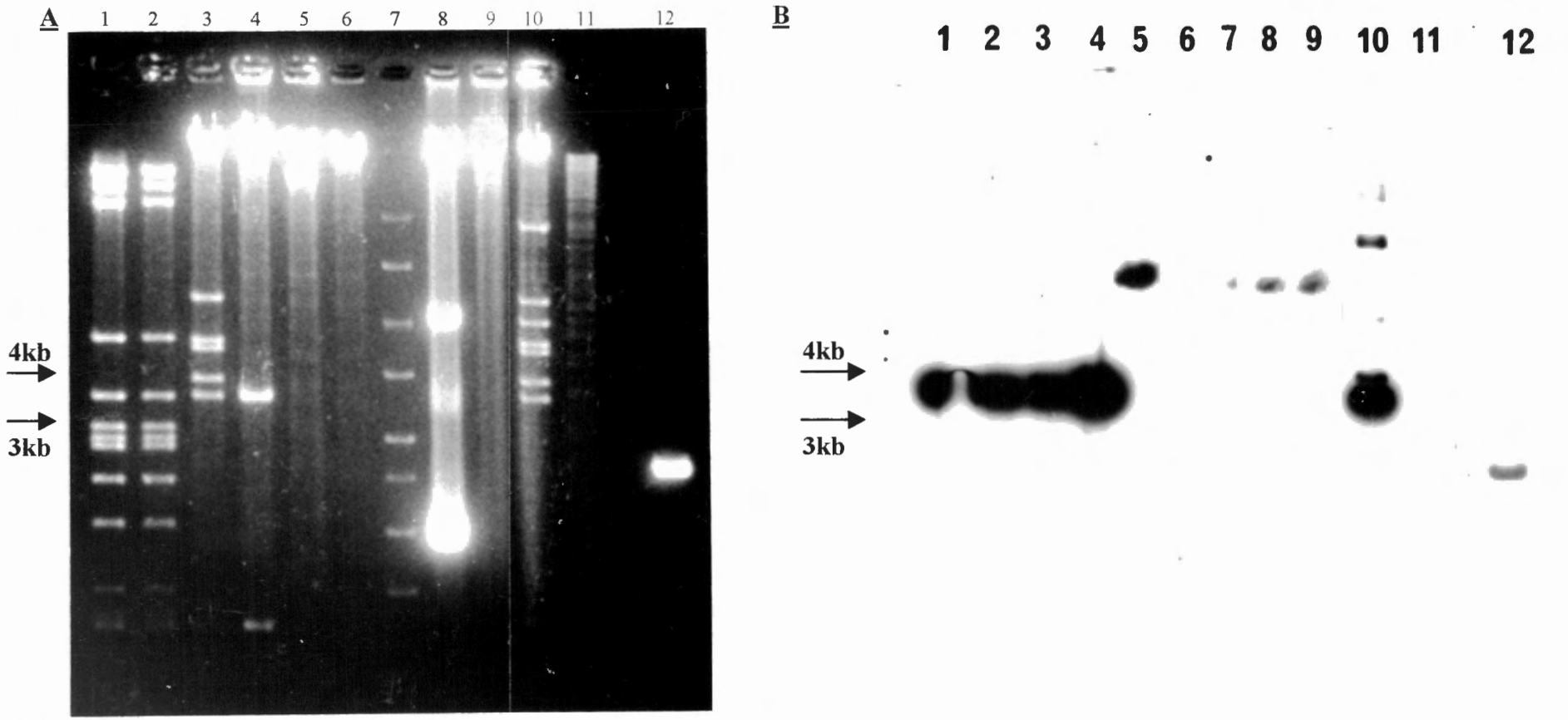



Fig 5.5 (iii) Hybridisation of SHV probe to *Bam*HI digested plasmid DNA extracted from the transconjugants.

**A** *Bam*HI restriction profiles following electrophoresis

**B** Autoradiograph of DNA shown in A After hybridisation the membranes wereexposed to Curix X-ray film for 2 hours.

**lanes 1 – 6**: Plasmid from transconjugants of K68, K69, M26, M30, M40 & M44-1, respectively

**lane 7**: Molecular weight marker (10kb BioMarker). The position of 2 of the fragments is illustrated.

**lanes 8 – 10**: Plasmids from transconjugants of M44-2, M46 & E49, respectively.

**lane 11**: Genomic DNA from *E. coli* DH5α (negative control)

**lane 12**: pUC19 (negative control)

Correlating these results with the results of both the slot blot hybridisation studies and the PCR assays is difficult, firstly because of the problems experienced with the chromosomal beta lactamase genes in *K. pneumoniae*, and secondly because of the difficulties encountered in performing the PCR assay itself [chapters 3 and 4]. However, the results of the studies on donor isolates other than *K. pneumoniae* correlate with the results obtained with this hybridisation. Specifically, the plasmid from the transconjugant of an isolate of *E. cloacae* (E49) was shown to contain an SHV-related gene as was the respective donor.

## 5.4 DISCUSSION

After excluding the donors K54 and M40, ampicillin resistance was transferred from all 27 donors to the recipient, although 9 of the transconjugants could not be cultured further. It is interesting that of the remaining 18 transconjugants, only 8 were shown to produce an ESBL, and it is assumed that the ampicillin resistance acquired by the other 10 transconjugants is due to the acquisition of a TEM- or SHV-related gene without extended spectrum activity. Given that all the donors, apart from M46, were shown to contain ESBLs [chapter 2], it is perhaps surprising that as many as 10 of them contain a non-ESBL TEM- or SHV-related beta lactamase as well as an ESBL. The finding of ESBL transfer in only 8 of the 18 (44%) conjugation experiments is slightly higher than described in other work. Hibbert-Rogers *et al.* (1995) demonstrated transfer of ceftazidime resistance from only 31% of clinical isolates of *Enterobactericeae*. It must be remembered that 9 of the transconjugants in this study could not be cultured, and the figure of 44% may be falsely elevated.

The transfer of ampicillin resistance from all 27 of the donors in this study is inconsistent with other published work. Gaur *et al.* (1992) demonstrated ampicillin resistance transfer in 14,7% thermotolerant coliforms (including *Klebsiella* spp. and *E. coli.*). In a study of multiply antibiotic resistant members of the *Enterobacteriaceae*, ampicillin resistance (with or without other antibiotic resistance determinants) was transferred from 20 of the 35 isolates (Levy *et al.*, 1985). A study by Leung *et al.*

(1997) showed transfer of beta lactam resistance from only 5 out of 80 strains of *K. pneumoniae*. The beta lactamase genes that were transferred encoded both ESBLs (SHV-5 and SHV-2 or –7) and non-ESBLs (SHV-1 and TEM-2). The fact that ampicillin resistance was transferred from all of the donors in this study may have been influenced by the selection of donors based on the presence of either a TEM- or SHV-related gene. Additionally, 9 of the transconjugants could not be subcultured, so stable resistance transfer was only demonstrated in 18 of the 27 conjugations.

As mentioned previously [3.4], *P. agglomerans, S. marscecens* and *E. cloacae* have chromosomal beta lactamase genes, which would not ordinarily be transferable. The finding, therefore, of self-transmissible beta lactam resistance determinants in these isolates (M46 and E49) is surprising since the presence of extra beta lactamase genes seems superfluous.

Hybridisation showed that plasmids from 12 of the transconjugants contained SHV related genes. The plasmids from the transconjugants of K42 and K63 (both *K. pneumoniae*) were not shown to contain SHV related genes while the hybridisation and PCR studies on the donors demonstrated the presence of these genes. This supports the supposition that chromosomal SHV-related genes as well as plasmid mediated SHV-related genes in the *K. pneumoniae* donors were detected by the PCR assay (with primers SHV-A and SHV-B) [4.3.5] and hybridisation with the SHV probe [3.4]. Of course, it is possible that these two donors (K42 and K63) may contain SHV-related beta lactamases that are not on self-transmissible plasmids, explaining the failure to detect such genes in the transconjugants.

Of the 8 transconjugants with demonstrated ESBL activity (using the Etest ESBL test), 3 were shown to contain only an SHV-related gene. This is highly suggestive of the fact that the SHV-related gene in these plasmids encodes a beta lactamase with extended spectrum activity, bearing in mind the *caveat* that a different beta lactamase (unrelated to TEM or SHV) may be responsible for the resistance to ceftazidime. The ceftazidime MIC of 1 of these transconjugants (from the cross with K46) is >32μg/ml, while the ceftazidime MICs of the other two (from crosses with K50 and M30) are 6μg/ml. Although a ceftazidime MIC of 6μg/ml does not indicate resistance to this

antibiotic, the Etest ESBL test does indicate the presence of an ESBL in these 2 transconjugants (albeit on a borderline level in transconjugant K50).

With respect to the 9 transconjugants which did not express ESBL activity, 7 were found to contain only an SHV-related gene (or genes). This suggests that the donors (K4, K8, K16, K68, K69, M26 and E49) contain a self transmissible plasmid encoding SHV-1, and an additional gene, which was not transferred, encoding an ESBL. Two of these 7 donors, K68 and K69, contain both TEM- and SHV-related genes, and it is possible that the TEM-related genes, which were not transferred, encode the enzymes with extended spectrum activity in these 2 strains. However, the presence of a second, non-transmissible SHV-related ESBL in these 2 donors cannot be excluded, and the presence of 2 SHV-related enzymes in *K. pneumoniae* isolates has been described Johnson *et al.*, 1992).

It is gratifying that the results of the hybridisation with the TEM probe to the plasmid DNA correlated well with the results of the PCR and hybridisations performed on the donor strains, although the discrepancies in the results of isolates K54 and M40 are disappointing. TEM-related genes were shown to be present in 7 of the plasmids extracted from the transconjugants.

Only TEM-related genes were found in 3 of the plasmids from the 9 ESBL producing transconjugants, which, again, strongly suggests that these TEM-related genes encode enzymes with extended spectrum activity. All 3 of these transconjugants (from crosses with K42, K63 and M46) have ceftazidime MICs of >32μg/ml. Of the 9 transconjugants with no ESBL activity, 2 had only TEM-related genes demonstrated, indicating that these genes were probably encoding either the TEM-1 or -2 beta lactamase. These 2 donors (M44-1 and M44-2, both *Salmonella* sp.) contain both TEM- and SHV-related genes, and in this situation it would seem likely that the SHV-related genes in the donors encode the ESBLs. Hammami *et al.*, (1991) described the presence of both TEM-1 and SHV-2 genes in isolates of *Salmonella wien*, although neither of these genes was shown to be transferable. A variety of SHV-related ESBLs have been described in *Salmonella* species (Garbarg-Chenon *et al.*, 1989; Hammami *et al.*, 1991), and the presence of TEM-1 in both typhoidal and non-typhoidal *Salmonella*

isolates is also well described (Kariuki *et al.*, 1996; Shanahan *et al.*, 1998). Again, the possible presence of another, non-TEM or -SHV-related enzyme should also be considered in these Salmonella isolates, as was described by Gazouli *et al.* (1998).

The plasmids from 2 of the transconjugants (from crosses with K29 and K43) contain both SHV- and TEM-related genes, and ESBL activity was demonstrated in both of these transconjugants. It is again highly probable that the ESBL gene in these 2 transconjugants is either TEM- or SHV-related, although it would be impossible to deduce which gene encodes the ESBL. The results of the hybridisation studies on the transconjugants, along with the PCR and hybridisation study results on the respective donors, are shown in Table 5.3.

Table 5.3

Results of PCR and hybridisation studies on the donors, and hybridisation studies on the respective transconjugants.

| **Original Isolate** | | **Donor** | | | | | **Transconjugant** | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Presence of ESBL | Hybridisation | | PCR | | Presence of ESBL | Hybridisation | |
| K4 | *K. pneumoniae* | Yes | SHV | | SHV | | No | SHV | |
| K8 | *K. pneumoniae* | No | SHV | | SHV | | No | SHV | |
| K16 | *K. pneumoniae* | Yes | SHV | | SHV | | No | SHV | |
| K29 | *K. pneumoniae* | Yes | SHV | TEM | SHV | TEM | Yes | SHV | TEM |
| K42 | *K. pneumoniae* | Yes | SHV | TEM | SHV | TEM | Yes | | TEM |
| K43 | *K. pneumoniae* | Yes | SHV | TEM | SHV | TEM | Yes | SHV | TEM |
| K46 | *K. pneumoniae* | Yes | SHV | | SHV | | Yes | SHV | |
| K50 | *K. pneumoniae* | Yes * | SHV | TEM | SHV | TEM | Yes | SHV | |
| *K54* | *K. pneumoniae* | *Yes* | *SHV* | | *SHV* | | *No* | *SHV* | *TEM* |
| K63 | *K. pneumoniae* | Yes | SHV | TEM | SHV | TEM | Yes | | TEM |
| K68 | *K. pneumoniae* | Yes | SHV | TEM | SHV | TEM | No | SHV | |
| K69 | *K. pneumoniae* | Yes | SHV | TEM | SHV | TEM | No | SHV | |
| M26 | *K. pneumoniae* | Yes | SHV | | SHV | | No | SHV | |
| M30 | *K. pneumoniae* | Yes | SHV | | SHV | | Yes | SHV | |
| *M40* | *E. cloacae* | *Yes* | *SHV* | | *SHV* | | *Yes* | *SHV* | *TEM* |
| M44-1 | *Salmonella* sp. | Yes | SHV | TEM | SHV | TEM | No | | TEM |
| M44-2 | *Salmonella* sp. | Yes | SHV | TEM | SHV | TEM | No | | TEM |
| M46 | *S. marcescens* | No | | TEM | | TEM | Yes | | TEM |
| E49 | *E. cloacae* | Yes | SHV | | SHV | | No | SHV | |

* - The result of the ESBL Etest on isolate K50 was borderline for ESBL presence.

The results of the conjugation studies on isolate K54 and M40 (italicised) have been ignored

The results of the hybridisation indicate that SHV-related genes are more common in the clinical isolates, particularly *K. pneumoniae*, than TEM-related genes. Of 13 *K. pneumoniae* isolates, 11 contain plasmid encoded SHV-related genes and 4 contain plasmid encoded TEM-related genes. Of these, 3 of the SHV-related genes, and 2 of the TEM-related genes might encode ESBLs. In addition, the TEM related gene originating from the transconjugant of donor M46 may also encode an ESBL. Although TEM-1 is the commonest plasmid mediated beta lactamase found in the *Enterobacteriaceae* (Du Bois *et al.*, 1995), SHV-related ESBLs have on occasion been shown to be more common than their TEM-related counterparts (Jacoby & Han, 1996).

*Bam*HI mapping showed some similarities amongst the transferred plasmids, in that 2 different profiles were common to some of the plasmids [5.4.3]. Some similarities could be observed between those plasmids that did not share a common profile, most notably a ~3,5kb *Bam*HI generated fragment. The SHV probe hybridised to this fragment in plasmids from 11 of the transconjugants. It is interesting that the plasmid extracted from the transconjugant of isolate E49 (*E. cloacae*) showed some similarities [5.4.3] to the plasmid extracted from the transconjugant of isolate M26 (*K. pneumoniae*). However, the focus of this research was not to determine the relatedness of the plasmids, and further studies on the plasmids were not carried out.

While transmissibilty of resistance, as demonstrated by the conjugation sudies, would be strong evidence in support of a plasmid located resistance gene, it must be remembered that non-transmissability does not necessarily exclude a plasmid-related resistance determinant. Resistance genes may be present on plasmids that need to be mobilised, and these studies were not performed as part of this research. It is interesting that in 9 of the conjugation experiments in our study, transconjugants could not be cultured further in selective media, suggesting that these plasmids may be unstable in *E. coli*.

It is also important to bear in mind that the beta lactamase genes may be located on transposons, either on plasmids or integrated in the chromosome. Conjugation experiments (as performed in this study) may not necessarily demonstrate transfer of

resistance carried on transposons, although, *in vivo,* such elemens may well be transferred (on plasmids). A variety of beta lactamase genes, encoding both extended spectrum and non-extended spectrum beta lactamases, have been described on transposons. Heritage *et al.* (1992) described the occurrence of a TEM-12 gene on a transposon (Tn*841*). Similarly, an SHV-3 gene has been found on a plasmid containing genes for aminoglycoside and tetracycline resistance, and the authors postulated that this association may indicate that the genes are located on a transposon (Nicolas *et al.*, 1989). The ability of resistance to be transferred may also be due to a conjugative transposon rather than a plasmid, although this is probably less likely since no ESBLs have yet been described on conjugative transposons. Although no TEM- and SHV-related ESBLs have yet been described on integrons, it seems unlikely that this situation will last, considering that at least one ESBL gene (TEM-3) has been described in close association with an integron (Mabilat *et al.*, 1992). Given the association between integrons and the dissemination of antibiotic resistance genes (Hall & Collis, 1995), the possible movement of ESBL genes onto integrons would be most unsettling.

From the point of view of the isolates in this study, what is important is that transmissability *in vitro* is a good pointer to resistance being transferrable *in vivo* with consequent spread of resistance. As mentioned previously this can have profound short and long term clinical implications. In the short term it would open the door to further infection or colonisation by resistant organisms in the presence of selective antibiotic pressure. The long term clinical importance of transferable resistance is related to the increased incidence and spread of multi-drug resistant organisms, with potentially higher morbidity and mortality of the patients along with increased health care costs.

# CHAPTER 6

# GENERAL CONCLUSIONS

The major aim of this study was to evaluate and compare the use of 2 different molecular methods for the detection of TEM- and SHV-related beta lactamase genes in clinical isolates of Gram negative bacilli. The methods evaluated in the study were hybridisation and PCR. In addition, during the course of the study, non-molecular methods for detecting ESBL production were assessed, and the self-transmissibility of resistance from those isolates shown to contain either TEM- or SHV-related beta lactamase genes was investigated.

The standard test used to detect the presence of an ESBL in clinical isolates is the double disc diffusion test [1.4.4.1-i]. This test was performed on all 45 isolates in this study, and the results compared to those of the Etest ESBL test, which has been shown to be more sensitive in detecting ESBL production (Cormican *et al.*, 1996). Of the 45 isolates tested, 27 were shown to produce ESBLs. Using the double disc test as it is performed in the clinical laboratory at Groote Schuur Hospital, the double disc test detected 19 ESBL producers. On repeating the test with the discs closer together, an additional 5 were detected. By and large, however, this test would not be repeated in a clinical laboratory, and the production of ESBLs in a proportion of isolates may go undetected by the double disc test. This is in contrast to the Etest method, which failed to detect only one ESBL producer, which, interestingly enough, was detected using the double disc test. These findings support previous work which has shown that the double disc test is not 100% sensitive (Cormican *et al.*, 1996). The findings also suggest that an alternative method may be required in the clinical laboratory to maximise ESBL detection.

Using a probe amplified from the TEM-1 gene and specific for TEM related genes, 12 isolates were shown to contain the corresponding gene/s in genomic DNA which had been transferred to a nylon membrane. When similar experiments were carried out on

colony blots, equivocal results were obtained, suggesting that such blots are not useful for hybridisation assays. A PCR assay, using primers specific for TEM-related genes, was performed on the 45 isolates, and TEM-related genes were consistently demonstrated in 11 of the isolates. A TEM-related gene was demonstrated in a $12^{th}$ isolate, but in only 2 of the 3 assays. These 12 isolates correspond perfectly to the 12 isolates shown by hybridisation to contain TEM-related genes, although the failure of PCR to detect the gene in one of the isolates, in one assay, illustrates that this technique may not be as reliable as hybridisation. However, the PCR assays, and the preparation of the template in particular, are far easier to carry out than hybridisation studies, and thus more suitable for use in a clinical laboratory. The only equipment required is a PCR thermocycler, apparatus for agarose gel electrophoresis and a transilluminator for visualisation of DNA in the gels.

ESBL activity was present in 10 of the 12 isolates shown to contain TEM-related genes suggesting that the TEM-related genes may encode an ESBL. However, the presence of another ESBL-encoding gene in these isolates cannot be excluded. The 2 non-ESBL producing isolates (*P. agglomerans* and *S. marcescens*) may contain a TEM-1 or TEM-2 gene. Alternatively, an ESBL (related or unrelated to TEM), which was not detected due to the activity of AmpC, may be present in one or both of these isolates.

These 12 isolates were conjugated with *E. coli* ($Nal^R$), and resistance to ampicillin was transferred from all 12 to the recipient. In 1 instance, the transconjugant could not be cultured further, suggesting instability of the plasmid in *E. coli,* and plasmid DNA could not be extracted from a second transconjugant. Plasmids from the remaining 10 transconjugants were characterised using Southern hybridisation, and TEM-related genes were detected in 7 of these. The fact that ESBL activity was detected in 5 of these 7 transconjugants is highly suggestive of a TEM-related ESBL. On the other hand, the TEM-related genes in the 2 transconjugants that did not produce an ESBL would be either TEM-1 or TEM-2. Interestingly, one of the transconjugants shown to produce an ESBL was derived from a cross with *S. marcescens*, which did not have detectable ESBL activity. The failure of both the Etest and double disc test to detect an ESBL in this donor was thought to be due to the activity of AmpC, as mentioned

earlier. The 3 transconjugants that did not contain TEM-related genes were all derived from crosses with *K. pneumoniae* isolates.

Using PCR and hybridisation techniques to detect SHV-related genes in the clinical isolates, the results were not as clear-cut. The presence of chromosomal genes such as LEN-1, very similar to SHV-1, in *K. pneumoniae* complicated both the hybridisation and PCR studies. Efforts to design an oligoprobe that would not hybridise to LEN-1, the known chromosomal gene, were unsuccessful and equivocal studies were always obtained in hybridisation studies involving *K. pneumoniae*. However, 4 of the other isolates (2 *Salmonella* spp. and 2 *E. cloacae*) were shown to contain SHV-related genes using this technique. All 4 of these also produced an ESBL. This suggests that the ESBL produced by each of the 2 *E. cloacae* isolates may be encoded by an SHV-related gene. The same conclusion cannot be reached with regard to the 2 *Salmonella* isolates, since each of these also contained a TEM-related gene.

The PCR assay, using primers specific for SHV-related genes, was also complicated by chromosomal SHV-related genes in *K. pneumoniae*. The assays did indicate the presence of SHV-related genes in the same 4 non-*Klebsiella* isolates shown by hybridisation to contain SHV-related genes. However, parameters resulting in a reliable assay could not be determined, and further work is necessary.

Three of the isolates (M40 being excluded) shown to contain SHV-related genes, and 22 *K. pneumoniae* isolates were conjugated with *E. coli* J53 ($Nal^R$). Ampicillin resistance was transferred from all these isolates to the recipient, but 8 of the transconjugants could not be cultured further. Hybridisation studies on the plasmids of the remaining 16 (no plasmid could be extracted from 1 transconjugant) showed the presence of SHV-related genes in 12 of the transconjugants. The Etest ESBL test demonstrated ESBL activity in 5 of the 12, again strong evidence that the ESBL is encoded by the SHV-related gene. No ESBL activity was present in the other 7 transconjugants, indicating that the SHV-related gene detected in the plasmids from these organisms is SHV-1. The 4 transconjugants that did not contain SHV-related genes arose from crosses with 2 isolates of *K. pneumoniae* and the 2 *Salmonella* spp.

Taken together, 17 transconjugants were studied, 8 of which had detectable ESBL activity. Both TEM- and SHV-related genes were detected in 2 of the 8. In this instance it is impossible to deduce which gene is encoding the ESBL, notwithstanding the fact that the ESBL in one or both of them may in fact be related to the presence of a non-TEM non-SHV gene. The remaining 6 ESBL-producing transconjugants contained either a TEM- or an SHV-related gene, suggesting that the ESBL in these organisms is encoded by the respective gene. Of the 9 transconjugants without ESBL activity, 7 contained an SHV-related gene (presumably SHV-1) and 2 contained a TEM-related gene (either TEM-1 or TEM-2).

Both TEM- and SHV-related beta lactamase genes were identified in clinical isolates of the *Enterobacteriaceae* from Groote Schuur Hospital, and some encode ESBLs. Of the 45 isolates studied, 12 (26,7%) contained TEM-related genes. Although these genes were commonest in isolates of *K. pneumoniae*, they were also present in other members of the *Enterobacteriaceae*. Plasmid mediated SHV-related genes could not be reliably detected in all the isolates. However, combining the results of the studies performed on the 22 non-*Klebsiella pneumoniae* isolates as well as the studies on the transconjugants derived from *K. pneumoniae*, 15 out of 35 isolates (42,8%) contained SHV-related genes. Of these 15 SHV-related genes, 11 were found in *K. pneumoniae* isolates. This, combined with the identification of most of the TEM-related genes in *K. pneumoniae*, is consistent with the fact that SHV- and TEM-related beta lactamases, in particular ESBLs, appear to have a predilection for *K. pneumoniae* (Philippon *et al.*, 1989; Livermore, 1995; Stratton, 1996b). However, the presence of plasmid mediated TEM- and SHV-related beta lactamases in other members of the *Enterobacteriaceae* should not be ignored.

The results showed that PCR can be easily and reliably used, as an epidemiological tool, to detect TEM-related genes in Gram negative bacilli in a clinical laboratory. More work is needed to develop a reliable PCR assay for the detection of SHV-related genes: with respect to both reproducibility as well as ability to differentiate between plasmid and chromosomally located SHV-related genes in *K. pneumoniae*. A recent study showed numerous chromosomal genes in different strains of *K. pneumoniae*, most having a very high degree of homology to a plasmid mediated SHV-1 gene

(Hægmann *et al.*, 1997). Given this, designing a PCR assay which discriminates plasmid from chromosomal SHV-related genes may well prove to be a Herculean task. An alternative would be to utilise the PCR assay that discriminates SHV-related ESBL genes from the non-ESBL encoding counterparts (Nuesch-Inderbinen *et al.*, 1996).

# APPENDIX A

# SOLUTIONS AND MEDIA

**All solutions were made up volumetrically unless otherwise indicated.**

### 50% Acrylamide/2,5% N-N bismethyleneacryalmide (bis-acryalamide)

Work wearing gloves and in a fume hood.

Dissolve 250g acrylamide and 12,5g bis-acrylamide in 500ml sterile water and filter through Whatman filter paper. Store at 4°C protected from light.

### Ammonium Persulphate 10%

Dissolve 1g of ammonium persulphate in 10ml of sterile water. Store aliquots at 4°C protected from light.

### Choloroform-Isoamyl Alcohol

Mix choloroform and isoamyl alcohol in a ratio of 24:1 (chloroform:isoamyl alcohol) Store protected from light at room temperature.

### Denaturing Solution (1,5M NaCl; 0,5M NaOH)

Mix 300ml 5M NaCl and 50ml of 10N NaOH. Make up to 1000ml with distilled water and store at room temperature.

### Dorset's Egg Agar

Dissolve 2,6g nutrient broth base in 200ml distilled water, place in a 1 litre Schott bottle and autoclave at 15 psi for 15 minutes. Once cooled, add about 14 eggs (600ml), dispense into sterile Bijou bottles and inspissate in a steamer.

**EDTA 0,5M, pH 8,0 (Ethylene diamine tetra-acetic acid)**

Add 93,05g EDTA to about 300ml water and stir vigorously. Add NaOH pellets until the pH has reached 8,0. Once the EDTA has dissolved, make up the volume to 400ml. Autoclave the final solution at 15 psi for 15 minutes and store at room temperature.

**Ethanol 70%**

Add 350ml of 100% ethanol to 150ml sterile water. Store at 4°C.

**Ethidium Bromide (10mg/ml)**

Work in a fume hood and wearing gloves.

Dissolve 0,1g ethidium bromide in 10ml sterile water by shaking well. Store at 4°C protected from light.

**Hexadecyl Trimethyl Ammonium Bromide/Sodium Chloride (CTAB/NaCl)**

Add 4,1g NaCl and 10g CTAB (hexadecyl trimethyl ammonium bromide) to about 80ml distilled water, and heat to about 60°C to facilitate dissolution. Once the CTAB has dissolved, make the volume up to 100ml with distilled water and autoclave at 15 psi for 15 minutes. Store at room temperature. The solution may need to be gently heated (to about 40°C) before it can be used as it is very viscous at low ambient temperatures.

**Hydrochloric Acid (HCl) 0,25M**

Add 20ml concentrated HCl to 980ml sterile water and store at room temperature.

**IPTG (Isopropyl-D-thio-galactopyranoside) 200 mg/ml**

Mix 2g IPTG in about 8ml sterile water and dissolve. Make up to 10ml with water, and aliquot 1ml volumes. Store at -20°C until needed. Add 50µl per 100ml agar.

### Lithium Chloride (LiCl) 4M

Dissolve 42,4g LiCl in 250ml water and sterilise by autoclaving at 15 psi for 15 minutes. Store at room temperature.

### Magnesium Chloride ($MgCl_2$) 1M

Dissolve 20,3g $MgCl_2$ in a final volume of 100ml. Autoclave at 15 psi for 15 minutes and store at room temperature.

### Magnesium Sulphate ($MgSO_4$) 1M

Dissolve 24,7g $MgSO_4$ in a final volume of 100ml distilled water. Autoclave at 15 psi for 15 minutes and store at room temperature.

### MacConkey Agar

Add 20g peptone and 5g sodium taurocholate to 1000ml water and dissolve by heating. Add 20g agar and dissolve it by steaming. Adjust the pH to 7,5 and add 100ml lactose (10% aqueous solution) and approximately 3,5ml neutral red solution (2% in 50% ethanol). Heat with free steam (~ 100°C) for 1 hour then autoclave at 15 psi for 15 minutes. The agar can be poured into Petri dishes once it has cooled to about 50°C.

### Mueller Hinton agar

Emulsify 1,5g starch in a small volume of cold water and pour this into 300ml of beef infusion. Add 17,5g casein hydrosylate and 10g agar and make the volume up to 1000ml with distilled water. Heat at ~100°C to dissolve the constituents, then adjust the pH to 7,4. Autoclave at 15 psi for 15 minutes and pour into Petri dishes once cooled to about 50°C.

### Neutralising Solution (1,5M NaCl; 0,5M Tris-HCl pH 7,4)

Combine 300ml 5M NaCl and 500ml 1M Tris-HCl (pH7,4). Make up to 1000ml with distilled water and store at room temperature.

**NNB Buffer 10x**

Dissolve 162g Tris base, 27,5g boric acid and 9,3g EDTA in about 800ml water. Make the volume up to 1000ml with distilled water and store at room temperature.

**Phenol**

Work with gloves and in a fume hood.

Add 600mg 8-hydroxy quinoline, 7,5ml NaOH (2M) and 6ml Tris-HCl (1M pH7,6) to 500g crystallized phenol. Leave to liquefy overnight (or at 40°C) until the solution is clear. Aliquot and store at 4°C.

**Phenol/Chloroform**

Mix phenol (as prepared above) and choloroform-isoamyl alcohol in the ratio of 1:1. Store protected from light at 4°C.

**Physiological saline (0,85% w/v)**

Dissolve 8,5g NaCl in about 900ml of distilled water and make up to 1000ml with distilled water. Autoclave at 15 psi for 15 minutes and store at room temperature.

**Potassium Acetate 5M**

Dissolve 98,2g potassium acetate in about 180ml distilled water and make up the volume to 200ml. Store at 4°C

## Primary Wash Buffer

Dissolve 180g urea in 400ml of water by gently warming the solution (about 50°C). Add 20ml SDS (10%) and SSC according to the stringency required (see below). Make up to 500ml with distilled water and store at 4°C.

| Final Concentration SSC | Volume 20xSSC to be added |
|---|---|
| 0,1x | 2,5ml |
| 0,2x | 5ml |
| 0,3x | 7,5ml |
| 0,5x | 10ml |
| 0,5x | 12,5ml |

## Salt Saturated Isopropanol

Mix isopropanol with a ¼ volume of 5M NaCl, and use the top phase. Store at room temperature.

## Sodium Acetate 3M, pH6,0

Dissolve 204,05g sodium acetate in 400ml distilled water and adjust the pH to 6,0 with glacial acetic acid. Make up to a final volume of 500ml with distilled water and store at 4°C.

## Sodium Chloride (NaCl) 5M

Dissolve 146,25g sodium chloride in 400ml distilled water and make up the volume to 500ml. Autioclave at 15 psi for 15 minutes and store at room temperature.

## Sodium Dodecyl Sulphate (SDS) 10%

Work with gloves and in a fume hood.

Dissolve 50g SDS in 400ml distilled water and adjust the pH to 7.2 with hydrochloric acid. Make up to a final volume of 500ml with distilled water and store at room temperature.

### Sodium Hydroxide (NaOH) 10N

Dissolve 400g of sodium hydroxide pellets in about 800ml distilled water and make up the volume to 1000ml with distilled water. Store in a plastic container at room temperature.

### Salt Sodium Citrate (SSC) 20x

Dissolve 175,3g NaCl and 88,2g tri-sodium citrate in about 800ml distilled water. After adjusting the pH to 7,0 make up to a final volume of 1000ml. Autoclave at 15 psi for 15 minutes and store at room temperature. Dilute with distilled water to achieve the required concentrations.

### Tris-Acetate-EDTA (TAE) buffer (50x)

Dissolve 242,0g Tris base along with 57,1ml glacial acetic acid and 100,0ml EDTA (0,5M) in about 800ml distilled water and make up the volume to 1000ml with distilled water. Autoclave at 15 psi for 15 minutes and store at room temperature. Dilute 1:50 to make TAE running buffer for electrophoresis.

### TE (Tris-EDTA) Buffer

Add 1ml Tris (1M pH 8,0) and 0,2ml EDTA (0,5M pH8,0) to 98,8ml distilled water. Autoclave at 15 psi for 15 minutes and store at room temperature.

### Tracking Dye

Dissolve 25mg bromophenol blue, 4,0g sucrose and 0,4ml EDTA (0,5M pH 8,0) in 10ml sterile water. Store at room temperature.

### Tris-HCl 1M

Dissolve 121g Tris in about 800ml distilled water. Adjust the pH with hydrochoric acid to the required level, and make up to 1000ml with distilled water. Autoclave at 15 psi for 15 minutes and store at room temperature.

### Tryptone Water

Add 10g tryptone and 5g NaCl to distilled water. Adjust the pH to 7,3 and make up to a final volume of 1000ml. Aliquot 5ml volumes into sterile test tubes and autoclave at 15 psi for 15 minutes.

### TSB broth

Add 10,0g polyethylene glycol (PEG), 1ml $MgCl_2$ (1M), 1ml $MgSO_4$ (1M) to 100ml YT broth. Warm slightly to dissolve the PEG (about 35°C – 40°C) and allow to cool. Add 5ml dimethyl sulphoxide. Use fresh.

### Xgal (5-bromo-4-chloro-3-indolyl-β-D galactoside) 20mg/ml

Dissolve 40mg Xgal in 1ml of dimethylsulphoxide (DMSO) and then add 1ml sterile water. Store at -20°C. Use 0,5ml (10mg) solution per 100ml agar.

### YT (Yeast Tryptone) broth

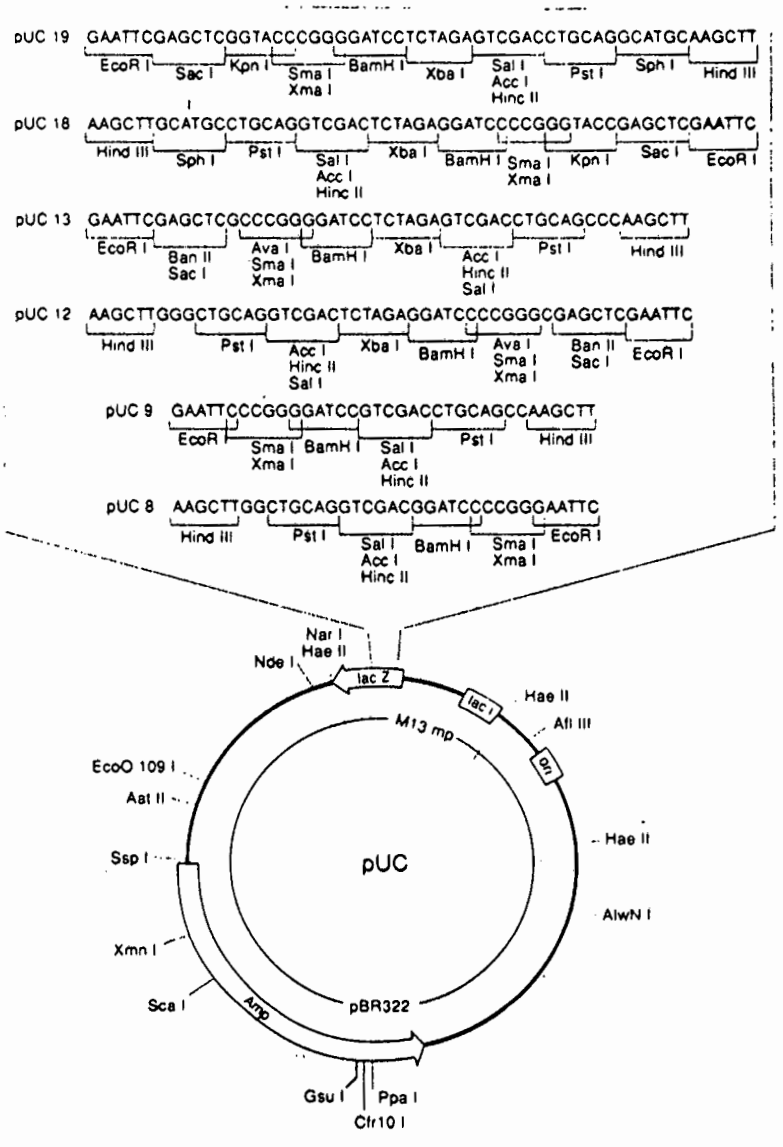
Dissolve 16g tryptone, 10g yeast extract and 5g NaCl in 1000ml distilled water. Dispense to the appropriate containers and autoclave at 15 psi for 15 minutes. Store at room temperature

### YT (Yeast Tryptone) agar

As for YT broth but add 15g agar per litre of broth before autoclaving. Store at room temperature.

# APPENDIX B

## PLASMID VECTOR pUC19




Restriction map of the plasmid vector pUC19, detailing the restriction enzyme recognition sites in the multiple cloning site (MCS) (Brown, 1991b)

# APPENDIX C

# MOLECULAR WEIGHT MARKERS

***Molecular Weight Marker VI***

(Boehringer Mannheim)

| | |
|---|---|
| Fragment sizes (bp) | 2176 |
| | 1766 |
| | 1230 |
| | 1033 |
| | 653 |
| | 517 |
| | 453 |
| | 394 |
| | 298 |
| | 234 |
| | 220 |
| | 154 |

***10kb BioMarker***

(Bioventures)

| Fragment sizes (kb) | 10 |
|---|---|
| | 7 |
| | 5 |
| | 4 |
| | 3 |
| | 2,5 |
| | 2 |
| | 1,5 |
| | 1 |

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## Part I