THE RESERVOIR OF PLASMID-ENCODED BETA-LACTAMASES IN COMMENSAL AEROBIC FAECAL BACTERIA IN BRITAIN AND SOUTH AFRICA

by

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Abstract

There is increasing evidence to suggest that the normal non-pathogenic commensal flora, of the healthy individual, may act as a reservoir of antibiotic resistant determinants. In order to investigate this potential gene pool further, three commensal faecal flora surveys examining three separate populations have been completed. Firstly, 100 faecal specimens were obtained from healthy members of the community in Edinburgh and were examined for the presence of antibiotic resistant determinants amongst the lactose-fermenting, aerobic faecal bacteria. A further 100 faecal specimens, which had been submitted to clinical diagnostic laboratories from general practitioners in Edinburgh and found not to contain any pathogens, were investigated as before. In developing countries, the carriage of antibiotic resistance amongst pathogens has been reported as being significantly higher than in the developed countries. Consequently, to make such a comparison about non-pathogenic commensal faecal flora, the third survey was carried out in the black communities of South Africa; 361 faecal specimens were obtained and examined for the presence of antibiotic resistant faecal flora.

The ampicillin resistant bacteria, from each survey, were purified and investigated further. Conjugation experiments identified the transferability of the resistance determinants. In those strains able to transfer the antibiotic

resistance genes, plasmid preparations and restriction profiles were prepared. From such work, the presence of single or epidemic plasmids within the given community could be identified. The causative β-lactamase mediating resistance within this population was thus determined.

A follow up survey was carried out in Edinburgh approximately two years after the initial survey. Faecal specimens were obtained from five people who had participated in the "healthy" commensal flora survey. Previously, their specimens were found to harbour transferable ampicillin resistant determinants; the responsible plasmids were isolated and identified. Whether or not ampicillin resistant bacteria had been maintained was assessed prior to investigation of the resistance mechanism employed; persistence of a single plasmid type was sought.

The TEM-1 β-lactamase, present on a wide variety of plasmids, was ubiquitous amongst the ampicillin resistant strains isolated from the three surveys.

"Science is built up of facts, as a house is built up of stones; but an accumulation of facts is no more a science than a heap of stones is a house."

Henri Poincaré

(1854 - 1912)

in *Science and Hypothesis*

Contents

Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

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Dedication

For their unending love and support, I dedicate this thesis to my parents, Iris and Cyril Shanahan.

Publications

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Abbreviations

Ac	amoxycillin/clavulanate
Amox	amoxycillin
CAZ (Caz)	ceftazidime
Chrom	chromosomal β-lactamase
Clav	clavulanic acid
Cm	Chloramphenicol
CTZ (Ctz)	cefotaxime
CXM (Cxm, Cf)	cefuroxime
C. freu	*Citrobacter freundii*
C. div	*Citrobacter diversus*
EDTA	ethylenediaminetetraacetic acid
Ent / Cit	*Enterobacter / Citrobacter* species
his	histidine
ID_{50}	dose giving 50% inhibition
IEF	iso-electric focusing
Kb	kilobases
Klebsiella	*Klebsiella* species
Kn	kanamycin
K. oxy	*Klebsiella oxytoca*
K. pneu	*Klebsiella pneumoniae*
MIC	minimum inhibitory concentration
Nit	nitrofurantoin
pI	Iso-electric point
pro	proline
psi	pounds per square inch
SDS	sodium dodecyl sulphate
Sm	streptomycin
Sp	spectinomycin
Su	sulphamethoxazole
Tc	tetracycline
Tp	trimethoprim
trp	tryptophan

Chapter 1

Introduction

1.1. Antimicrobial Chemotherapy

The ability to control infections with antibiotics has revolutionised the practice of medicine. Early considerations of the nature of infectious disease were made by Fracastoro (1546) but it was not until the publications of Henle (1840) and then Koch (1876) that the first evidence that a specific micro-organism could cause a specific disease in an animal, became apparent. Control of disease was initiated with the use of various antiseptics and disinfectants (Semmelweis, 1850; Lister, 1867). It was, however, with the development of chemotherapy that great advances were made in the treatment of infections.

The term chemotherapy expressed the belief of Paul Ehrlich that infectious disease could be combated by treatment with synthetic chemicals. Indeed, as a result of his extensive screening of dyes, the first man-made compound

recognised as being able to produce a curative effect, Trypan Red, was found in 1904. This was followed by the examination of organochemical compounds and the discovery of salvarsan (Ehrlich, 1913) which, together with its derivative neo-salvarsan, became the standard treatment for syphilis at that time. However, this arsenical-based compound was not very selective. Further progress in the therapy of bacterial infections was slow until Domagk (1935) reported that Prontosil rubrum was active against infections; the effective antibacterial agent was subsequently identified as sulphanilamide (Tréfouël, 1935). The sulphonamides were enormously successful drugs which enjoyed widespread clinical use (Greenwood, 1983). Their success stimulated interest in bacterial chemotherapy and in the work performed a few years earlier on penicillin by Fleming.

Interest in penicillin was initiated when the antibacterial action of a Penicillium and its potential importance was recognised by Fleming (1929). He serendipitously, observed the inhibitory effect of *Penicillium notatum* on a Staphylococcus plate. However, a considerable period of time elapsed before the responsible active substance, penicillin, was extracted (Chain *et al.*, 1940). The initial clinical trials (Abraham *et al.*, 1941), which have since been recounted (Fletcher, 1984), revealed the clinical usefulness of penicillin in man. An extensive screening of the soil for micro-organisms which might produce other antibacterial agents followed which led to the identification of streptomycin and neomycin (Waksman and Lechevalier, 1949).

In 1945, Brotzu isolated an antibiotic producing strain of *Cephalosporium acremonium* mould from a sewage outfall (Kuemmerle *et al.*, 1983). Seven antibiotics were isolated from the culture, namely cephalosporin P1, P2, P3, P4, P5 (Burton and Abraham, 1951), cephalosporin N (Newton and Abraham, 1954) and later cephalosporin C. The isolation of the cephalosporin C (Newton and Abraham, 1955) was a significant event as this antibiotic was found to have a wide antibacterial activity. In addition, it was found to be equally effective against penicillin sensitive staphylococci and also against the penicillin resistant staphylococci which had emerged at that time. Penicillin resistant strains of *Staphylococcus aureus* were noted within a few years of the introduction of penicillin and, unfortunately, these resistant strains soon became a major clinical problem. Penicillinase (β-lactamase), the hydrolytic enzyme mediating resistance, predates the introduction of penicillin into widespread clinical use. The first identification of this enzyme was from a strain of *Escherichia coli* (Abraham and Chain, 1940) and only later were these enzymes found in strains of *S. aureus*, the bacteria actually being treated with the antibiotic (Kirby, 1944).

The appearance of antibiotic resistant bacteria encouraged firstly, the development of the semi-synthetic penicillins, with either an increased spectrum of bacterial activity or with modifications rendering them stable to resistant strains, and secondly, a continued progress in the discovery of natural, novel

β-lactam products (Kuemmerle *et al.*, 1983). Many β-lactam antibiotics have been listed to date although only approximately 50 of them have clinical use (Hood, 1991). Although not discussed here, bacterial resistance has also developed to the other classes of antimicrobial agents (Amyes and Gemmell, 1992).

1.2. Development of β-Lactam Antibiotics

1.2.1. Semi-Synthetic Penicillins

Although the original penicillin F and its replacement, penicillin G (benzylpenicillin) were both clinically successful products, variants were sought. Penicillin V (phenoxymethyl penicillin), an early derivative of penicillin G, was obtained in 1948. This agent was available in 1954 for oral administration as it was resistant to hydrolysis by gastric enzymes (Selwyn, 1980).

Many semi-synthetic derivatives of penicillin have been developed as a direct result of the isolation, in 1957, of the penicillin "nucleus" (6-aminopenicillanic acid, or 6-APA) (Figure 1), which was biosynthesised in large quantities by Beecham Research Laboratories (Batchelor *et al.*, 1959).

Figure 1 & 2. Basic penam (left) and cephem (right) nuclei with R and S positions in which modifications are possible (Pickering and Murray, 1984a).

Figure 1.

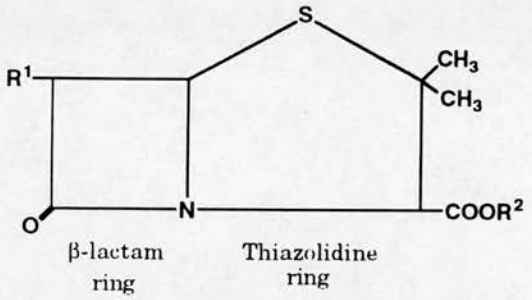
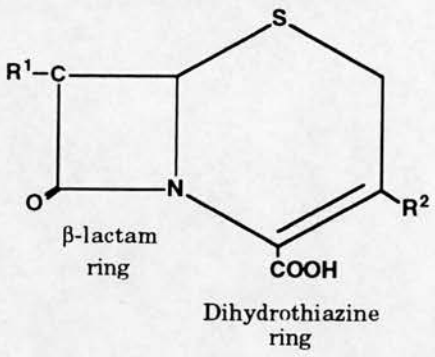


Figure 2.



This molecule consists of the β-lactam ring attached to a five member thiazolidine ring (Greenwood, 1983) and although alone it is virtually devoid of antibacterial action, simple acylation substitutions on the nucleus with different carboxylic acids, chlorides or mixed anhydrides have created compounds with important pharmacological and antibacterial differences (Pickering and Murray, 1984a).

The first semi-synthetic penicillin to be employed in clinical practice, methicillin (Rolinson *et al.*, 1960), was active against the penicillin-resistant staphylococci. Similar derivatives, oxacillin and cloxacillin (Doyle *et al.*, 1961; Knudsen *et al.*, 1962) followed but although they were resistant to staphylococcal penicillinases, they have little antibacterial activity against Gram-negative bacteria (Pickering and

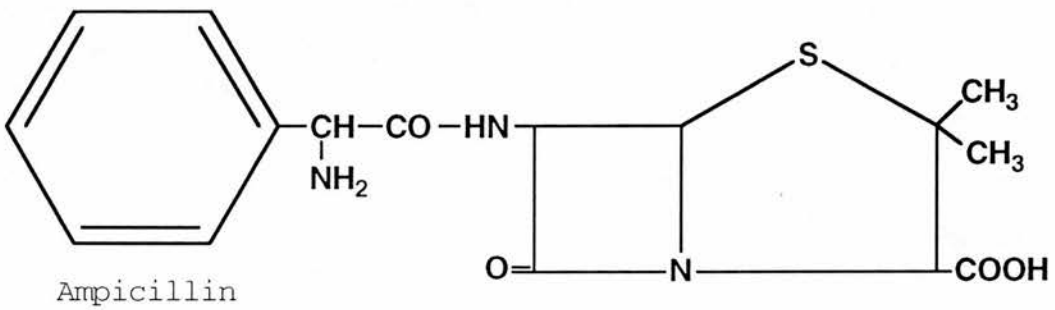
Murray, 1984a). The fact that there is only one site for chemical modification on the penicillin molecule (C6), limits the possibilities for both broad spectrum activity and for penicillinase stability. The development of ampicillin was, therefore, of particular interest on account of its broad spectrum activity against Gram-positive and Gram-negative organisms (Rolinson and Stevens, 1961). Further to this, carbenicillin, ticarcillin and piperacillin were developed specifically with antipseudomonal activity (Selwyn, 1982).

1.2.2. Ampicillin

Ampicillin, 6-D(-)aminophenylacetamidopenicillanic acid (Figure 3), was introduced in 1961 (Rolinson and Stevens, 1961). It is more effective against Gram-negative bacteria such as *E. coli*, *Shigella* spp., *Salmonella* spp., and *Proteus mirabilis* than penicillin G since it is able to penetrate the outer membrane with greater efficiency (Neu, 1979). Ampicillin was created as a result of an insertion of an amino group in the α position of the benzylpenicillin side chain (Rolinson and Stevens, 1961). In 1972, an insertion of a hydroxyl group in the ampicillin molecule at the para position of the benzyl side chain resulted in the formation of amoxycillin (Sutherland *et al.*, 1972). Although ampicillin and amoxycillin share the same spectrum of bacterial activity, (Neu, 1974; Neu, 1979; Neu, 1986) the additional hydroxyl group in amoxycillin alters the ability of the drug to be absorbed from the gastrointestinal

tract; between 40-60% of ampicillin given orally is absorbed compared with 70% of amoxycillin given orally (Pickering and Murray, 1984a).

Figure 3. Structure of ampicillin



Ampicillin

1.2.3. Semi-Synthetic Cephalosporins

Similarly, many semi-synthetic cephalosporins have now been marketed. While cephalosporin C possesses a β-lactam ring it is attached to a six membered dihydrothiazine ring (Figure 2) (Newton and Abraham, 1955). In contrast to the penam nucleus, the 7-aminocephalosporanic acid or 7-ACA nucleus is not a naturally occurring substance; it is only obtained from the side chain of cephalosporin C (Rolinson, 1988). Advantageously, it has several sites for modifications and, as a result, thousands of cephalosporin antibiotics have been produced (Chauvette *et al.*, 1962; Muggleton *et al.*, 1964). Therapeutically important modifications have been made at the sulphur atom

at position 1, the acyl group attached to C7 (R1), C7 itself and the moiety attached to C3 (R2) (Donowitz and Mandell, 1988).

The first derivatives of cephalosporin C included cephalothin (Chauvette *et al.*, 1962) which became available in the United States in 1964. This was followed quickly with the introduction of cephaloridine by Glaxo in the United Kingdom (Muggleton *et al.*, 1964). The success of these early cephalosporins was, however, jeopardized by β-lactamases. Consequently, new cephalosporins, with increased β-lactamase stability and an expanded spectrum of antibacterial activity eg. cefuroxime (O'Callaghan *et al.*, 1976; Brogden *et al.*, 1979), were developed, mainly as a result of modifications to the 7-beta-acyl position. Similarly, in order to combat increasing resistance, ceftizoxime, cefmenoxime and cefodizime were all launched (reviewed by Hoover, 1983). Finally the latest cephalosporins, such as cefotaxime, ceftazidime and ceftriaxone, exhibited a much higher antibacterial activity over an even broader range of bacterial species. The major advantages of this newest group are the β-lactamase stability and more favourable pharmacological properties (Pickering and Murrray, 1984b). The release of ceftazidime, in particular, by Glaxo in 1981 (Muggleton, 1981) was considered a major breakthrough; the antimicrobial was resistant to most of the β-lactamases which had been identified at that time (Simpson *et al.*, 1982) and *Pseudomonas aeruginosa* was included in its range of activity (Klingeren, 1981; Labia *et al.*, 1981).

1.3. Mechanism of Action of β-Lactams

β-lactam antibiotics have a bactericidal mode of action. They bind to and inactivate penicillin binding proteins (PBPs) which lie on the inner surface of the bacterial cell membrane (Spratt, 1975;1980; Tomasz, 1986). The PBPs, which are transpeptidases, carboxypeptidases and endopeptidases, catalyse the cross linking of the cell wall polymer, peptidoglycan. The function of peptidoglycan is to maintain the strength of the bacterial cell wall which it is able to do because it possesses a mesh structure of polysaccharide strands which are cross-linked by peptide bonds (Donowitz and Mandell, 1988; Livermore, 1991). The β-lactams inhibit the formation of these bonds, weakening the wall (Tipper and Strominger, 1965) and are able to inactivate endogenous inhibitors of bacterial autolysins (Höltje and Tomasz, 1975). Consequently, β-lactams can cause bacterial lysis. Different β-lactams have different affinities for the various PBPs; binding with some specific PBPs causes a more detrimental effect than with others. While inactivation of PBPs 4, 5, and 6 by β-lactams is not lethal to bacteria, as these particular PBPs are not essential for bacterial viability, binding with PBPs 1a and 1b will result in cell death (Tomasz, 1986; Donowitz and Mandell, 1988). If low dose or long periods of intermittent penicillin therapy are given, the effect of the antibiotic is quite different; the autolytic system is inhibited with the result that, while cells divide, they cannot separate and so form long filaments (Lorian and Sabath, 1972).

1.4. Mechanisms of Resistance to β-Lactams

It is estimated that the introduction of antibiotics added 10 years to the life expectancy of Americans (Donowitz and Mandell, 1988); however, they are not the panacea they once promised to be. Each drug has the potential to create adverse reactions in the recipient (Murray and Moellering, 1978) and bacteria have responded to the challenge of antibiotics by developing various mechanisms of resistance (Amyes and Gemmell, 1992). Such adaptations have complicated the treatment of infections as resistant bacteria are indeed associated with increased risk of morbidity and mortality (Holmberg *et al.*, 1987; Murray, 1991; Sanders and Sanders, 1992). The problem of resistance has, in some cases, forced the clinician to select the more toxic or the more expensive alternative drug (Jacoby and Archer, 1991). Three mechanisms of resistance have developed to β-lactam antibiotics:

1.4.1. Impermeability

The presence of a cell envelope, consisting of exopolysaccharide, in some bacteria may act as a primary barrier to the penetration of β-lactam antibiotics (Piddock and Wise, 1985). Gram-negative bacteria possess an outer membrane which provides the next obstacle to the entrance of antibiotics. β-lactams

traverse this outer membrane layer through the water filled protein porin channels (Jaffe *et al.*, 1982; Hancock, 1988a). The size, charge and hydrophobicity of the agent will determine the ease of passage through the membrane (Nikaido *et al.*, 1983). However, alterations in the porins, by mutation, can prevent β-lactams from penetrating the outer membrane and ultimately reaching the target PBPs on the inner membrane (Livermore, 1991). Amongst Enterobacteria, a simultaneous consequence of such adaptations is difficulty in nutrient uptake and, on some occasions, difficulty in attaching to mammalian cells as a result of the altered surface (Nikaido, 1989; Livermore, 1991). *Ps. aeruginosa* has a high intrinsic resistance to most commonly used antibiotics owing to their low rate of permeation across the outer membrane; permeation is 12-100 fold lower than permeation of the same compound in *E. coli* (Hancock, 1988b).

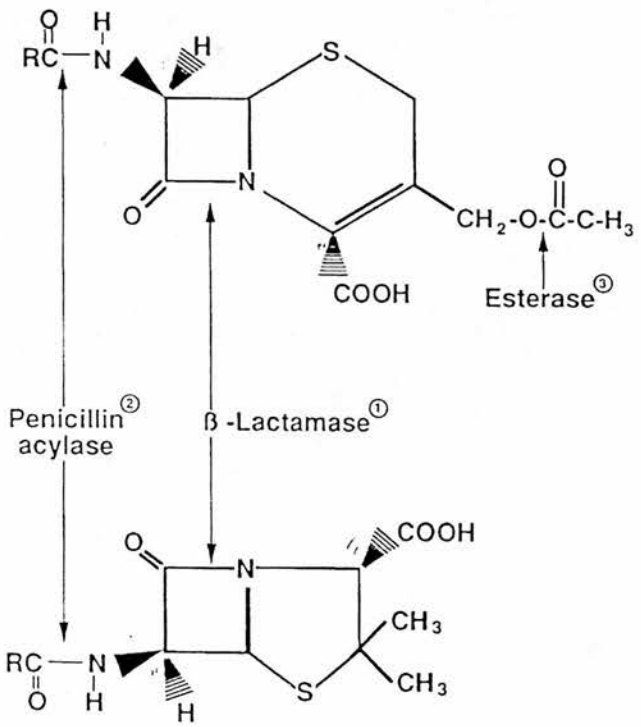
1.4.2. Altered Target

Resistance may arise as a result of mutations which are responsible for the lack of affinity between a β-lactam and its target PBPs (Hedge and Spratt, 1985). Often metabolically important PBPs are selected for modification (Piddock and Wise, 1985). Alternatively, there may be the acquisition of supplementary PBPs which are not associated with sensitive strains and are incompatible for binding the β-lactam drug (Hartman and Tomasz, 1984; Ubukata *et al.*, 1985).

1.4.3. Production of β-Lactamases

The production of β-lactamase is the most important mechanism of resistance to β-lactam drugs (Hamilton-Miller, 1982; Medeiros, 1984; Pratt, 1989). β-lactamases hydrolyse the cyclic amide bond of β-lactam antibiotics (Figure 4) resulting in a product which is antibiotically inactive.

Figure 4. Action of β-lactamases (Sykes, 1982)



Essentially β-lactamases act by firstly, binding to the β-lactam substrate in a noncovalent Michaelis complex. The enzyme/substrate complex may at this stage, dissociate or alternatively commit itself to a hydrolytic reaction. With most

clinically important β-lactamases, the latter procedure involves acylation of the complex in which the carboxyl of the opened β-lactam ring is linked to the hydroxyl group on the serine side chain. This reaction is then terminated with the deacylation of this covalent complex with the release of ring-open product (Livermore, 1993). The products of penicillin hydrolysis are penicilloates which are stable and easily recognised compounds. In contrast, the products of cephalosporin hydrolysis are cephalosporoates which are unstable and differ depending on the nature of the C-3 substitute (Sykes, 1982). The β-lactam antibiotics may also experience the removal of acyl side-chains by amino acid acylases (Figure 4). This enzymatic degradation of β-lactam antibiotics is, however, of little importance in antibiotic resistance. These antibiotics may act as substrates for a number of other hydrolytic enzymes but none of which, particularly in the clinical situation, are as significant as β-lactamases (Sykes, 1982).

The three major factors of rate of penetration of drug into the cell, interaction of drug with β-lactamase in the periplasmic space and the ability of the drug to bind to and inactivate the target PBPs, all determine the susceptibility of Gram-negative bacteria to any given β-lactam antibiotic. Each of the three factors are, none the less, interdependent upon the others (Sanders and Wiedemann, 1988). As the production of β-lactamases is the most important mechanism of resistance (Medeiros *et al.*, 1988; Roy *et al.*, 1989), they will be discussed in more detail.

1.5. β-Lactamases

1.5.1. Origin of β-Lactamases

It had been suggested that β-lactamases arose from penicillin-sensitive D-alanyl-D-alanine peptidases involved in bacterial wall peptidoglycan metabolism (Kelly *et al.*, 1986). This would not be altogether surprising since both groups of proteins interact with β-lactam compounds in much the same way. β-lactamases are unbound proteins which rapidly acylate and deacylate β-lactams. This results in a hydrolysed drug and the regeneration of a free, active enzyme. PBPs meanwhile, are membrane bound proteins that rapidly acylate but only slowly deacylate β-lactams resulting in hydrolysed drug bound covalently to the PBP, which is itself inactivated (Spratt, 1980). Therefore, while the activity of the β-lactamase represents drug resistance, the affinity of the β-lactam for PBPs determines drug susceptibility. The proposal that a PBP mutated to a free unbound form many millennia ago and then mutated to a form that rapidly hydrolysed β-lactams is not so unlikely (Sanders and Wiedemann, 1988).

1.5.2. Classification of β-Lactamases

Classification of β-lactamases was initiated in the early 1960's when penicillin inactivating enzymes were divided into two broad groups, the penicillinases and

the amidases (Ayliffe, 1963). Some years later, the classification scheme was developed and the β-lactamases were classified on the basis of their "substrate profile" ie. the spectrum of hydrolytic activity against a range of β-lactam substrates (Sawai *et al.*, 1968). A further modification was made, still dividing the enzymes into groups according to substrate profile, but in addition parameters such as antisera, enzyme inhibition with p-chloromercuribenzoic acid (pCMB) and cloxacillin, and electrophoretic mobility were included (Jack and Richmond, 1970). As subsequent β-lactamases were discovered, this classification scheme did not provide sufficient discrimination of the enzymes. Consequently, the system was adapted further (Richmond and Sykes, 1973) to produce the first classification to be recognised and extensively used. The β-lactamases were classified by the following criteria: substrate and inhibitor profiles, genetic locus (plasmid or chromosomally mediated) and nature of enzyme expression (constitutive or inducible).

When detailed guidelines for the detection and assay of β-lactamases (Sykes and Matthew, 1976) were applied, a large number of new enzymes were identified. This did, however, result in great confusion as far as the classification schemes were concerned. The limitations of the available scheme included the heterogeneity among enzymes in the same class, the difficulty in classifying an enzyme if the genetic locus implicated had not been localised and finally the difficulty in determining inhibitor profiles under certain conditions (Medeiros,

1984). In 1980, a completely new classification scheme was created (Ambler, 1980) which was based on the molecular characteristics of the proteins or the genes coding for them. Three groups were established, Class A, B and C. Since then two additional groups, Class D and E have been added (Bicknell *et al.*, 1985; Huovinen *et al.*, 1988a). The latest classification scheme is based primarily on biochemical characteristics employing substrate and inhibitor profiles in addition to physical data (Bush, 1988a; 1989a; 1989b; 1989c). The intention of the new system was to allow meaningful comparison between well established β-lactamases and those recently identified.

Changes in medical practice resulting in the appearance of new β-lactamases, the continued discovery and synthesis of β-lactamase substrates and inhibitors and in addition, the development of sophisticated technology such as isoelectric focusing and amino acid analysis, have necessitated the need for the modifications in the classification systems (Sykes, 1982; Bauernfeind, 1986).

A crude distinguishing factor in classification systems is the position of the genetic locus; the genes controlling the production of β-lactamases may be either chromosomally or plasmid encoded:

1.5.3. Chromosomal β-Lactamases

Virtually all Gram-negative bacteria have a chromosomal gene called *amp C*

that encodes a β-lactamase more active in hydrolysing cephalosporins than penicillins (Medeiros, 1984). These chromosomal β-lactamases are species specific but nevertheless, they may resemble one another closely. However, the manner in which they are produced varies considerably and is reflected in their ability to confer resistance (Livermore, 1987). While the production of chromosomal β-lactamase is usually under low level constitutive control in *E. coli* with little contribution to β-lactam resistance, some clinical isolates possessing the ability to make large amounts of chromosomal enzyme, have been identified (Lindberg and Normark, 1986; Korfmann and Wiedemann, 1988). This hyperproduction, which is extremely high but rather unstable, may result from an alteration in the promoter for *amp C* allowing more efficient expression (Jacoby and Archer, 1991).

A variety of nonfastidious Gram-negative bacilli produce chromosomal enzymes which are characteristically inducible (Sanders and Sanders, 1985; 1986). β-lactamase induction may simply be described as a transient elevation in β-lactamase synthesis which occurs when a β-lactam drug is present. The synthesis of the enzyme returns to a low level when the inducer is removed (Livermore, 1987). β-lactamases may be induced by one of two mechanisms (Sanders, 1983; Sanders and Sanders, 1986); either there might be a spontaneous mutation of the wild type organism to a stably derepressed state (Gwynn and Rolinson, 1983; Curtis *et al.,* 1986). Such mutants may be termed

"constitutive", "stably derepressed" or "enzyme hyperproducers" or alternatively, high level β-lactamase formation might be a result of reversible derepression of the β-lactamase in response to a β-lactam inducer (Sanders, 1983). Following derepression, it has been speculated that these enzymes may mediate resistance by a non-hydrolytic mechanism (Sanders, 1984; Livermore, 1985).

1.5.4. Plasmid β-Lactamases

The concept of transferable, extrachromosomal elements that contain antibiotic resistance genes, R-plasmids, was first recognised in Japan (see Watanabe *et al.*, 1963). At that time, the impact of such plasmids was not fully appreciated but it was not long before their potential to facilitate widespread antibiotic resistance was realized (Murray and Moellering, 1978); in *E. coli*, the production of plasmid-determined β-lactamases is the most important cause of resistance to β-lactam antibiotics (Roy *et al.*, 1989). Although the massive use of antibiotics from the 1940's onwards encouraged the selection and dissemination of R-plasmids, it did not create them; a tetracycline-streptomycin R-plasmid in an *E. coli* was found prior to the clinical introduction of these drugs (Smith, 1967). R-plasmids have now been identified in almost all bacteria (Murray and Moellering, 1978).

The first plasmid-mediated resistance reported to the β-lactam agents was transmissible ampicillin resistance identified in a strain of *Salmonella*

typhimurium (Anderson and Datta, 1965). The synthesis of penicillinase by cultures carrying the R-plasmid was shown to be under direct control of those R-plasmids (Datta and Kontomichalou, 1965). Plasmid-mediated β-lactamases are commonly expressed constitutively (Medeiros, 1984) and may be divided into two groups:

1.5.4.1. Broad-Spectrum β-Lactamases

The most prevalent type of plasmid-mediated β-lactamase in Gram-negative bacilli, is the TEM β-lactamase. Two evolutionary variants of this β-lactamase, designated TEM-1 (pI 5.4) and TEM-2 (pI 5.6) have been distinguished by analytical isoelectric focusing (Matthew *et al.*, 1975). They differ from each other by a single amino acid as determined by amino acid and DNA sequencing (Ambler, 1980); there is a substitution of glutamine for lysine at position 39 following Ambler notation (Ambler *et al.*, 1991). TEM β-lactamases have been identified on plasmids with a wide variety of incompatibility properties and from a broad taxonomic range of bacteria isolated from various continents (Hedges *et al.*, 1974; Medeiros, 1984; Simpson *et al.*, 1986; Sousa *et al.*, 1991; Roy *et al.*, 1992). The ubiquity of the TEM structural gene, named TnA for Transposon-ampicillin, results from firstly, the success of the coded enzymes, which may be described as fully efficient (Christensen *et al.*, 1990) and secondly from the ability of the gene to transpose (Hedges and Jacob, 1974; Rubens *et al.*, 1976; Heffron *et al.*, 1977); transposition, thought to be non site specific

(Wiedemann, 1986), may be from plasmid to plasmid, from plasmid to chromosome and then again from chromosome to plasmid. While the transposon TnA includes Tn1, Tn2 and Tn3, the TEM-1 β-lactamase is encoded by the blaTEM-1 gene that is present on Tn2 and Tn3 specifically, whereas TEM-2 is encoded by Tn1 (Sutcliffe, 1978; Chen and Clowes, 1987).

The nucleotide sequence of the ampicillin resistant (TEM) gene in *E. coli* has been determined and a 27000 dalton protein of 286 amino acids identified, although the first 23 amino acids are involved in signalling for secretion and do not appear in the mature enzyme (Ambler and Scott, 1978; Sutcliffe, 1978). Furthermore, the crystalline structure of TEM-1 has recently been elucidated providing an insight into structure-function relationships (Jelsch *et al.*, 1992).

SHV-1 is a broad spectrum β-lactamase also isolated with some regularity. It shares 68% sequence homology with TEM-1 (Sutcliffe, 1978; Barthélémy *et al.*, 1988a). It is thought that SHV-1 β-lactamase gene evolved as a chromosomal gene in *Klebsiella* spp. and was later incorporated into a plasmid (Medeiros, 1984). It is mainly detected in *Klebsiella* spp. (Shannon *et al.*, 1990b; Reig *et al.*, 1993). Other broad-spectrum plasmid-mediated β-lactamases have been found only on isolated occasions and cannot be considered to be major mediators of β-lactam resistance.

While considered to be broad-spectrum β-lactamase because they hydrolyse both penicillins and cephalosporins, none of these β-lactamases are capable of

hydrolysing the extended-spectrum β-lactam drugs such as cefotaxime or ceftazidime. This might be expected when it is considered that extended-spectrum antibiotics were developed in part because of their resistance to the common plasmid-mediated broad-spectrum enzymes such as TEM-1 (Palzkill and Botstein, 1992a).

1.5.4.2. Extended-Spectrum β-Lactamases

By the mid 1980s resistance to the extended-spectrum antibiotics had been reported (Knothe *et al.*, 1983; Kliebe *et al.*, 1985). Transferable resistance to extended-spectrum β-lactams arose from subtle amino acid changes in the common plasmid-mediated Ambler Group A β-lactamases, namely TEM-1, TEM-2 and SHV-1 (Philippon *et al.*, 1989; Payne and Amyes, 1991; Sanders and Sanders, 1992). For example, analysis of the nucleotide sequence of the bla T-3 gene, which mediates the production of the TEM-3 β-lactamase, revealed differences in two positions from that of the TEM-2 β-lactamase; lysine (TEM-3) for glutamic acid (TEM-2) at position 164 and serine (TEM-3) for glycine (TEM-2) at position 238 (Ambler notation) (Sougakoff *et al.*, 1988a). The β-lactamases designated TEM-3 to TEM-7 all share the same phenotype (exhibiting activity against cefotaxime) and DNA-DNA hybridisation has indicated they are all variants of the TEM structural gene (Sougakoff *et al.*, 1988b). The first β-lactamase to appear, conferring resistance to cefotaxime, was recognised as being a modification of SHV-1 (Knothe *et al.*, 1983). This enzyme was named SHV-2 as it

was closely related to SHV-1 (Korfmann *et al.*, 1986). SHV-2 differs from the amino acid sequence of SHV-1 (Barthélémy *et al.*, 1988b) at position 238 (Ambler notation) where glycine is replaced by serine.

The earliest known extended-spectrum β-lactamase was a TEM-1 derived (TEM-E2) enzyme identified from a strain of *Klebsiella oxytoca* in Liverpool in 1982 (Payne *et al.*, 1990). The enzyme was unique in possessing the ability to hydrolyse ceftazidime. Other "ceftazidimases" have been reported (Sirot *et al.*, 1987; Bauernfeind and Horl, 1987). The substitutions usually occur in the active site pocket of the enzyme indicating that the newly introduced changes influence the binding and catalysis of the antibiotics (Palzkill and Botstein, 1992b). Mutagenesis experiments suggest the TEM-1 β-lactamase does, however, exhibit a high level of tolerance to amino acid substitutions; 44% of mutants with random substitutions retained some β-lactamase activity (Palzkill and Botstein, 1992b). It is clear that consequences for the interactions between the β-lactam and β-lactamases are not the same for different substitutions (Collatz *et al.*, 1990). The subsequent expanded spectrum has been attained at a cost of reduced hydrolytic activity against ampicillin (Bush and Singer, 1989).

Since the first emergence of TEM and SHV substituted β-lactamases, there has been a proliferation of extended-spectrum β-lactamases, many of which have been isolated in France (Philippon *et al.*, 1989) where there is a higher usage of the newer generation cephalosporins. However, many of the novel enzymes such as

TEM-9 (Mabilat *et al.*, 1990) have only been isolated in a single hospital at a single time (Bush, 1989a). Epidemic spread throughout the bacterial populations is not common except in a few isolated cases. In Clermont-Ferrand, France there was "an epidemic of antibiotic multiply-resistant strains of *K. pneumoniae* producing a new beta-lactamase" which was designated, CTX-1 (Sirot *et al.*, 1987). Recently, an outbreak of infections caused by an SHV-5 producing strain of *K. pneumoniae* was reported (Bauernfeind *et al.*, 1993). The spread of a single extended-spectrum β-lactamase is dependent on the pathogenicity of the strain carrying the plasmid, the stability of the plasmid concerned, the presence of other influencing resistance markers on the plasmid and finally, the consistency of the selective pressure to stabilise the resistant plasmid (Payne and Amyes, 1991).

The question has been raised as to whether certain plasmids have properties that promote gene evolution (Jacoby and Sutton, 1991). As a single extended-spectrum β-lactamase has not predominated in a similar fashion to TEM-1, a pandemic strain carrying such an enzyme is highly unlikely. Variations in the prevalence of the extended-spectrum β-lactamases must be related to local epidemiological factors, general preventive methods and antibiotic policies (Sirot *et al.*, 1992). A number of surveys have assessed the prevalence of the extended-spectrum β-lactamases in hospitals (Sirot *et al.*, 1988; Jarlier *et al.*, 1988; Sirot *et al.*, 1992). At present they are not a "major threat" to antimicrobial chemotherapy but are more of a "minor inconvenience" (Payne

and Amyes, 1991). The introduction of new oral cephalosporins is hoped to be a viable option (Wise, 1992) particularly in the management of outpatient infections (Cullmann, 1992). It has been stated that these new agents will not enhance the selection pressure for extended-spectrum β-lactamases directly as they are highly stable to the 'classical β-lactamases' (Cullmann, 1992). However, it might be suggested that, on the contrary, an increased usage of these agents in the community will certainly increase the selection pressure.

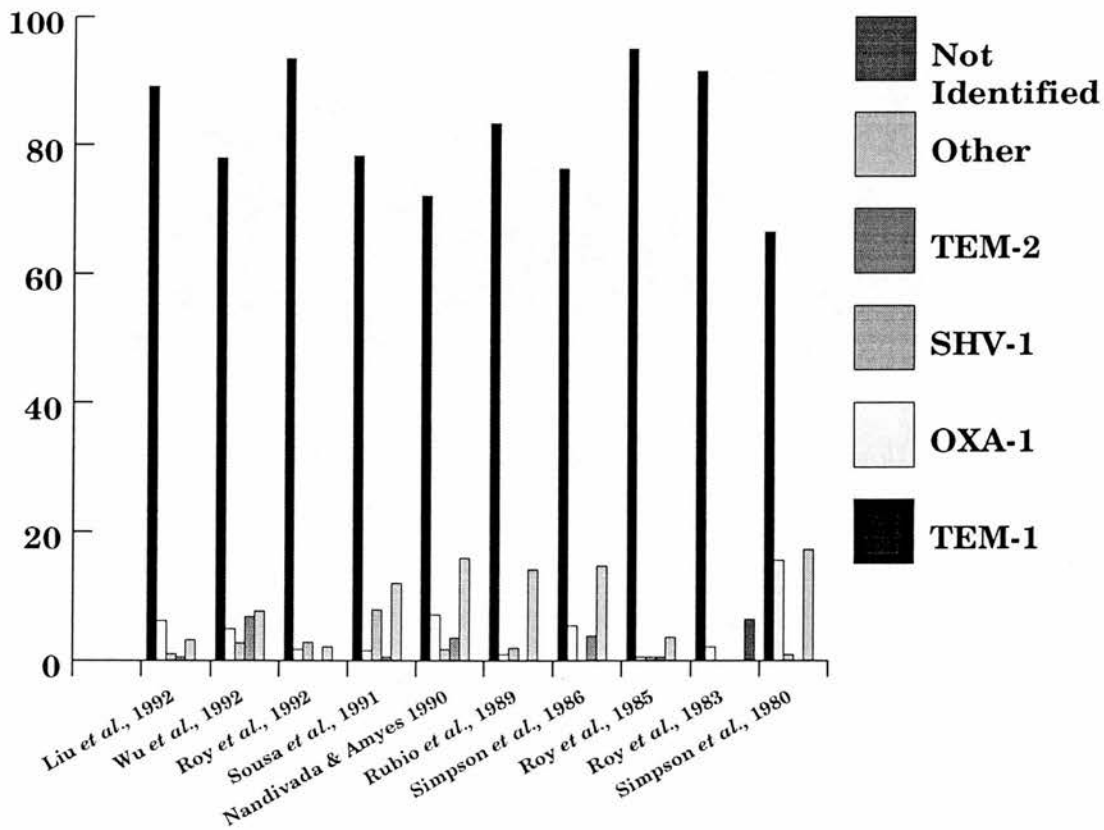
Recently, new enzymes, BIL-1 (Woodford *et al.*, 1990; Payne *et al.*, 1992) and MIR-1 (Papanicolaou *et al.*, 1990) have been identified. These enzymes are resistant to expanded-spectrum cephalosporins caused by plasmid-mediated β-lactamases but are not inhibited by clavulanic acid. These enzymes are Ambler class C chromosomal β-lactamases which have, for the first time, migrated onto plasmids (Payne *et al.*, 1992). Many other extended-spectrum β-lactamases unrelated to either TEM or SHV have been reported (Jacoby and Medeiros, 1991).

1.5.5. Epidemiology of Plasmid-Mediated β-Lactamases

A number of studies have been conducted to identify the β-lactamases contributing to β-lactam resistance in clinical Gram-negative bacterial populations (Simpson *et al.*, 1986; Jouvenot *et al.*, 1987; Reid *et al.*, 1988; Redjeb *et al.*, 1988; Thomson *et al.*, 1992b; Thomson and Amyes, 1993a). As a result of such work, the clinical potential of any novel β-lactam agents can be assessed

(Simpson *et al.*, 1980). Prior to the use of isoelectric focusing (Matthew *et al.*, 1975) β-lactamases were frequently divided into groups according to their susceptibility to various β-lactam drugs (Medeiros *et al.*, 1974). Since the detection method of β-lactamases has been improved (Matthew *et al.*, 1975), the characterisation and prevalence of the different enzymes involved in resistance has been monitored and their epidemiology recorded (Matthew, 1979; Jacoby and Sutton, 1985; Wiedemann *et al.*, 1989). The distribution of plasmid-mediated β-lactamases identified in various surveys is reflected in Figure 4.

Figure 4. Distribution of plasmid-mediated β-lactamases in *E coli*.



Some clinical surveys have examined isolates by DNA hybridisation with a TEM probe revealing TEM-1 to be the most prevalent β-lactamase (Huovinen *et al.*, 1988b; Cooksey *et al.*, 1990; Burman *et al.*, 1992). However, it should be remembered that a complete TEM-1 gene probe is not discriminatory enough to distinguish between TEM and TEM derived genes and therefore oligotyping may be preferable for complete characterisation and molecular epidemiology of β-lactamases (Mabilat and Courvalin, 1990).

The TEM-1 enzyme is not limited to *E. coli*. It has proliferated throughout all Enterobacteriaceae and other species such as *Vibrio* spp. (Hedges *et al.*, 1977) and *Pseudomonas* spp. (Huovinen *et al.*, 1988a). It was found to be the principal mechanism of resistance in *Haemophilus influenzae* strains (Reid *et al.*, 1987) and in *Neisseria gonorrhoeae* (Elwell *et al.*, 1977). The ubiquitous nature of the TEM β-lactamase exemplifies the slow but efficient spread of the resistance gene in the environment (Wiedemann *et al.*, 1989) and reflects its widespread success. The ubiquitous nature is of concern, however, as this enzyme exhibits a remarkable capacity to overcome advances in antibiotic chemotherapy; the TEM-1 β-lactamase is recognised as a progenitor of resistance to third generation cephalosporins (Philippon *et al.*, 1989; Payne and Amyes, 1991).

1.6. Overcoming β-Lactamase Resistance

There have been two main strategies employed to overcome resistance mediated by β-lactamases. Firstly, the development of new β-lactam antibiotics which are not hydrolysed by most β-lactamases as a result of modifications in the structure. This has been discussed previously. As a result of the associated clinical resistance problems, a second strategy became more attractive, namely the use of β-lactamase inhibitors which, when co-administered with a β-lactam, prevent inactivation by β-lactamases (Pratt, 1989; Sutherland, 1990; Thomson and Amyes, 1992b; Moellering, 1993).

1.6.1. β-Lactamase Inhibitors

1.6.1.1. Olivanic Acids

In 1967, Beecham Pharmaceuticals initiated a screening programme of micro-organisms for the presence of β-lactamase inhibitors. The first likely compounds were isolated from a strain of *Streptomyces olivaceus* (Brown *et al.*, 1976) and named olivanic acids. Unfortunately, although certain pathogens such as *K. pneumoniae* and *S. aureus* could be rendered susceptible to ampicillin or amoxycillin in the presence of the olivanic acids, there was little effect against other pathogens because of poor penetration through the bacterial cell wall and their rapid metabolism (Moellering, 1991).

1.6.1.2. Clavulanic Acid

Further screening led to the discovery of clavulanic acid which was produced from a strain of *Streptomyces clavuligerius* (Brown *et al.*, 1976). While clavulanic acid is a progressive potent inhibitor of β-lactamases, it has weak antibacterial activity (Brogden, 1981; Selwyn, 1982). The structure is distinct from penicillins and cephalosporins. It possesses an oxazolidine ring fused to the β-lactam, it lacks an acylamino side-chain and it has an unusual substituent, a β-hydroxyethylidine group at C-2 (Brown, 1981).

Clavulanic acid binds with a β-lactamase resulting in the formation of the acyl enzyme complex (Bush and Sykes, 1986; Bush, 1988b). The effectiveness of clavulanic acid as a β-lactamase inhibitor depends on the β-lactamase involved (Reading and Cole, 1977). Most constitutively produced plasmid- and chromosomally-mediated enzymes are readily inhibited. These enzymes are widespread in Enterobacteriaceae, *P. aeruginosa* and *H. influenzae*. In contrast, inducible β-lactamases are less readily inhibited because the affinity between enzyme and inhibitor is poor (Rolinson, 1991); such enzymes are produced by *Serratia* spp., *Enterobacter* spp. and *Citrobacter* spp. (Brown, 1981). Clavulanic acid itself is a weak inducer and had little activity against these Class 1 (Richmond and Sykes) β-lactamases (Moosdeen *et al.*, 1986).

Clavulanic acid has been marketed in combination with amoxycillin

(Augmentin) in an amoxycillin:clavulanic acid 2:1 ratio since 1981. Doses of clavulanic acid exceeding 200mg are not well tolerated and so the formulation is usually 250mg:125mg amoxycillin:clavulanic acid (Leigh *et al.*, 1981). While also available in a 5:1 ratio, this formulation is currently being reassessed. Clavulanic acid may be associated with mild side effects such as diarrhoea.

A number of *in vivo* studies have been carried out in order to assess the efficacy of this β-lactam/β-lactamase inhibitor combination; early success was reported when 85% of patients made a complete recovery after treatment with the combination against Gram-negative bacteria resistant to penicillin (Martinelli *et al.*, 1981). This success has continued (Cherubin *et al.*, 1991; Cherubin, 1992; Barry *et al.*, 1993). In addition, *in vitro* studies report low levels of resistance (Martinez *et al.*, 1987; Williams *et al.*, 1988; French and Ling, 1988; Thomson *et al.*, 1992a).

Shortly after the introduction of "Augmentin", a formulation of potassium clavulanate with ticarcillin (Timentin) was marketed by Beecham Laboratories and has been evaluated (Labia *et al.*, 1986). While clavulanic acid in combination with amoxycillin is administered orally, in combination with ticarcillin, administration is parenteral. It seems that there is also synergy between clavulanic acid and the newest cephalosporins. This might be promising in the therapy of serious infections caused by bacteria with multi-resistance to β-lactam antibiotics (Redjeb *et al.*, 1988).

The effectiveness of clavulanic acid may relate to the moderate synergism when combined with other β-lactam antibiotics or it may have a better penetration rate than other inhibitors.

1.6.1.3. Sulbactam

Sulbactam (45,899) (English *et al.*, 1978) is a penicillanic sulphone which has been formulated with ampicillin and in some countries with cefoperazone. Like clavulanate, sulbactam inhibits a wide range of β-lactamases but in general it is less potent than clavulanate (Wise *et al.*, 1980; Jacobs *et al.*, 1986). When a clinical study compared their activity in mixed infections of anaerobes and aerobes, amoxycillin performed better in combination with clavulanic acid rather than sulbactam (Gisby and Beale, 1988). The activity of sulbactam combinations against *E. coli* isolates with known amounts of TEM-1 β-lactamase was found to be very weak. The concentration of sulbactam needed to potentiate the β-lactam did depend, however, on the amount of β-lactamase present (Livermore, 1993). In common with clavulanate, sulbactam possesses a weak antibacterial activity alone (Brown, 1981).

1.6.1.4. Tazobactam

Tazobactam (YTR 830) is an analogue of sulbactam with activity comparable with clavulanate (Jacobs *et al.*, 1989). It inhibits a wide range of commonly encountered β-lactamases including plasmid-mediated types and limited Class I

chromosomal enzymes (Aronoff *et al.*, 1984; Gutmann *et al.*, 1986; Moosdeen *et al.*, 1986; Bauernfeind, 1990; Livermore and Seetulsingh, 1991). *In vitro* activity seems very promising for the clinical use of the combination piperacillin and tazobactam (Kuck *et al.*, 1989; Livermore and Seetulsingh, 1991). Indeed a recent survey reported 89% of Enterobacteriaceae that were resistant to piperacillin, were restored to susceptibility by tazobactam (Acar *et al.*, 1993). Furthermore, piperacillin/tazobactam exhibit comparable susceptibility rates to other β-lactam agents with β-lactamase inhibitors against anaerobic bacteria (Appelbaum, 1993). Recent investigations have suggested that inactivation of TEM-1 β-lactamase by tazobactam may be pH dependent (Livermore and Corkill, 1992).

1.6.1.5. BRL 42715

BRL 42715 enhances the activity of the β-lactams in strains that constitutively express Richmond and Sykes Class I β-lactamases and also in strains expressing extended-spectrum plasmid-mediated β-lactamases (Piddock *et al.*, 1993). It is a potent inhibitor of both common chromosomal and plasmid-mediated β-lactamase whatever the level of expression (Coleman *et al.*, 1989). This compound, a substituted penem, is a more potent β-lactamase inhibitor than clavulanate, sulbactam or tazobactam (Rolinson, 1991; Livermore and Seetulsingh, 1991 and Appelbaum *et al.*, 1992). BRL 42715 is, however, unstable (Piddock *et al.*, 1993) and consequently is not being developed further for clinical use (Appelbaum *et al.*, 1992; Zhou *et al.*, 1993).

At present, inhibitors are only available in the combined form in the U.K. If the new extended-spectrum β-lactamase enzymes become a clinical problem, the case may arise for their use as individual agents, leaving the final choice of the β-lactam to the clinician (Thomson and Amyes, 1992b). Analysis of therapy with β-lactamase inhibitors has indicated that for clavulanic acid, single dose therapy with a lower cost, better patient compliance, diminished pressure on intestinal flora and fewer side effects is preferable (Raz *et al.*, 1991). The inhibitors seem to display an acceptable safety profile as determined in clinical trials (Martinelli *et al.*, 1981; Kuye *et al.*, 1993).

1.6.2. Mechanism of Action

β-lactamase inhibitors may act in either a competitive or non-competitive manner. If the former, the inhibitors may function in a reversible manner where there is a non-chemical interaction between the substrate and inhibitor. Consequently, if the inhibitor is removed, the enzyme may act on the substrate. Alternatively, the inhibitor may function in an irreversible manner whereby the enzyme is inactivated; the enzyme is incapable of catalysing the conversion of substrate to product even after removal of excess inhibitor (Bush and Sykes, 1983). In this situation, the inhibitors inevitably destroy themselves and so the term "suicide inhibitor" has been adopted (Wise, 1982). True suicide inhibition requires a branched reaction pathway whereby the covalent ester reacts either

to yield hydrolysed inhibitor and reactivated enzyme or to give a permanently inactivated product in which the inhibitor, or a fragment of it, remains permanently attached to the β-lactamase (Livermore, 1993). In contrast, in non-competitive inhibition, the enzyme will be bound at a point removed from the catalytic site and so distort the enzyme rendering it non-functional (Wise, 1982).

1.6.3. Resistance to β-Lactamase Inhibitors

Decreased susceptibility to β-lactam/β-lactamase inhibitor combinations such as amoxycillin/clavulanate and ticarcillin/clavulanate have been reported (Martinez *et al.*, 1987; French and Ling, 1988). This may be attributed to either an increased frequency in hyperproduction of TEM-1 β-lactamase (Martinez *et al.*, 1987; Page *et al.*, 1989; Shannon *et al.*, 1990a) or, alternatively, as a result of an enzyme mediating such resistance (Thomson and Amyes, 1992a; Vedel *et al.*, 1992). Examining each:

Irrespective of the β-lactamase and inhibitor type, it is easier to protect a β-lactam antibiotic against the activity of a smaller rather than a larger amount of β-lactamase (Livermore, 1993). This fact is highlighted for *E. coli* strains that produce TEM-1. The TEM-1 β-lactamase is expressed constitutively but the amount varies 100-fold amongst different strains. Amoxycillin/clavulanate resistant *E. coli* were found to produce 3 times as much TEM-1 as the standard sensitive strain (Williams *et al.*, 1988). Clear correlations have been established

between β-lactamase quantity and levels of resistance to the β-lactam inhibitor combinations (Sanders *et al.*, 1988; Thomson *et al.*, 1990; Seetulsingh *et al.*, 1991). The exact mechanism involved in the hyperproduction of the TEM-1 β-lactamase is unclear but several possibilities have been proposed. These include plasmid copy number; plasmid rearrangement may produce small multicopy plasmids and consequently gene duplication (Martinez *et al.*, 1987; Martinez *et al.*, 1989; Shannon *et al.*, 1990a; Nandivada *et al.*, 1990; Reguera *et al.*, 1991). For example, amoxycillin/clavulanate resistant *E. coli* isolates from Madrid were found to produce between 4 and 50 fold more TEM-1 than did a control strain with a single copy of the TEM-1 gene (Martinez *et al.*, 1987). The degree of gene amplification within plasmids and also the promoter efficiency may also be of some importance (Page *et al.*, 1989; Seetulsingh *et al.*, 1991). It is not known whether strains displaying this mechanism of resistance arose as a result of use of amoxycillin plus clavulanic acid or already existed in the bacterial population before the introduction of inhibitor combinations (Page *et al.*, 1989; Thomson and Amyes, 1992b).

Increasing incidences of hyperproducing TEM-1 strains may be restricted to local outbreaks caused by a ubiquitous strain or plasmid, since there has been no increase in the frequency of such strains in clinical isolates (Seetulsingh *et al.*, 1991; Thomson and Amyes, 1993a). In addition, amoxycillin/clavulanate resistant nosocomial *E. coli* which were analysed for hyperproduction may have

comprised a single strain (Williams *et al.*, 1988). Hyperproduction may be a great energy drain on the cell and therefore tend to be an inefficient mechanism of resistance.

As mentioned previously, lack of susceptibility of certain isolates eg. *Serratia marcescens*, to a β-lactam plus a β-lactamase inhibitor may result from lack of penetration of the antibiotic or the inhibitor into the cell. On the other hand, the strains may produce an inducible cephalosporinase against which the combination is relatively inactive (Bush *et al.*, 1991). Occasional antagonism of the β-lactam activity has been attributed to the induction of chromosomal cephalosporinases (Livermore *et al.*, 1989). These features of induction are further complicated if strains produce a second plasmid-mediated β-lactamase. The success of therapy is uncertain when multiple β-lactam antibiotics are used in such situations (Bush *et al.*, 1991).

It has been suggested that mutation to clavulanic acid resistance in the TEM-1 β-lactamase would deprive the enzyme of its catalytic ability to hydrolyse β-lactam antibiotics (Thomson and Amyes, 1992b). However, such enzymes have been selected *in vitro* (Manavathu *et al.*, 1990; Bonomo *et al.*, 1992). The emergence of a clavulanic acid resistant TEM β-lactamase was first reported in a clinical strain in Scotland (Thomson and Amyes, 1992a). The plasmid encoded enzyme was named TRC-1: TEM resistant to clavulanate. The pI value of TRC-1 is lower than for TEM-1. While TRC-1 has a raised resistance to inhibition by

clavulanic acid, as assessed by ID_{50} values, it is associated with only a moderate increase in MIC to amoxycillin plus clavulanic acid. Interestingly, mutation of TEM-1 to TRC-1 also appears to be accompanied by a decrease in resistance to amoxycillin (Thomson and Amyes, 1993a). In addition a plasmid-mediated enzyme, TRI (TEM resistant to β-lactamase inhibitors), has been isolated from clinical strains in France and found to possess features comparable to TRC-1 (Vedel *et al.*, 1992). The identification of a mutant derived from OHIO-1 (Bonomo *et al.*, 1992) with increased inhibitor resistance, reveals that mutations can occur amongst the SHV family of enzymes and are not restricted to the TEM enzymes. Analysis of mutants resistant to inhibitors indicate that this resistance is a result of an alteration in the amino acids at the active site; the inhibitor is not able to acylate the β-lactamase irreversibly. The enzyme is inactivated less efficiently, either as a result of decreased binding of the inhibitor or, alternatively, the inhibitor is hydrolysed in a reaction yielding product and free enzyme (Bonomo *et al.*, 1992). It seems that the amino acids methionine, at position 69, and arginine, at position 244, are crucial in the inhibition pathway (Oliphant and Struhl, 1989; Belaaouaj *et al.*, 1991; Delaire *et al.*, 1992). It is unclear if the emergence of these new enzymes will have a significant impact in the clinical setting. At present they still appear to be rare.

1.7. The Impact of Antibiotic Therapy

1.7.1. Acquisition of Resistant Determinants

The therapeutic use of antibiotics undoubtedly favours the selection of resistant bacteria (Hamilton-Miller, 1990; Sanders and Sanders, 1992). It is evident, however, that resistant determinants evolved before the advent of the antibiotic era. A commensal flora study of people living in an "antibiotic-virgin" community in North Borneo indicated this, as from four subjects six strains of *E. coli* were found to possess transferable drug resistance (Davis and Anandan, 1970). In addition, a study of remote rural Xhosa communities in South Africa, with minimal exposure to antibiotics, found that 19% of the coliform bacteria examined, carried antibiotic resistant determinants (Burt and Woods, 1976).

1.7.2. Reservoirs of Antibiotic Resistant Determinants

E. coli is a ubiquitous micro-organism which is found in large numbers in the gastrointestinal tract of every individual where it forms a part of the normal gut flora (Turck *et al.*, 1969). The potential exists for this *E. coli* to play a pathogenic role in urinary tract infections (Turck *et al.*, 1969; Bettelheim *et al.*, 1971; MacGowan *et al.*, 1993) and other infections (Tullus *et al.*, 1988). Indeed *E. coli* has remained the most frequently isolated nosocomial pathogen in U.S. hospitals, accounting for

approximately 18.6% of hospital acquired infections (Cooksey *et al.*, 1990).

The normal human gastrointestinal microflora is a remarkably stable ecosystem (Nord *et al.*, 1993). However, changes in the microfora may occur if there are physiological or ecological disturbances in the digestive tract. The most common cause of disturbance is the administration of antimicrobial agents since the antimicrobials might be incompletely absorbed if orally administered (Nord, 1990). In addition, they might be secreted in the saliva or bile or from the intestinal mucosa (Nord and Heimdahl, 1986). The influence of an antimicrobial agent on the normal microflora is mostly not beneficial to the patient as the indigenous micro-organisms are suppressed, thereby, often permitting potential pathogens to overgrow (Nord and Heimdahl, 1986; Nord *et al.*, 1993). Furthermore, the emergence of antimicrobial resistant strains is promoted either through mutation or transfer of resistant factors between the bacteria (Nord *et al.*, 1993). It seems that whilst R-plasmid transfer may occur amongst the faecal flora of subjects not taking antibiotics, transfer is much more likely when antibiotics are being administered (Anderson *et al.*, 1973a, 1973b; Hawkey, 1986). While it was thought coliform flora of the gut return to a predominantly sensitive state within 10 days or so of the end of treatment, it now appears that, when treatment is terminated, any antibiotic resistant strains may either rapidly disappear or alternatively, they may be maintained for months (Hartley and Richmond, 1975). Persistence of antibiotic resistant

microflora may be the result of an indiscriminate use of antibiotics in the community. It has been suggested that the nature of the strain, rather than the plasmid being carried, is of more influence in determining its survival. For example, whether or not the strains possess the ability to stick to the intestinal wall (Hartley and Richmond, 1975). However, it has become apparent that plasmid carriage may impair bacterial function and, therefore, survival (Anderson, 1974). The survival capacity of *E. coli* carrying R-plasmids is uncertain. Both increased and decreased survival rates have been shown (Kelch and Lee, 1978; Hartley and Richmond, 1975). The gastrointestinal tract is not alone in containing bacteria that harbour antibiotic resistant determinants. They have been detected in organisms from a number of skin sites in hospitalised patients (Larson *et al.*, 1986).

The acquisition of resistant bacteria may be achieved through the ingestion of contaminated water (Young, 1993). The frequency of antibiotic resistant determinants has been assessed in both non-polluted (Jones, 1986) and polluted water (Al-Jebour and Al-Meshadani, 1985; Kaspar and Burgess, 1990). The ingestion of contaminated food is another method of acquiring resistant determinants (Levy, 1986). The flow of resistant genes from both animal to animal and from animal to man has been extensively discussed (Smith, 1968; Shooter *et al.*, 1970; Anderson *et al.*, 1975; Levy *et al.*, 1976; Linton, 1977; Rowe and Threlfall, 1984; Linton, 1986; Corpet, 1987).

A number of specific reservoirs have been associated with the emergence and steady maintenance of antibiotic resistant bacteria and have been studied in some detail:

1.7.3. Clinical Practice

An enormous number of studies have been undertaken to assess the level of antibiotic resistance in clinical practice (Ma *et al.*, 1983; Mayer, 1986; Kresken and Wiedemann, 1986; O'Brien *et al.*, 1986). The carriage of resistance determinants differs considerably, firstly amongst different bacterial species, secondly with the type of antibiotics in use and thirdly, and most noticeably, it differs in different countries.

1.7.3.1. Developing Countries

It is well established that the level of antibiotic resistance in the developing countries is substantially higher than in developed countries (Farrar, 1985). Ampicillin, in particular, will be considered in the following discussions of surveys as firstly, it is the most widely used antibiotic in these countries and indeed the world (Selwyn, 1982) on account of its relatively low cost following the expiry of its patent (Young *et al.*, 1989). Secondly, ampicillin resistance among clinical isolates of *E. coli* is a useful indicator of resistance arising from antibiotic selection of resistant strains among naturally susceptible species (Sanders and Sanders, 1992).

In South India in 1984, 82% of Enterobacteriaceae causing bacteriuria were resistant to ampicillin (Young *et al.*, 1989; Nandivada and Amyes, 1990). In a study conducted in Northern India between 1984-86, 90% of the isolates collected from man which were responsible for urinary tract infection were resistant to one or more antimicrobial agents; 43.5% of isolates recovered from both man and animals were resistant to ampicillin (Singh(b) *et al.*, 1992). Amongst children presenting with diarrhoea in the Sudan, 91% of the *E. coli* isolated were resistant to ampicillin (Shears *et al.*, 1987). Examination of enteropathogenic *E. coli* strains from a nosocomial outbreak in Kenya revealed ampicillin resistance to be 100% (Senerwa *et al.*, 1991). Similarly, a high incidence of ampicillin resistance (70.9%) was also found in northern Tanzania (Gillespie *et al.*, 1992). A survey of eight African countries reported 69% of the *E. coli* were ampicillin resistant (Dowse and Prigent, 1991). Ampicillin resistance was, however, reported to be surprisingly low in Zaire (38%), Peru (37%) and Belize (0%) but high in Sudan (90%) (Shears *et al.*, 1988a). As 20 individuals or less were included in each of these studies, the resistance levels cannot be considered to be truly representative of the whole community. High levels of antibiotic resistance in developing countries is promoted by "over the counter" buying of drugs, indiscriminate use of antimicrobial agents and poor sanitary conditions which facilitates the spread of resistant determinants (Kunin *et al.*, 1987; Shears *et al.*, 1988a).

1.7.3.2. Developed Countries

In comparison, in the United Kingdom, the prevalence of ampicillin resistance, specifically, was much lower; it was found to be 18.8% in the Charing Cross hospital in London and 24.1% in Nottingham (Simpson *et al.*, 1980). A variation in drug resistance in various cities was revealed in a large study of Gram-negative isolates, mostly from urinary tract infections. In Glasgow, Dundee and Edinburgh, 71.9%, 45.2% and 48.5% of the isolates respectively, were resistant to ampicillin (Reid *et al.*, 1988). Two large surveys have been carried out in order to assess the changes in the antibiotic sensitivities of urinary pathogens, firstly between 1971-82 and secondly between 1971-89. During this period the resistance of *E. coli* to ampicillin in general practice increased from 8.6% to 24.4% to 34.2% in 1971, 1981 and 1989 respectively. A similar pattern was observed in the hospital where *E. coli* resistance to ampicillin increased from 15.6% to 35.7% to 43.1% in the same years respectively (Grüneberg, 1984; Grüneberg, 1990). A recent comprehensive study of general antibiotic resistance of clinical urinary isolates reported ampicillin resistance to have in fact fallen from 48.5% to 33% in Edinburgh and from 71.9% to 40.2% in Glasgow (Thomson *et al.*, 1992a). In contrast, a survey of *E. coli* isolated from blood from 1969-91 indicated ampicillin resistance to have increased from 22% to 40% (Shannon *et al.*, 1992).

In the United States, a steady state of ampicillin resistance has been maintained. During a 12 year survey, *E. coli* isolated from patients showed no

significant change in resistance to ampicillin (25.8% mean) (Atkinson and Lorian, 1984). A survey of Gram-negative bacteremia isolates examined a few years later showed that ampicillin resistance (29.5% in community acquired organisms and 37.3% in nosocomially acquired organisms) has only slightly increased (McGowan *et al.*, 1989).

A number of other studies have been carried out in other parts of the world. In a university hospital and a general practice survey in Denmark the resistance levels found in *E. coli* during a seven year period fluctuated only between 16-19% suggesting little change in the selective pressure (Møller, 1989). In a European collaborative study, resistance in Gram-negative bacilli isolated from blood indicated ampicillin resistance in *E. coli* to be slightly lower in northern Europe (28%) than in south Europe (46%) (Dornbusch *et al.*, 1990). In an extensive survey of Gram-negative bacteria isolated in Dutch hospitals, 42% of *E. coli* were ampicillin resistant (Buirma *et al.*, 1991). A higher level of resistance has been observed to the aminopenicillins (55.3%) in pathogenic strains of *E. coli* from Portugal (Sousa *et al.*, 1991) while a decreasing susceptibility to ampicillin of urinary isolates has been found in Israel; resistance ranging from 49% in 1981 to 72% in 1985 (Alon *et al.*, 1987).

1.7.4. Farming Practice

The acquisition of antibiotic resistance may be associated with transfer of the

determinants from animals to man (Levy, 1986). Therefore the presence of a reservoir of antibiotic resistant determinants in animal practice is not surprising (Levy *et al.*, 1987; Moellering, 1990). The development of antibiotic resistance factors in animals has been encouraged through the use of antibiotics in animal feeds (Smith, 1968). Such practice creates a selective pressure which, although lower than that associated with drugs used in the treatment of clinical disease, is of a longer duration. This exercise was mainly aimed at reducing animal disease and also promoting animal growth. If, however, the Swann report, produced in 1969, intended to reduce antibiotic resistance in *E. coli* in the human populace by restricting the use of antibiotics in animal feed, it seems to have failed (Richmond and Linton, 1980). Intensive farming practices particularly allow the proliferation of resistant determinants.

1.7.5. Travel to Developing Countries

A number of studies have now been conducted which indicate that travel itself (Levy, 1982) is associated with the acquisition of resistant *E. coli.* One such study revealed the emergence of high level resistance to trimethoprim-sulphamethoxazole in strains of *E. coli* isolated from 95% of students from the United States while either taking trimethoprim alone, or in combination with sulphamethoxazole, for prophylaxis of travellers' diarrhoea while in Mexico (Murray *et al.*, 1982). This survey was of particular interest as approximately

one third of travellers from the United states to the developing countries will contract travellers' diarrhoea (Wanger *et al.*, 1988). A second such study observed the increase in resistance to mecillinam from 5% of pre-travel *E. coli* to 42.9% of post-travel *E. coli* (Stenderup *et al.*, 1983). The emergence of resistant faecal *E. coli* in travellers not taking prophylactic antimicrobial treatment has also been shown. After three weeks in Mexico, trimethoprim resistant *E. coli* in the faecal flora of the individuals screened had risen from none to 57% (Murray *et al.*, 1990).

1.7.6. Day Care Centres

An increasingly recognized reservoir of resistant determinants is that of day care centres (Reves *et al.*, 1987; Reves *et al.*, 1990). A number of factors contribute to promote the spread of resistant determinants in this reservoir including ill children attending the centre (Goodman *et al.*, 1984), the lower standard of hygiene associated with children, the close contact between children and the wide use of antibiotics amongst that age group (Singh *et al.*, 1990). In addition, the prophylactic use of antimicrobial agents in the community may encourage the establishment of such reservoirs (Murray *et al.*, 1982; Moellering, 1990). Although children are not representative of the community at large, they may represent one avenue through which resistance determinants may spread into the community.

1.7.7. Epidemiology of Resistant Plasmids

Many studies have analysed the resistant strains isolated from the various reservoirs and examined them for the presence of plasmids. Characterization of the plasmids has established if common or epidemic plasmids are present in given communities. In particular strains from the developing world have been assessed (Crosa *et al.*, 1977; Murray and Rensimer, 1983; Rudy and Murray, 1984; Levy *et al.*, 1985; Shears *et al.*, 1988a; 1988b; Shears *et al.*, 1989). In addition, much work had been carried out on the strains isolated from day care centres (Gordillo *et al.*, 1992; Singh(a) *et al.*, 1992; Fornasini *et al.*, 1992).

1.7.8. Commensal Flora Surveys

Non-pathogenic commensal flora of the healthy individual may act as a reservoir of antibiotic resistant determinants (Gross *et al.*, 1982; Hawkey, 1986; Shears *et al.*, 1988a). This is of considerable concern as firstly, enteric organisms may cause either an endogenous infection, particularly in the urinary tract (Brumfitt *et al.*, 1971; Anderson *et al.*, 1973a; Søgaard, 1975), or secondly, the resistance determinants may transfer to any invading pathogens (Thomson *et al.*, 1993). Naturally the treatment of consequent infections will, therefore, be complicated.

To date there has been a dearth of information in this particular field. Although a

number of surveys have been conducted in order to establish the prevalence of resistant strains in the gastrointestinal tract, few have taken advantage of recent advances in molecular techniques to characterize the mechanisms of resistance involved. The first survey of this kind was reported in 1966 (Smith and Halls, 1966). While 62% of the individuals studied carried resistant *E. coli*, 17% of which were resistant to ampicillin, unfortunately only 24 people were included in the survey which, while providing some indication of resistance amongst commensals, could not be referred to as a true representation of the community at large.

A number of surveys have sought to assess the carriage of antibiotic resistance in the human gut by examining faecal specimens on admission to hospital. In a study examining 100 patients on admission to hospital, *E. coli* resistant strains were found in 52% of the patients, with 17% being resistant to ampicillin (Datta, 1969). In Edinburgh, 36% of the volunteers, who were submitted for chemotherapy treatment carried resistant coliforms in their faecal flora (Shaw *et al.*, 1973) while in Denmark, amongst 95 patients admitted to a male urological ward, 67% contained resistant *E. coli* strains (Søgaard, 1975). Each of these three studies assessed people without bacterial infection but whose health was compromised in some other way; the connection with the hospital naturally biases this group particularly as these participants may have received other drug treatments which may have affected the normal gut flora.

The first comprehensive survey to examine truly healthy volunteers was

conducted on 100 Irish infants. Resistant bacteria were isolated in 81% of the cases with ampicillin resistant strains in 62% of them (Moorhouse, 1969). A survey conducted in Sweden (Lidin-Janson *et al.*, 1977) identified 102 out of 709 *E. coli* strains from children which were resistant to one or more antibacterial agent. However, as these surveys only examined children, the results may not be considered to be indicative of the whole community, especially as the spread of resistant determinants through the faecal/oral route is higher in this group. This point is clarified by a survey which examined intestinal coliform flora of both healthy children and adults in an urban and rural community. A much higher proportion of resistant strains was found in the former population (67%) rather than the latter (46%) (Linton *et al.*, 1972).

A further two studies have examined only children. Firstly, a study which although interestingly spanned three continents, in addition to examining just children, only a few numbers were included in the study so the results can not be a true reflection of the total population in each area (Lester *et al.*, 1990). Secondly, assessment of faecal flora of children in day care centres was carried out relatively recently (Reves *et al.*, 1990). Although this study did not take the whole community into consideration, it did however identify a new reservoir of resistant determinants in its own right.

Two commensal flora studies have examined student populations. As students only represent a small section of the community, it may be that their faecal flora

is not representative of the total community. However, in the Netherlands, 76% of the students contained flora resistant to ampicillin (Bonten *et al.*, 1990) while in Nigeria, where only trimethoprim resistance was assessed, 38.1% of the strains were resistant to this antimicrobial (Lamikanra *et al.*, 1989).

Another select population group examined was that of healthy staff at hospitals in Germany, Amman, Mexico and Columbia. While the carriage rate of ampicillin resistance was not reported, the β-lactamases contributing to ampicillin resistance were investigated (Simpson *et al.*, 1986).

To date few surveys have overcome the problems of population selection. The first study to examine a full cross section of the healthy community was carried out amongst different age-groups in Dutch urban communities (Degener *et al.*, 1983). Of the specimens found to contain *E. coli*, 26% were resistant to ampicillin. More recently, a study in Boston of both patients and ambulatory volunteers found 34.9% of the latter population contained ampicillin resistant flora (Levy *et al.*, 1988). In a survey, carried out in South India, the highest incidence of antibiotic resistance in any bacterial population was identified. Nearly 100% of the samples contained strains resistant to both ampicillin and trimethoprim (Amyes *et al.*, 1992). A further study examined two different areas in an industrialised country. High prevalence of resistance was found in both populations, ranging from 28% for trimethoprim to 89% for ampicillin (Bonten *et al.*, 1992). The most recent survey to be conducted was based in Jeddah; 40.4%

of the faecal bacteria from healthy urban and rural dwellers were resistant to ampicillin (Ismaeel, 1993). It is, however, difficult to draw comparisons between these surveys on account of differences in initial screening procedures and also in the concentration of antibiotic employed to determine resistance.

Each of the studies discussed is associated with one or more of the following complicating factors:

1) Biased study group - A number of the surveys examined selected groups only, such as children (Moorhouse, 1969; Reves *et al.*, 1990; Lester *et al.*, 1990), students (Lamikanra *et al.*, 1989; Bonten *et al.*, 1990), or patients on admission to hospital (Datta, 1969; Shaw *et al.*, 1973; Søgaard, 1975). The implications of which have been discussed previously.

2) Small sample size - Insufficient sample numbers subtract from the validity of some studies (Smith and Halls, 1966; Lester *et al.*, 1990).

3) Lack of analysis - None of the conducted surveys have addressed themselves to the responsible mechanism mediating resistance to ampicillin.

4) Lack of comparison - Only one research group (Lester *et al.*, 1990) examined the carriage of resistance in different parts of the world. This is necessary for a true comparison.

As the healthy members of the community represent the largest reservoir of

bacteria resistant to antimicrobial agents (Lester *et al.*, 1990) accurate information regarding the resistance levels within it will help in the choice of antimicrobial regimens. Therefore, in order to establish the carriage of resistance determinants in this particular reservoir, it is necessary for surveys to be conducted by one team employing the same experimental procedure, in both developing and developed countries. Furthermore, the biases associated with the previous studies should be eliminated.

Aims

1) To establish the carriage of antibiotic resistance determinants in the aerobic commensal flora of selected healthy populations in South Africa and in Edinburgh.

2) To investigate the biochemical and molecular basis for the beta-lactam resistance in the bacterial reservoirs studied.

Chapter 2

Materials and Methods

2.1. Bacterial Strains

The bacterial strains used were as follows:

Escherichia coli K12 J62-2 - pro^- his^- trp^- lac^- rif^R (Bachmann, 1972).

Pseudomonas aeruginosa - NCTC 10662.

Staphylococcus aureus - NCTC 6571.

Escherichia coli - NCTC 10418.

Standard β-lactamase producing strains:

Escherichia coli K12 J53-2 containing plasmid R1 (TEM-1) (Hedges *et al.*, 1974).

Escherichia coli K12 J53-2 containing plasmid RP4 (TEM-2) (Hedges *et al.*, 1974).

Escherichia coli K12 J53-2 containing plasmid R1010 (SHV-1) (Petrocheilou *et al.*, 1977).

Escherichia coli K12 J53-2 containing plasmid R455 (OXA-1) (Dale and Smith, 1974).

All the stains were maintained at -70°C in nutrient broth with 10% glycerol v/v.

2.2. Media

2.2.1. Complex Media

The complex media used were Nutrient Broth No.2 (CM67), Columbia Base Agar (CM331), Diagnostic Sensitivity Test Agar (CM261), Isosensitest Broth (CM473), Isosensitest Agar (CM471), Agar Bacteriological No.1 (L11), and MacConkey agar (CM7b) all from Oxoid, Basingstoke, Hants. Mueller Hinton Broth was obtained from Difco, Michigan.

2.2.2. Minimal Media

Double strength minimal salts medium (DM) was prepared as described by Davis and Mingioli (1950) (Table 1). Single strength minimal medium (DM) was prepared by diluting double strength DM with an equal volume of distilled water prior to autoclaving. Diluents were prepared by distributing single strength DM into 9.9ml and 4.5ml aliquots and then autoclaving at 15psi for 15 minutes. Supplement solutions for incorporation in the minimal medium are given in Table 2.

Table 1. Preparation of double strength Davis Mingioli basal medium (DM)

K_2HPO_4	14.0g
KH_2PO_4	6.0g
Tri-Sodium citrate.$2H_2O$	0.9g
$MgSO_4.7H_2O$	0.2g
$(NH4)_2SO_4$	2.0g

These ingredients were all dissolved in a litre of distilled water in the order given and then 50ml quantities were distributed and autoclaved at 15psi for 15 minutes.

Table 2. Supplement solutions

Solution	Source	Strength Prepared	Final Concentration	Mode of Sterilisation
D-glucose	Sigma	200mg/ml	2.8mg/ml	Autoclaving
L-proline	BDH	5mg/ml	50µg/ml	Steaming
L-histidine	BDH	5mg/ml	50µg/ml	Steaming
L-tryptophan	BDH	2mg/ml	50µg/ml	Steaming

Autoclaving was at 15psi for 15 minutes. Steaming was for 30 minutes.

2.2.3. Preparation of Plates

2.2.3.1. Complex Media

Complex media were made up according to the manufacturer's instructions and were autoclaved at 15psi for 15 minutes. With trimethoprim selection plates (Amyes and Gould, 1984), the compounds listed in Table 3 were added to the Agar No. 1 prior to autoclaving. The plates, each containing approximately 15mls of agar, were poured while the agar was still molten. With antimicrobial drug sensitivity plates, the agar was allowed to cool to 50°C before the appropriate antimicrobial drugs were added and the plates poured. After setting, all the plates were dried, inverted at 55°C for 20 minutes.

Table 3. Preparation of Trimethoprim Selection Plates

Mueller Hinton Broth	21.0g
Lactose	10.0g
Bile salts	5.0g
Neutral red indicator	0.075g
Agar No.1	12.0g

All the ingredients were added to 1L distilled water prior to autoclaving at 15psi for 15 minutes.

2.2.3.2. Minimal Media

Minimal DM media plates were prepared as follows. The required amino acid supplements (to give a final concentration of 50μg/ml), 1.4ml of 20% D-glucose and the appropriate antimicrobial drugs were added, aseptically, to 50ml of double strength DM media. Sterile distilled water was added to bring the total volume up to 60mls. This mixture was added to 40mls of molten bacteriological agar No.1 and mixed gently before pouring.

2.3. Materials

2.3.1. Chemotherapeutic Drugs

The chemotherapeutic drugs used are listed in Table 4. Allowance was made for the potency of those drugs combined with an inert base. All were supplied sterile and prepared as indicated. Compounds were made up freshly as required. In contrast, the non chemotherapeutic drug, nitrocephin (Glaxo), was stored, in solution, in the dark at -20°C until required.

2.3.2. Buffers

Sodium phosphate buffers were prepared as described in Data for Biochemical Research (Oxford University Press, 1974). The buffer used routinely for β-lactamase

Table 4. Chemotherapeutic drugs

Chemotherapeutic drug	Method used to dissolve	Supplier
Ampicillin (Sodium salt)	H_2O	Sigma/SKB
Amoxycillin (Sodium salt)	sodium hydrogen carbonate	SKB
Clavulanic acid (Lithium salt)	H_2O	SKB
Cephaloridine	H_2O	Glaxo
Cefuroxime (Sodium salt)	H_2O	Glaxo
Ceftazidime (Sodium salt)	H_2O	Glaxo
Cefotaxime (Sodium salt)	H_2O	Rousell
Nalidixic acid (Sodium salt)	H_2O	Sigma
Ciprofloxacin (Hydroch loride monohydrate)	H_2O	Bayer
Tetracycline (Hydroch loride)	H_2O	Lederle
Gentamicin (Sulphate)	H_2O	Sigma
Trimethoprim (Lactate)	H_2O	Wellcome
Chloramphenicol	Alcohol + H_2O	Boehringer Mannheim
Rifampicin (Sodium formaldehyde sulphoxylate)	H_2O	Ciba Geigy
Benzyl penicillin (Sodium salt)	H_2O	Sigma
Streptomycin (Sulphate)	H_2O	Upjohn
Spectinomycin (Dihydrochloride)	H_2O	Sigma

preparation and assay was 50mM sodium phosphate buffer, pH7.0.

Plasmid isolation buffers (Takahashi and Nagano, 1984):-

Solution A - 400mM Tris-acetic acid
20mM disodium EDTA

Solution B - 3M sodium acetate - acetic acid

Solution C - 10mM Tris - acetic acid
2mM disodium EDTA

Solution D - 1M sodium acetate
10mM Tris-acetic acid
2mM disodium EDTA

Lysing solution - 4% SDS
100mM Tris

2.3.3. Standard Markers

The standard molecular weight proteins used to calibrate Sephadex gel filtration columns are shown in Table 5. For DNA gels, Lambda phage pre-cut with restriction enzyme, *Hin* d III was employed.

Table 5. Proteins used as molecular weight markers

Protein	Molecular mass daltons	Supplier
Ovalbumin	45000	Sigma
Chymotrypsinogen	22500	Sigma
Cytochrome C	12384	Sigma

2.4. Methods

2.4.1. Population Description

2.4.1.1. Survey 1

Between December 1990 and April 1991, a total of 100 faecal specimens were collected from the "healthy" population in Edinburgh. Four separate population groups participated in the survey including:-

(i) members of the Edinburgh University Catholic Chaplaincy Centre (33)

(ii) office workers in the city of Edinburgh (9)

(iii) registered members of a general practice in Loanhead, Edinburgh (34)

(iv) medical students at the University of Edinburgh (24)

The figure in parenthesis indicates the number of faecal specimens obtained from the associated group. Candidates were excluded from the study if they had received any medical treatment in the three weeks prior to sampling. Information regarding the sex and age of the volunteer was requested.

2.4.1.2. Survey 2

Between November 1990 and February 1991, a total of 100 faecal specimens were collected from the clinical diagnostic laboratories at the Medical School, Edinburgh. The specimens had been submitted by general practitioners in the Edinburgh region and were found not to contain any of the following pathogens: *Salmonella* spp, *Shigella* spp, *Campylobacter* spp. or *E. coli* 0157.

2.4.1.3. Survey 3

The survey was conducted between January and March 1992; a total of 361 faecal specimens were obtained. Survey participants consisted of healthy volunteers resident in the Transvaal, South Africa. The study examined eight separate population groups from both urban and rural areas (Table 6). In the urban area, the four groups comprised infants attending either a childminder or a creche in South Western Townships (SOWETO), urban children and urban teenagers attending a school in Kagiso, a town on the West Rand and urban adults who resided in SOWETO. The rural population was composed of infants attending a "well baby" clinic at Middleplaas in the KaNgwane district, rural children and rural teenagers attending a school at Hekpoort in the Magaliesburg district and lastly adults resident in KaNgwane district. Notably, the rural adults were all associated with the hospital at Shongwe Mission and consequently were in contact with patients. This specific population consisted of healthy mothers feeding sick children, mothers attending a nutrition centre for children and finally escorts to patients visiting the out patients department.

Candidates were excluded from the study if they had received any medical treatment in the three weeks prior to sampling. Parental consent was obtained for all persons involved of less than 18 years. A completed questionnaire (Figure 6) accompanied each specimen.

Figure 6. Questionnaire used in South Africa Survey

VOLUNTEER DETAILS

NAME :

AGE :

SEX :

ADDRESS :

RECENT MEDICAL HISTORY

Have you been to hospital or a clinic in the last week? Yes/No

If yes, what was your reason for making the visit?

RECENT THERAPY

Have you had any treatment in the past month?

Injections

Tablets

Capsules

Liquids

DIET

Do you eat meat?

If so, how often?

FAMILY

How many in the same family?

How many in the same house?

ANCILLARY QUESTIONS

Where do you get your water from?

Do you keep any animals?

Table 6. Population groups sampled in South Africa survey

Group	Age (years)	Area	No. Sampled
Rural Infants	0 - 5	Shongwe	50
Rural Children	6 - 11	Hekpoort	47
Rural Teenagers	12 - 19	Hekpoort	36
Rural Adults	>19	Shongwe	50
Urban Infants	0 - 5	SOWETO	45
Urban Children	6 - 11	Kagiso	42
Urban Teenagers	12 - 19	Kagiso	47
Urban Adults	>19	SOWETO	44

2.4.2. Sample Processing

Rectal swabs or freshly passed faecal specimens were deposited in small plastic containers for immediate transport to the laboratory. Following the protocol established for such studies (Amyes *et al.*, 1992), each faecal specimen was streaked directly onto a series of Oxoid MacConkey Agar plates containing different antibiotics as well as a control plate with no antibiotic. In addition, the specimens were also plated onto Modified Difco Mueller Hinton Agar plates

containing trimethoprim. The antibiotics employed differed in each of the surveys (Table 7). The plates were incubated overnight at 37°C. Each plate was scored for the presence or absence of bacterial colonies which were subsequently classified as either lactose fermenters or lactose non-fermenters. All non-mucoid lactose fermenters were purifed and kept for further study. Non-mucoid strains were selected in an attempt to eliminate mucoidy as a resistance mechanism and thus more readily identify the proportion of plasmid-mediated resistance. Some *Klebsiellae* strains appeared mucoid on Isosensitest agar but not on MacConkey medium. Therefore some of the non-mucoid lactose-fermentors isolated were in fact *Klebsiellae*.

Table 7. Selective plates included in the surveys

Antibiotic	Concentration µg/mL	Survey 1	Survey 2	Survey 3
Ampicillin	10	+	+	+
Cephaloridine	10	+	+	NT
Cefuroxime	4	+	+	NT
Ceftazidime	2	+	+	NT
Nalidixic acid	10	+	+	+
Ciprofloxacin	1	+	+	NT
Tetracycline	10	+	+	NT
Gentamicin	4	+	+	+
Trimethoprim	10	+	+	+
Chloramphenicol	10	NT	NT	+

+, tested NT, not tested

Survey 1 = Edinburgh healthy faecal flora survey
Survey 2 = Edinburgh diagnostic faecal flora survey
Survey 3 = South Africa healthy faecal flora survey

2.4.3. Water Sample Collection and Processing (Survey 3 only)

From each study centre, except for Kagiso, a 100ml sample of the normal drinking water was collected in a sterile bottle. After immediate transportation to the laboratory, the water was filtered and the filter pad placed on an Oxoid MacConkey agar plate containing no antibiotic. The plate was incubated overnight at 37°C. Each plate was examined for the presence of bacterial colonies which were subsequently identified.

2.4.4. Identification of Bacterial Species

Bacterial colonies were identified either by standard biochemical analysis as described in Practical Medical Microbiology (Churchill Livingston, 1989). Strains were allocated into one of four groups; *E. coli*, *Klebsiella* spp., *Enterobacter* spp., or other enterobacteria. Alternatively, the API 20E microtube system (API System, France) was employed.

2.4.5. Plasmid Transfer

Isolates were tested for their ability to transfer their resistance determinants by the method previously described (Amyes and Gould, 1984). The conjugation experiments employed the rifampicin resistant *E. coli* K12 strain J62-2 as the recipient. Donor and recipient strains were grown up statically overnight in

4.5ml of nutrient broth at 37°C. After overnight growth, 0.1ml of the donor culture and 1ml of the recipient culture were combined in 4.5ml of prewarmed nutrient broth. The mixture was incubated at 37°C for 18 hours. Cells were harvested by centrifugation at 4000g for 15 minutes (Heraeus Christ Bactifuge) and resuspended in 5.6ml basal DM medium. The suspension was subsequently diluted to 10^{-1}, 10^{-2} and 10^{-4} in DM medium. 0.1ml of each of the dilutions and of the neat culture was spread on minimal media DM containing rifampicin (25mg/L) and either ampicillin (10mg/L) or trimethoprim (10mg/L) selective plates. Such plates only allowed growth of a plasmid containing recipient strain. The plates were incubated for 48 hours at 37°C after which the resultant single colonies were purified on the same selective plates. The auxotrophic requirements of the transconjugants were confirmed on selective DM plates, lacking histidine. Controls consisted of spreading donor and recipient strains on selective plates after centrifugation and resuspension in 4.5ml DM.

2.4.6. Minimum Inhibitory Concentration (MIC) Determination on Solid Agar

A 4.5ml Isosensitest broth aliquot was inoculated with a culture from a fresh Isosensitest Agar plate, and grown overnight at 37°C. Serial dilutions were made with 1 in 100 dilution steps in single strength DM resulting in a 1 in 10^{-4} dilution. Of this suspension, 2µl was spotted onto media containing varying concentrations of the drug with a multipoint inoculator (Denley). All plates were

incubated at 37°C for 18 hours. The concentration of the drug increased by a factor of two in the plates and the MIC was taken as the first concentration permitting no visible growth. A control plate lacking any drug was used in each case. In addition, NCTC strains, *E. coli*, *S. aureus* and *Ps. aeruginosa* were included as control strains.

2.4.7. Antibiogram Determination

The protocol for MIC determination on solid agar as described was followed except that 2µl of bacterial suspension was spotted onto a range of Isosensitest agar plates containing a single antibiotic at a given breakpoint value (BSAC guidelines). All plates were incubated at 37°C for 18 hours. As before, a control plate and control strains were used in each case.

2.4.8. Isolation of Plasmid DNA

Isolation of plasmid DNA was by the method of Takahashi and Nagano (1984). After 4.5ml nutrient broths were inoculated with a single bacterial colony and grown, shaking overnight, at 37°C, the cells were harvested by centrifugation at 4000g for 15 minutes (Heraeus Christ Bactifuge). The procedure described by the authors was then followed. A final volume of 100µl plasmid DNA solution was obtained. Plasmids were analysed by agarose gel electrophoresis.

2.4.9. Preparation of DNA for Endonuclease Restriction Digestion

Plasmid DNA for endonuclease restriction digestion was extracted as described previously (Takahashi and Nagano, 1984).

A further 350µl Solution C buffer and 50µl Solution D buffer were added to 100µl plasmid DNA solution. This solution was inverted 5 times prior to precipitation with 2 volumes of cold ethanol at -20°C for 10 minutes. The precipitate was collected by centrifugation at 4°C for 10 minutes at high speed (MSE Micro Centaur) after which the pellet was dried in a vacuum desiccator for 5 minutes. The pellet was dissolved in 60µl of a RNAse solution (50mg/L) (Sigma) and incubated at 65°C for 10 minutes.

The restriction endonuclease digest was carried out in a sterile eppendorf tube in a 37°C water bath for 4 hours. The restriction digest mixture consisted of:-

- 18µl plasmid DNA solution
- 2µl restriction endonuclease buffer (10 times strength, NBL Enzymes)
- 1µl restriction endonuclease (NBL)

Unless otherwise stated, restriction endonuclease *Eco* R1 in combination with restriction buffer "React 3" (NBL) were employed. The reaction was terminated by adding 10µl loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water). The sample was analysed by agarose gel electrophoresis.

2.4.10. Agarose Gel Electrophoresis

Horizontal slab gel electophoresis was performed by the method of Meyers *et al.* (1976). Plasmid DNA was electrophoresed overnight at 70V in a 0.7% (w/v) agarose gel (Sigma) while restricted DNA was electrophoresed overnight at 60V in a 0.8% (w/v) agarose gel. DNA was visualised by staining with ethidium bromide (0.5 mg in 1L) and examination on a flat bed UV source. Agarose gels were photographed with an IBI Polaroid Quick Shooter camera (Model N0. 46400) with a orange filter.

2.4.11. Large Scale β-Lactamase Preparation of Crude Cell Extracts

β-lactamase extracts were prepared as described by Simpson *et al.* (1980). Nutrient broth (1L) was inoculated with a bacterial colony and incubated, shaking, at 37°C overnight. Cells were harvested at 6000g for 15 minutes at 4°C (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Instruments). The pellet was resuspended in 10mls 50mM, pH7.0, sodium phosphate buffer. A further pellet was obtained after centrifugation at 4000g (Heraeus Christ Bactifuge) for 15 minutes. The final pellet was resuspended in 1ml 50mM, pH7.0, sodium phosphate buffer. The cells were disrupted by ultrasonication (8μm, 2 x 30 seconds separated with a 1 minute break) with constant cooling (MSE Soniprep 150, MSE Instruments, Crawley). The cell lysate was cleared by centrifugation at 32000g for 30 minutes at 4°C (Sorvall). The supernatant crude enzyme preparation was stored at -20°C until required.

2.4.12. Small Scale β-Lactamase Preparation of Crude Cell Extracts

A 10ml nutrient agar slope was inoculated with a bacterial culture and incubated at 37°C overnight. The overnight growth was washed off the slope with 1ml sodium phosphate buffer (50mM, pH7.0). The cells were disrupted by ultrasonication (8μm, 2 x 15 seconds separated with a 30 second break, Soniprep). The cell lysate was removed by centrifugation for 10 minutes at 4°C at high speed (MSE Micro Centaur). The cleared lysate was transferred to a fresh tube and stored at -20°C until required.

2.4.13. Assessment of β-Lactamase Activity in Small Scale Preparations

In a microtitre plate (Sterilin), 30μl of crude β-lactamase was added to 100μl nitrocephin solution (50μg/ml). The reaction time, or the time taken for a colour change from yellow to red, was taken as an indication of the β-lactamase activity of the enzyme preparation (Simpson *et al.*, 1980).

2.4.14. Analytical Isoelectric Focusing

Analytical isoelectric focusing (IEF) of β-lactamases was performed by the method of Matthew *et al.*, (1975). A glass plate was employed to support a thin layer of polyacrylamide gel containing ampholines on which crude β-lactamases

were focused. The composition of the gel is described in Table 8. Standard crude preparations of broad-spectrum β-lactamases of known pI values were used as controls.

Crude small scale β-lactamase preparations were applied to the surface of the gel at the anode end. A maximum of 12 β-lactamase samples could be loaded to each gel. The amount of enzyme loaded directly related to the enzyme activity as determined by nitrocephin testing ie. time in seconds for colour change equalled volume in µl loaded up to a maximum volume of 30µl. Isoelectric focusing was carried out at 4°C at 500V and 20mA, limited by constant power set at 1W for 18 hours (LKB 10-2000V Power Pack).

Table 8. Composition of polyacrylamide gels for analytical isoelectric focusing

Material	Supplier	Volume Used	Final Concentration
5% tetramethyl-ethylenediamine (TEMED) in distilled water	Sigma	0.2ml	0.25mg/L
40% ampholines w/v (pH 3.5-10 and pH 4-6)	LKB	2.0ml	12%w/v
Acrylamide (100g) plus methylene bisacrylamide (2.7g) in water (300mls)	BDH	9.0ml	acrylamide 70mg/L bisacrylamide 2mg/L
Distilled water		25.0ml	
Riboflavin (20mg/L)	Sigma	4.0ml	2mg/L

These ingredients were combined together immediately before the gel was poured.

Bands of β-lactamase activity were detected by staining the polyacrylamide gels with nitrocephin (500mg/L). A sheet of Whatman No.1 paper, which had been dipped in the solution of nitrocephin, was placed on the surface of the gel, taking care not to trap any air bubbles. The focused bands of β-lactamase activity appeared red on a yellow background.

The polyacrylamide gels were photographed on a light box employing an IBI Polaroid Quick Shooter camera (Model No. 46400) with a green filter.

2.4.15. Gel Filtration

An LKB (Bromma, Sweden) column (90 x $2cm^2$) filled with sephadex G75 (Pharmacia, Uppsala, Sweden) was maintained at 4°C in an LKB mini cold lab. A peristaltic pump (LKB) controlled the flow rate of the column buffer, sodium phosphate buffer (50mM, pH7.0), at 2ml per 10 minutes.

Samples for separation (1 - 2ml) were applied to the bottom of the column and eluted with sodium phosphate buffer. Fractions were collected with an LKB Ultrorac fraction collector. Elution was continued until a volume of buffer equivalent to the total column volume had passed through (180mls). The column was washed with sodium phosphate buffer for 12 hours between each run. Fractions containing β-lactamases activity were determined by testing samples with nitrocephin spotting solution (50mg/L); fractions with peak β-lactamase activity were pooled for further analysis.

2.4.16. Enzyme Molecular Weight Determinations

Calibration of the Sephadex column was based on the method of Andrews (1964). Standard proteins (Table 5), at a concentration of 5mg, were dissolved in 2ml 50mM sodium phosphate buffer (pH7.0) and applied to the Sephadex column. After elution and collection of the fractions, the position of the protein peaks was determined by measuring the absorbance of the fractions at 280nm.

2.4.17. Protein Estimations

Protein estimations were determined according to the method of Waddell (1956). All samples were suitably diluted in sterile water and the absorbance measured at 215 and 225nm. The protein concentration (mg/L), which was correlated with a standard curve of bovine serum albumin concentrations in a Perkin-Elmer Lambda 2 spectrophotometer, could then be calculated with the formula:

$$(215nm - 225nm) \times 165$$

2.4.18. Assays of β-Lactamase Activity

The ultraviolet absorption spectrum of penicillins and cephalosporins is altered if the cyclic amide bond in the β-lactam ring is hydrolysed. The wavelength at which maximum decrease in absorption occurs following hydrolysis is termed λ max. The rate of substrate hydrolysis is assessed by measuring the rate of decrease in

optical density (OD) at λ max (Reid, 1986). The wavelength employed therefore varied with the different β-lactams (Table 9).

Table 9. Wavelengths used in substrate hydrolysis

Substrate	Wavelength (nm)
Ampicillin	238
Cephaloridine	255
Benzyl Penicillin	238
Nitrocephin	384

β-lactamase activity was measured by spectrophotometric assay following the method of O'Callaghan *et al.* (1969). The assays were performed in a Perkin Elmer Lambda 2 spectrophotometer at 37°C. Penicillin and cephalosporin substrates were prepared at 10^{-2}M and 10^{-3}M respectively in 50mM sodium phosphate buffer (pH7.0). Test and blank cuvettes were prepared and equilibrated at 37°C. The test cuvette contained the following:

- 0.3ml substrate
- 2.6ml sodium phosphate buffer (50mM, pH7.0)
- 0.1ml β-lactamase preparation

The blank cuvette contained 2.9ml buffer but no substrate. The initial linear part of the reaction curve was used to obtain a value for change in OD per minute.

Calculation: $R = \frac{OD \times N \times \text{enzyme dilution}}{OD1 \times \text{time}}$

where

R = μmoles of substrate hydrolysed per minute per ml enzyme

OD = change in optical density

N = μmoles substrate in cuvette

(0.3 for cephalosporins, 3.0 for penicillins)

OD1 = optical density of intact substrate

2.4.19. Inhibition of β-Lactamase Activity

The effect of β-lactamase inhibitors on specific β-lactamases was determined employing the spectrophotometric assay (O'Callaghan *et al.*, 1969). In each assay, nitrocephin was employed as the substrate. After the β-lactamase inhibitor was added to the cuvette, the mixture was pre-incubated at 37°C for 5 minutes prior to the start of the assay. A range of inhibitor concentrations was employed in order to determine the concentration required for 50% inhibition (ID_{50}) of enzyme activity.

2.4.20. Mutation Studies

Mutation studies were carried out following the procedure described by

Thomson and Amyes (1993b). *E. coli* J62-2 transconjugants were inoculated into 100ml Isosensitest broths containing amoxycillin plus clavulanic acid in a 2:1 ratio at a range of concentrations. The concentrations included were one below the plate MIC of the experimental transconjugant, one on the MIC, one above the MIC and two above the MIC of the transconjugant. After 18 hours incubation at 37°C those cultures which had grown, were subcultured into fresh Isosensitest broth containing the same concentration of amoxycillin/clavulanate. This was repeated five times. After the final subculture, bacteria were harvested from 10ml of the culture by centrifugation at 4500g, for 15 minutes (Heraeus Christ Bactifuge). The pellet was resuspended in 10ml DM media and 0.1ml spread onto Isosensitest agar plates containing amoxycillin/clavulanate at the same concentration as the strain had been cultured in. Ten single colonies were purified onto the same media and then their β-lactamases were examined as described previously.

Chapter 3

Results

3.1. Survey of Antibiotic Resistance in the Aerobic Faecal Flora of the Healthy Population in Edinburgh.

There have been very few controlled studies conducted to determine the carriage of antibiotic resistance genes amongst the commensal faecal flora of healthy individuals selected from non-biased populations. Furthermore, there has been little investigation into the biochemical and molecular basis for the antibiotic resistance in such reservoirs.

3.1.1. Study Design

Between January and June 1991, a survey of antibiotic resistance in the aerobic faecal flora of the healthy population in Edinburgh was undertaken. Four separate

population groups in Edinburgh participated in the study (Table 10). A total of 100 specimens was collected. No volunteer was allowed to participate in the study if they had received any antimicrobial treatment in the three weeks prior to sampling. All participants were described as "healthy". In addition, to prevent bias, those volunteers chosen from general practice had no gastrointestinal condition. The study group consisted of 46 males, 45 females and 9 unknown and were equally distributed throughout the population groups (Table 10).

Table 10. The study populations included in the survey

Group	Number	Male	Female	Unknown
Medical Students	24	11	11	2
Office Workers	9	7	0	2
Catholic Chaplaincy Members	33	14	19	0
General Practice Members	34	15	15	4

3.1.2. The Carriage of Antibiotic Resistance in the Aerobic Faecal Flora

The carriage of antibiotic resistant determinants amongst the commensal faecal flora of the healthy population examined in Edinburgh, along with the antibiotic breakpoint sensitivity values used to determine resistance, is shown in Table 11. The protocol employed for the isolation of resistant

strains is as described in the Materials and Methods.

Table 11. Proportion of volunteers with antibiotic resistant bacteria in their commensal faecal flora

Antimicrobial Agents	Concentration (mg/L)	Number of volunteers with resistant bacteria				
		Students	OW	CCM	GPM	%
Ampicillin	10	8	4	8	22	42
Cefuroxime	4	10	2	10	17	39
Ceftazidime	2	0	0	0	0	0
Gentamicin	4	1	0	0	0	1
Tetracycline	10	10	2	9	6	27
Nalidixic acid	10	0	0	0	2	2
Ciprofloxacin	1	0	0	0	0	0
Trimethoprim	10	4	1	1	6	12

OW = Office Workers
CCM = Catholic Chaplaincy Members
GPM = General Practice Members

The highest incidence of resistance was identified against ampicillin as 42% of the specimens contained lactose-fermenting, aerobic bacteria not susceptible to the drug. Resistance to another β-lactam agent was also high; 39% of specimens contained cefuroxime resistant bacteria. This result may, however, not be real (see page 189). However, no resistance was found to the later generation cephalosporin, ceftazidime. Similarly, no resistance was

detected to ciprofloxacin while only a few specimens contained bacteria resistant to nalidixic acid (2%) and gentamicin (1%). Tetracycline resistance was present in 27% of specimens while trimethoprim resistance was found to be only 12%. There was no significant difference in the carriage of resistance found between the different population groups.

3.1.3. Identification of the Ampicillin Resistant Strains

The 42 ampicillin resistant strains that were obtained, were all identified employing the API 20E system (Table 12). As shown in Table 12, *E. coli* predominated amongst the isolates.

Table 12. Identification of the ampicillin resistant strains

Species	Number	Percentage
Escherichia coli	28	66.7
Klebsiella oxytoca	7	16.6
Klebsiella pneumoniae	2	4.8
Citrobacter freundii	3	7.1
Citrobacter diversus	1	2.4
Hafnia alvei	1	2.4

The ampicillin resistant strains were analysed further:

3.1.4. Sensitivity of Ampicillin Resistant Strains to a Range of β-Lactam Agents

The sensitivity of the ampicillin resistant strains to a range of β-lactam agents was tested. The minimum inhibitory concentration (MIC) of amoxycillin, amoxycillin plus clavulanic acid, cefotaxime, ceftazidime and cefuroxime, was determined. The results are shown in Table 13. Amongst the 42 isolates, 24 (57%) were highly resistant to amoxycillin (MIC > 512mg/L) and were predominantly *E. coli*. Amongst the *E. coli*, 14% were cefuroxime resistant (MIC > 4mg/L) whilst there was almost uniform sensitivity to cefotaxime and ceftazidime (MIC < 2mg/L). The addition of clavulanic acid restored the sensitivity of the *E. coli* to amoxycillin to an MIC below 32 mg/L in all but two cases. Those isolates identified as *Klebsiella* spp. had lower MIC's of amoxycillin and amoxycillin in combination with clavulanic acid than the *E. coli* and universal sensitivity to cefuroxime, cefotaxime and ceftazidime. The small number of *Citrobacter* spp. and *Hafnia* spp. identified showed variable resistance to amoxycillin, amoxycillin plus clavulanic acid and cefuroxime but no resistance to ceftazidime and cefotaxime.

3.1.5. Transfer of Ampicillin Resistant Determinants

The ability of the ampicillin resistant strains to transfer their resistance determinants by conjugation to the standard bacterial strain *E. coli* J62-2 and

Table 13. Minimum Inhibitory Concentration (mg/L) of ampicillin resistant isolates to a range of antimicrobials

Strain No.	Antimicrobial (mg/L)					✣β-lactamase	Species
	Amox	Amox + Clav	CTZ	CAZ	CXM		
PS 19	>1024	16	2	2	2	TEM-1	*E. coli*
PS 43	>1024	16	2	2	4	TEM-1	*E. coli*
* PS 47	>1024	16	2	2	2	TEM-1	*E. coli*
* PS 279	>1024	16	2	2	4	TEM-1	*E. coli*
PS 302	>1024	16	2	2	4	TEM-1	*E. coli*
* PS 307	>1024	16	2	2	2	TEM-1	*E. coli*
PS 322	>1024	16	2	2	4	TEM-1	*E. coli*
* PS 516	>1024	16	2	2	2	TEM-1	*E. coli*
* PS 529	>1024	16	2	2	4	TEM-1	*E. coli*
PS 12	1024	8	2	2	2	TEM-1	*E. coli*
* PS 17	1024	16	2	2	4	TEM-1	*E. coli*
PS 27	1024	32	2	8	32	Chrom	*E. coli*
* PS 31	1024	16	2	2	2	TEM-1	*E. coli*
* PS 275	1024	16	2	2	4	TEM-1	*E. coli*
* PS 277	1024	16	2	2	2	TEM-1	*E. coli*
* PS 283	1024	16	2	2	4	TEM-1	*E. coli*
PS 295	1024	8	2	2	8	TEM-1	*E. coli*
PS 305	1024	8	2	2	4	TEM-1	*E. coli*
PS 319	1024	8	2	2	4	TEM-1	*E. coli*
* PS 320	1024	16	2	2	4	TEM-1	*E. coli*
PS 327	1024	8	2	2	8	TEM-1	*E. coli*
* PS 512	1024	16	2	2	4	TEM-1	*E. coli*
* PS 520	1024	16	2	2	2	TEM-1	*E. coli*
* PS 503	512	16	2	2	2	TEM-1	*E. coli*
PS 526	512	2	2	2	2	TEM-1	*E. coli*
PS 332	256	32	2	2	16	TEM-1	*E. coli*
PS 530	32	2	2	2	2	N.I	*E. coli*
PS 336	16	2	2	2	4	Chrom	*E. coli*
PS 35	64	2	2	2	2	SHV-1	*K. oxy*
PS 40	64	2	2	2	2	N.I	*K. oxy*
PS 293	64	2	2	2	2	SHV-1	*K.pneu*
PS 318	64	2	2	2	2	K1	*K. oxy*
PS 508	64	4	2	2	2	SHV-1	*K. oxy*
PS 42	32	2	2	2	2	SHV-1	*K. oxy*
PS 308	32	2	2	2	2	SHV-1	*K. oxy*
PS 522	32	2	2	2	2	TEM-1	*K. oxy*
PS 312	32	2	2	2	2	N.D	*K.pneu*
PS 285	1024	8	2	2	2	TEM-1	*C. freu*
PS 21	256	64	2	2	4	Chrom	*C. freu*
PS 290	256	64	2	2	2	Chrom	*C. freu*
PS 50	16	16	2	2	16	TEM-1	*C. dive*
PS 3	32	16	2	2	2	TEM-1	*H. alvei*

* = Transferable strain - See section 3.1.5

✣β-lactamase = see section 3.1.8.

N.D.= Not detected

N.I. = Not identified

therefore to an isogenic background was tested. Amongst the isolates, 14 (33%) exhibited this ability. Each of the wild type transferable strains was noted as *E. coli* from the previous identification procedure and have been labelled in Table 13. Interestingly, each of the transferable strains mediated a high level of resistance to amoxycillin.

3.1.6. Antibiogram Analysis of Ampicillin Resistant Transconjugants

Each transconjugant was tested for its ability to confer resistance to a range of antimicrobials including amoxycillin (10mg/L), amoxycillin plus clavulanic acid (10:5mg/L), trimethoprim (10mg/L), gentamicin (4mg/L), cefuroxime (4mg/L), ceftazidime (2mg/L), chloramphenicol (10mg/L), tetracycline (10mg/L), streptomycin (10mg/L) and spectinomycin (10mg/L). As a result, an antibiogram profile for each transconjugant was established (Table 14). One profile, where resistance to ampicillin, cefuroxime, streptomycin and spectinomycin was mediated, was found to predominate.

3.1.7. Plasmid Analysis of Ampicillin Resistant Transconjugants

Plasmid extraction, as outlined in the Materials and Methods, was carried out in order to examine the plasmid content of each transconjugant. Although three transconjugants contained more than one plasmid, the remainder of the

Table 14. Analysis of the ampicillin resistant transconjugants

Transconjugant Number	Number of Plasmids	Size Kb	Restriction Pattern Group	Antibiogram
ES 20	1	80.9	A	Ap Cf Sm Sp
ES 59	3	80.9	A	Ap Cf Sm Sp
ES 68	1	80.9	A	Ap Cf Sm Sp
ES 75	1	84.1	A	Ap Cf Sm Sp
ES 76	1	82.9	A	Ap Cf Sm Sp
ES 60	1	87.9	B	Ap Cf Sm Sp Cm Tc
ES 62	1	87.0	C	Ap Cf Sm Sp Tc
ES 63	3	81.9	D	Ap Cf Sm Sp
ES 73	1	81.9	D	Ap Cf Sm Sp
ES 64	1	85.7	E	Ap Cf Sm Sp
ES 65	3	121.5	F	Ap Cf Sp
ES 67	1	46.5	G	Ap Cf Sm Sp Tp
ES 69	1	54.8	H	Ap Cf Sm Sp
ES 72	1	46.1	I	Ap Cf Sm Sp

Ap = Ampicillin, Cf = Cefuroxime, Sm = Streptomycin

Sp = Spectinomycin, Cm = Chloramphenicol, Tc = Tetracycline

Tp = Trimethoprim

transconjugants contained only a single plasmid (Figure 7, Table 14).

The relatedness of these plasmids was then investigated by endonuclease restriction analysis with *Eco* R1. As a result, the presence of common or closely related plasmid types were observed. From the endonuclease digestion pattern (Figure 8), the plasmids were assigned to a number of groups. Plasmids with closely related restriction digest patterns were assigned to the same category, that is, if they differed by no more than three bands. If the profiles differed, however, by four bands or more, the plasmids were assigned to a separate group.

As shown in Figure 8, a number of different plasmid types (9) were identified. A common plasmid type was, however, isolated on 5 occasions (Figure 8; Tracks A, B, I, M and N). Each of these plasmids conferred resistance to amoxycillin, [redacted], cefuroxime, streptomycin and spectinomycin suggesting the plasmid was the same. Another plasmid was present on two occasions (Figure 8; Tracks E and L) and appeared to be related to the common plasmid because the bands present in the common plasmid also featured in the related plasmids. Indeed, the profiles differed by only three bands. Furthermore, the antibiogram profiles were identical. The remaining seven plasmids occurred on single occasions and although the endonuclease restriction digest is quite different for each, the antibiogram profile revealed that, in common with the other plasmids isolated, resistance was mediated to amoxycillin, cefuroxime, streptomycin and

Figure 7. Agarose gel electrophoresis of plasmid DNA extracted from Edinburgh transconjugants

Tracks:-

A) ES 20; B) ES 59; C) ES 60; D) ES 62; E) ES 63; F) ES 64; G) ES 65; H) ES 67; I) ES 68; J) ES 69; K) ES 72; L) ES 73; M) ES 75; N) ES 76; O) λ pre-cut with *Hin* dIII

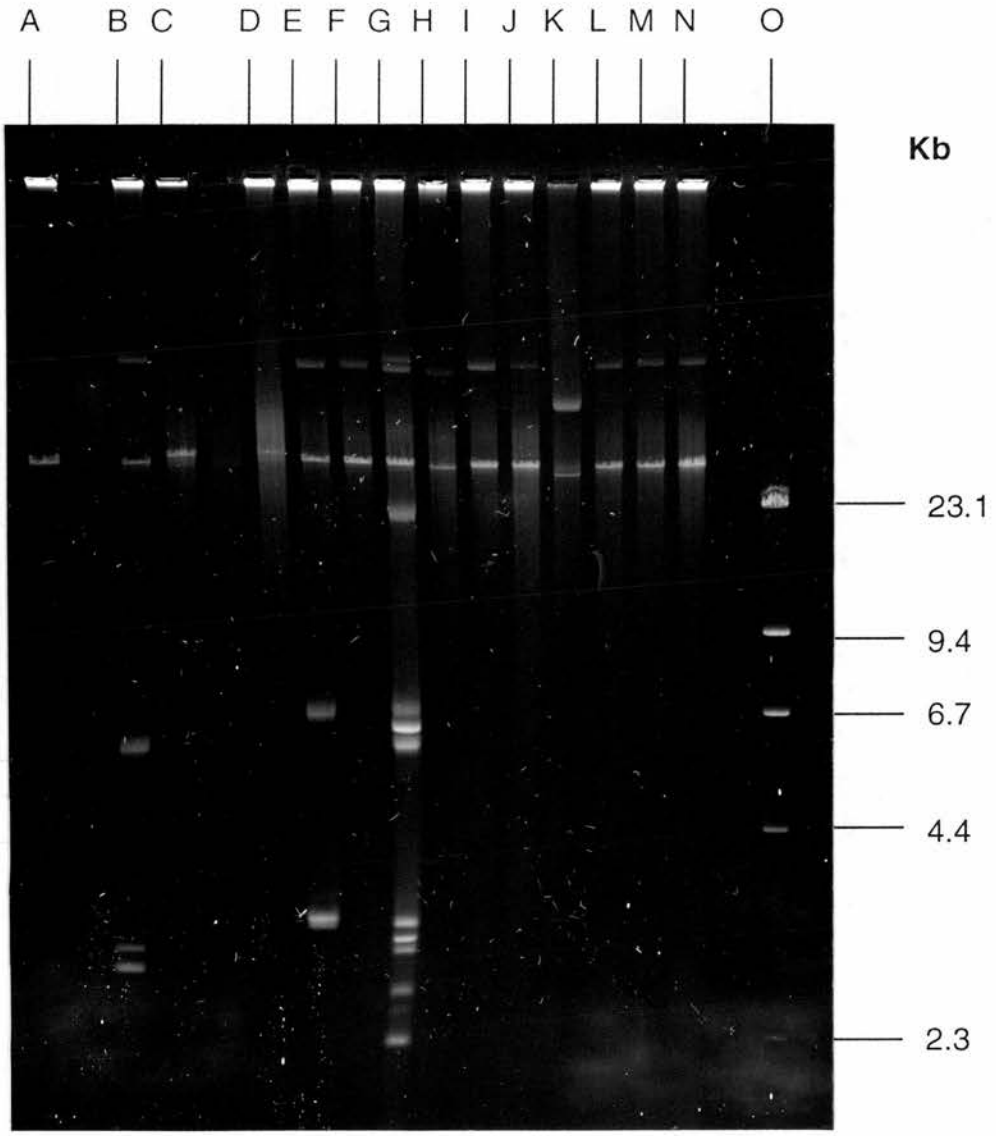
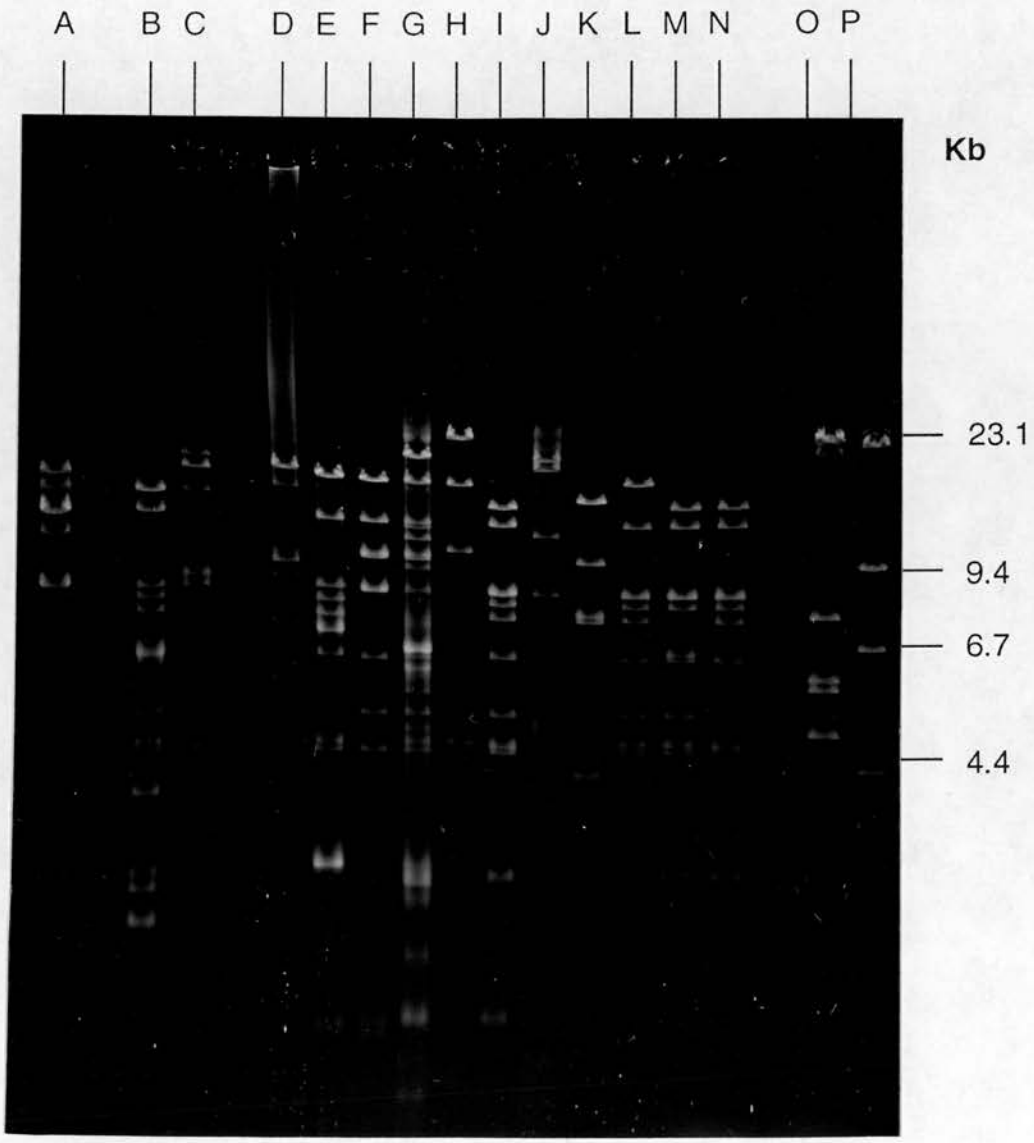


Figure 8. Agarose gel electrophoresis of restriction endonuclease digestion of plasmid DNA, with *Eco* R1, extracted from Edinburgh transconjugants

Tracks:-

A) ES 20; B) ES 59; C) ES 60; D) ES 62; E) ES 63; F) ES 64; G) ES 65; H) ES 67; I) ES 68; J) ES 69; K) ES 72; L) ES 73; M) ES 75; N) ES 76; O) λ pre-cut with *Eco* R1; P) λ pre-cut with *Hin* dIII



spectinomycin and in some cases, resistance was also conferred to chloramphenicol, tetracycline or trimethoprim. The analysis of the plasmids by restriction endonuclease digestion and the subsequent plasmid classification is shown in Table 14.

The distribution of the plasmid types throughout the study population groups is shown in Table 15. Interestingly, the occurrence of the common plasmid type (Group A) did not seem to be restricted to one population group suggesting that this plasmid type might be spread throughout the community.

Table 15. Distribution of the ampicillin resistant plasmids amongst the population groups

Source	Plasmid Group
Medical Students	A, C, G, H
Office Workers	A
Catholic Chaplaincy Members	B
General Practice Members	A, D, E, F, I

3.1.8. Distribution of β-Lactamases in the Ampicillin Resistant Strains

The different β-lactamases responsible for mediating resistance to ampicillin amongst the strains isolated, were determined by iso-electric focusing. All of the

transconjugants encoded the TEM-1 β-lactamase (Table 13; Figure 9) regardless of the plasmid type on which the gene was carried. In contrast, the non-transferable strains which were also examined (Table 13; Figure 10, 11, 12 and 13) encoded a variety of different β-lactamases. Amongst the non-transferable *E. coli*, only three isolates did not produce TEM-1; in one isolate no β-lactamase was detected, whilst in the other two isolates, chromosomal β-lactamases were identified. Amongst the *Klebsiella* isolates there was a greater heterogeneity of β-lactamase production. Whilst SHV-1 predominated amongst these isolates, TEM-1 and K1 were also found to be present. In addition, one isolate produced no enzyme at all. The *Citrobacter* spp. and *Hafnia* spp. produced only TEM-1 or chromosomal enzyme.

3.1.9. Specific Activity of the TEM-1 β-Lactamase and Resistance to Clavulanic Acid

It has been suggested that resistance to amoxycillin and amoxycillin plus clavulanic acid may be associated with the specific activity of the β-lactamase. Therefore, the specific activities of the β-lactamases in each of the transconjugants were determined (Table 16). When compared with the previously established MIC to amoxycillin and amoxycillin plus clavulanic acid, no relationship could be established between the two (Table 13). Similarly, with reference to Table 14, no significant correlation could be identified between the plasmid type and the specific activity of the β-lactamase.

Figure 9. Analytical iso-electric focusing of β-lactamases in the Edinburgh transconjugants

Tracks:-

A) ES 20; B) ES 59; C) ES 60; D) ES 62; E) ES 63; F) R1 (TEM-1 producer);

G) ES 64; H) ES 65; I) ES 67; J) ES 68; K) ES 69

These selected TEM-1 producing transconjugants are representative of all of the transconjugants examined.

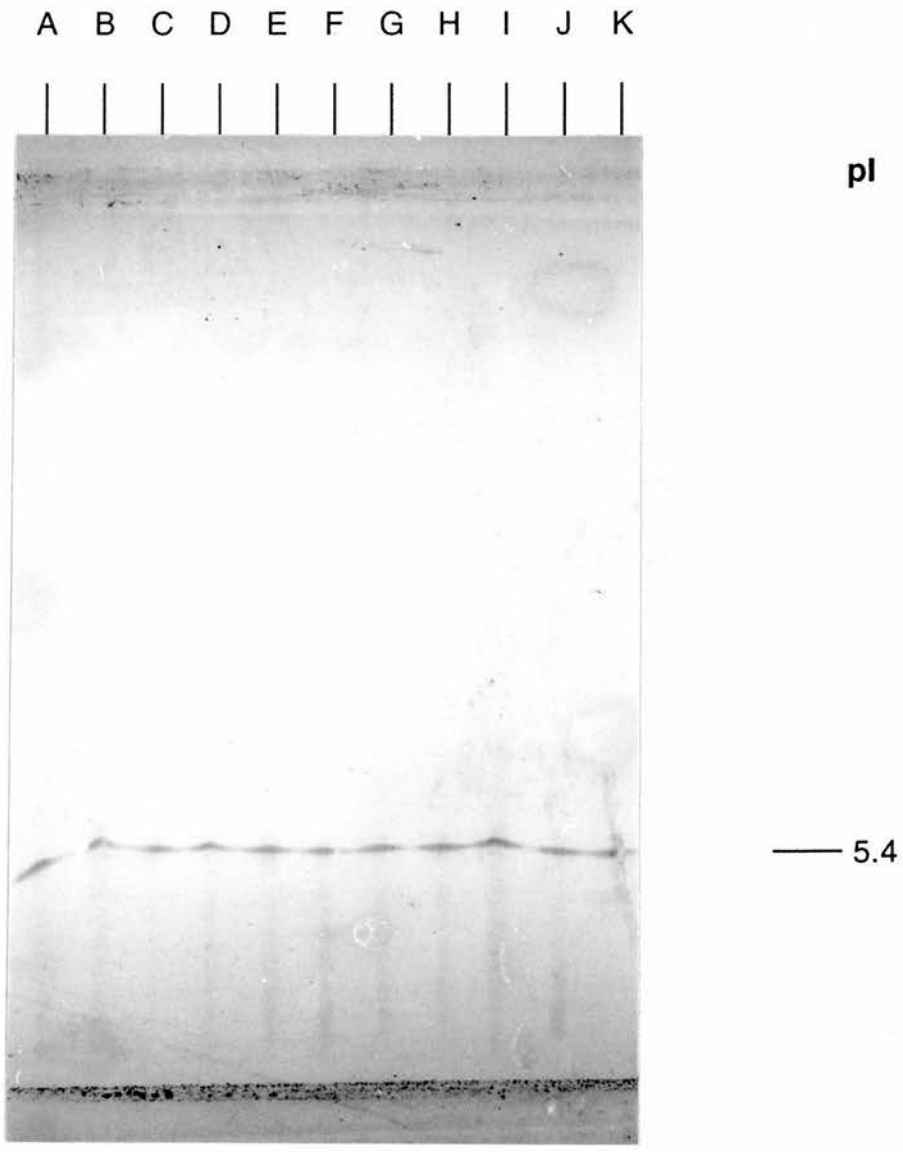


Figure 10. Analytical iso-electric focusing of β-lactamases isolated from wild type, non-transferable ampicillin resistant strains in Edinburgh

Tracks:-

A) PS 293; B) PS 308; C) PS 312; D) PS 19; E) PS 508; F) R1 (TEM-1 producer); G) PS 27; H) PS 42; I) PS 43; J) PS 285; K) PS 290

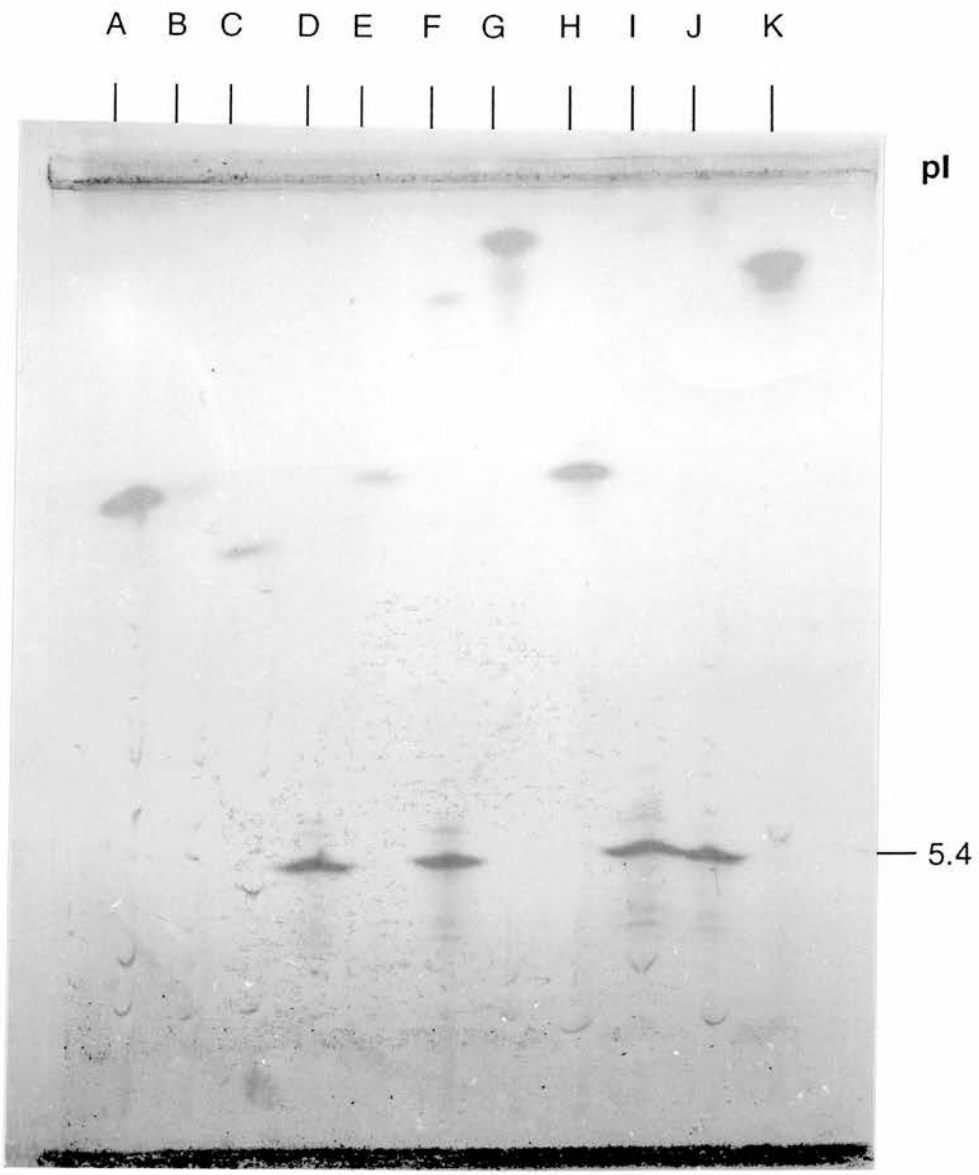


Figure 11. Analytical iso-electric focusing of β-lactamases isolated from wild type, non-transferable ampicillin resistant strains in Edinburgh

Tracks:-

A) PS 295; B) R1010 (SHV-1 producer); C) PS 318; D) PS 319; E) R455 (OXA-1 producer); F) PS 336; G) PS 21; H) RP4 (TEM-2 producer); I) R1 (TEM-1 producer); J) PS 327

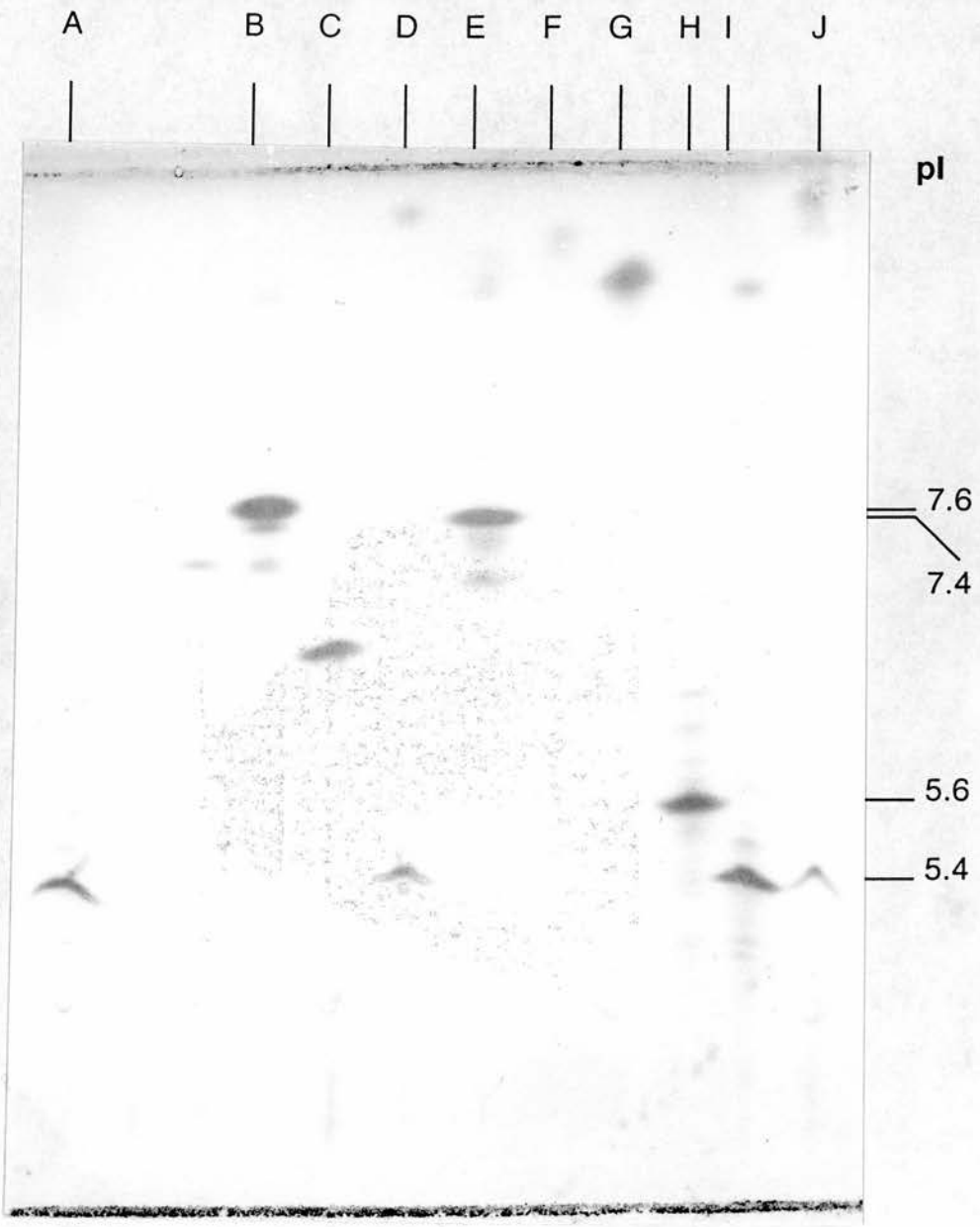


Figure 12. Analytical iso-electric focusing of β-lactamases isolated from wild type, non-transferable ampicillin resistant strains in Edinburgh

Tracks:-

A) PS 3; B) PS 50; C) PS 332; D) R1 (TEM-1 producer); E) PS 522; F) PS 526; G) PS 27; H) PS 35; I) R1010 (SHV-1 producer); J) PS 40

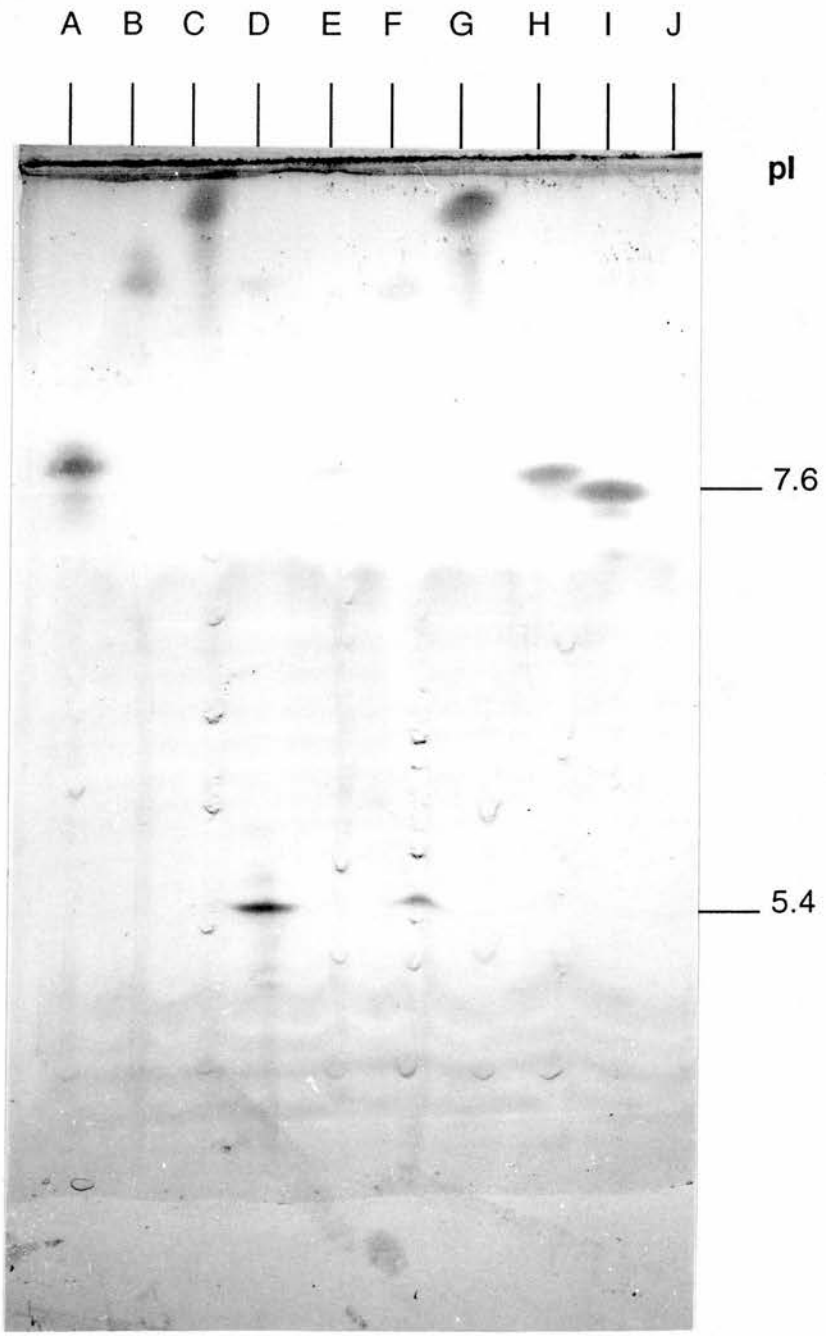
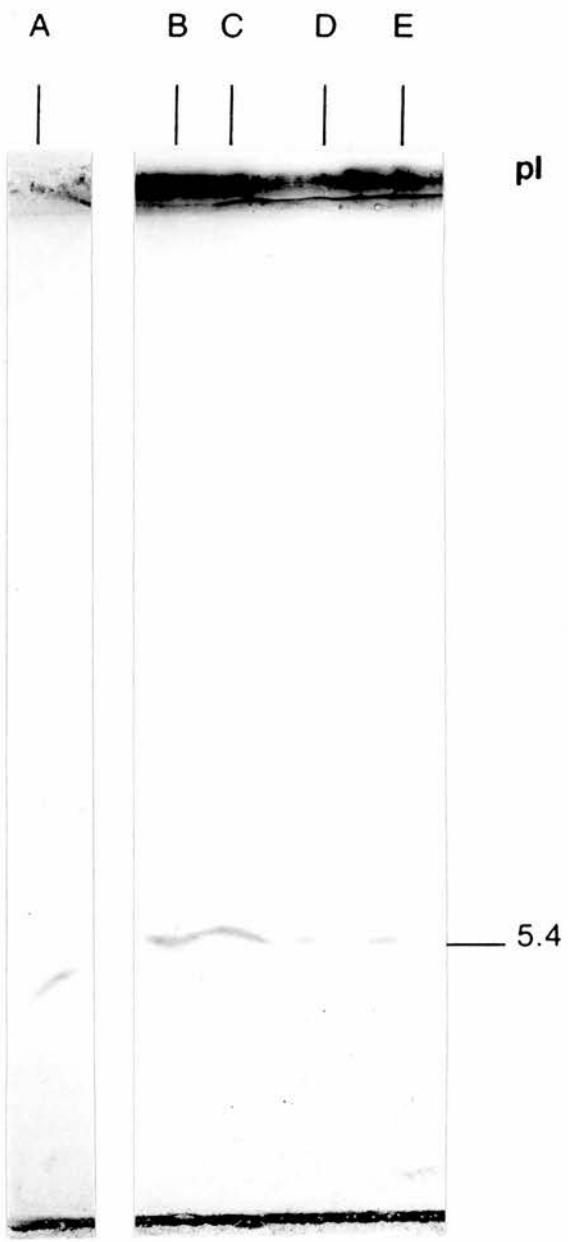


Figure 13. Analytical iso-electric focusing of β-lactamases isolated from wild type, non-transferable ampicillin resistant strains in Edinburgh

Tracks:-

A) PS 12; B) R1 (TEM-1 producer); C) PS 302; D) PS 305; E) PS 322;



The MIC of amoxycillin and amoxycillin plus clavulanic acid was determined for each ampicillin resistant transconjugant (Table 16). As a result, the level of resistance to ampicillin owing purely to the transferable resistance determinants was established. In comparison to the MIC values of wild type strains where 13 of the 14 transferable isolates were resistant to 1024mg/L amoxycillin (Table 13), the level of resistance in the transconjugants was much lower. Only two transconjugants were resistant to 512mg/L amoxycillin, while the remaining 12 transconjugants were resistant to 256mg/L amoxycillin. This suggests that the resistance levels in the original strains do not simply derive from the β-lactamase activity but are interdependent with the rate of penetration of the drug into the cell and the ability of the drug to bind to and inactivate its target, the penicillin-binding proteins. All the transconjugants had an MIC of 8mg/L to amoxycillin plus clavulanic acid. Yet again, no relationship could be established between these MIC values and the specific activity of the β-lactamases.

Table 16. The different specific activities and the sensitivities to amoxycillin and amoxycillin plus clavulanic acid of the ampicillin resistant transconjugants

Transconjugant Number	Specific Activity ⁜	Minimum inhibitory concentration mg/L	
		Amoxycillin	Amoxycillin + Clavulanic acid
ES 62 (PS 283)	0.0127	512	8
ES 67 (PS 275)	0.0355	512	8
ES 20 (PS 47)	0.0022	256	8
ES 59 (PS 307)	0.0193	256	8
ES 60 (PS 320)	0.0068	256	8
ES 63 (PS 503)	0.0289	256	8
ES 64 (PS 512)	0.0177	256	8
ES 65 (PS 516)	0.0181	256	8
ES 68 (PS 277)	0.0052	256	8
ES 69 (PS 279)	0.0042	256	8
ES 72 (PS 17)	0.0031	256	8
ES 73 (PS 31)	0.0057	256	8
ES 75 (PS 520)	0.0034	256	8
ES 76 (PS 529)	0.0028	256	8

⁜ = Δ μmoles nitrocephin hydrolysed / min/ mg protein

() = Original isolate number

3.2 Follow-up Survey of Ampicillin Resistance in the Aerobic Faecal Flora of the Healthy Population in Edinburgh

The high carriage rate of ampicillin resistant determinants amongst the commensal faecal flora of the healthy population in Edinburgh was surprising. In addition, the presence of an epidemic plasmid amongst these strains was also unexpected. To determine if ampicillin resistant bacteria are maintained in this population group, a follow-up survey was conducted.

3.2.1. Study Design

Two years after the original survey of the carriage of antibiotic resistance in the healthy population in Edinburgh, a follow-up survey was performed. Volunteers were those from the original survey, whose faecal specimen contained bacteria capable of transferring ampicillin resistance; five of the 14 candidates could be contacted and hence participated in the survey. These participants were evenly distributed between the various population groups, that is the general practice members (2), office workers (2) and Catholic Chaplaincy Centre (1). As before, there had been no antimicrobial therapy received by the participants in the three weeks prior to sampling.

3.2.2. The Carriage of Ampicillin Resistance in the Aerobic Faecal Flora

The carriage of ampicillin resistant determinants amongst the commensal faecal flora of the selected healthy population re-examined in Edinburgh is shown in Table 17. Amongst the five participants, three were found to contain ampicillin resistant non-mucoid aerobic lactose-fermenting bacteria. The protocol employed for the isolation of the ampicillin resistant strains is as described in the Materials and Methods.

Table 17. Volunteers in Edinburgh re-examined for presence of ampicillin (Ap) resistant bacteria (10mg/L) in their commensal faecal flora

Participants	Population Group	Sex	Ap Resistant Bacteria
No.1	OW	Male	++
No.2	OW	Male	_
No.3	GPM	Male	++
No.4	GPM	Male	++
No.5	CCC	Male	_

OW = Office Workers
GPM = General Practice Members
CCC = Catholic Chaplaincy Centre

++ = Growth
_ = No growth

3.2.3. Transfer of Ampicillin Resistant Determinants

The ability of the ampicillin resistant strains to transfer their resistance determinants by conjugation to the isogenic background of the standard bacterial strain *E. coli* J62-2 was tested. Amongst the three isolates, only one (No.3) transferred ampicillin resistance. In the initial study, the transconjugant obtained from bacteria isolated from this volunteer was ES 76.

3.2.4. Plasmid Analysis of the Ampicillin Resistant Transconjugants

To examine the plasmid contents of the new transconjugant and to establish if plasmid similarities with the original transconjugant (ES 76) existed, the plasmids from both transconjugants were extracted and restricted by endonuclease digestion as described previously. From the endonuclease digest pattern, shown in Figure 14, the newly isolated plasmid (Track B) was identical to the plasmid identified in ES 76 two years previously (Track A).

3.2.5. Distribution of β–Lactamases in the Isolated Ampicillin Resistant Strains

The different β-lactamases responsible for mediating resistance to ampicillin amongst all of the isolates, both transferable and non-transferable, were determined by iso-electric focusing. From Figure 15, it may be seen that as in

Figure 14. Agarose gel electrophoresis of endonuclease restriction digest of plasmid DNA obtained from Edinburgh transconjugants

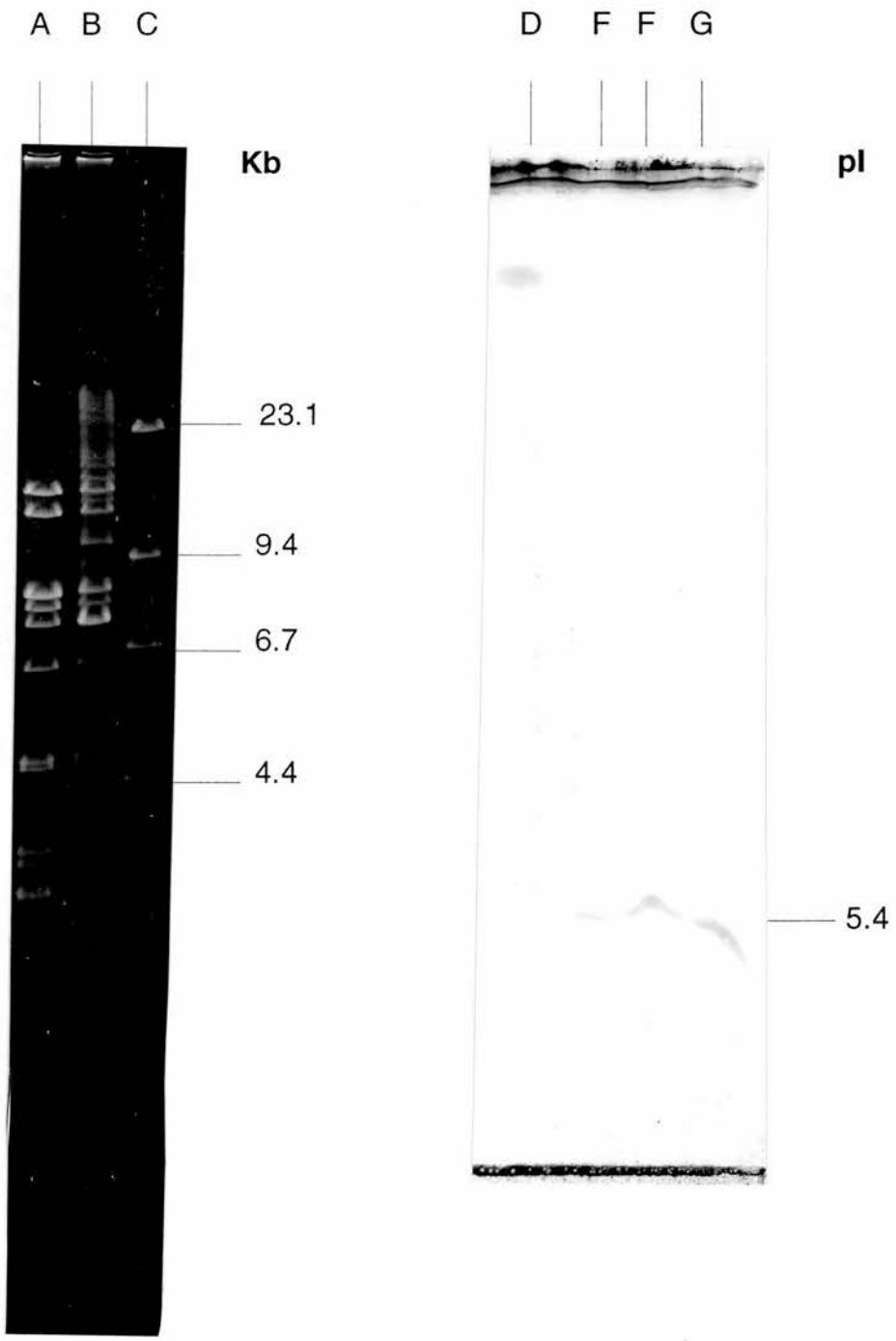
Tracks:-

A) ES 76; B) PS 1528; C) λ pre-cut with *Hin* dIII

Figure 15. Analytical iso-electric focusing of β-lactamases in the Edinburgh transconjugants

Tracks:-

D) PS 1527; E) PS 1528; F) PS 1529; G) R1 (TEM-1 producer)



the original survey, the transferable strain (Track E) produced TEM-1 β-lactamase. While one of the non-transferable strains also produced TEM-1 (Track F), the other (Track D) produced a chromosomal β-lactamase (pI 9.5).

3.2.6. Identification and Antibiogram Analysis of the Ampicillin Resistant Strains

The ampicillin resistant strains were all identified as *E. coli* employing the standard biochemical tests outlined in the Materials and Methods. The single transconjugant was tested for its ability to confer resistance to a range of antimicrobials including amoxycillin (10mg/L), trimethoprim (10mg/L), gentamicin (4mg/L), cephaloridine (10mg/L), cefuroxime (4mg/L), ceftazidime (2mg/L), chloramphenicol (10mg/L), tetracycline (10mg/L), streptomycin (10mg/L) and spectinomycin (10mg/L). The transconjugant was found to mediate resistance to ampicillin and streptomycin. In addition to ampicillin and streptomycin, the plasmid identified in ES 76 two years previously was able to confer resistance to spectinomycin and cefuroxime. It may be concluded therefore, that the newly isolated plasmid has since lost these specific resistance determinants. Alternatively, this variation might be explained if the break-point concentrations, employed in the testing, were very close to the actual MIC values of the strains. Under such circumstances, differences in the sensitivity of the strains may be expected if slightly different concentrations of antibiotic were used in each testing.

3.3. A Comparison of Plasmids Isolated from Normal Faecal E. coli with Plasmids Isolated from Clinical Urinary E. coli.

The genetic and biochemical basis for the development and spread of resistance is well documented in clinical settings. Unfortunately, until now, few of the studies on the normal *E. coli* from healthy populations have examined the mechanisms of resistance in the strains isolated (see Section 3.1.8). The value of such studies is obvious in establishing whether the resistance genes and plasmids in the normal flora are indeed the progenitors of those encountered in clinical isolates. A direct comparison was conducted in order to determine if the plasmids, mediating resistance to ampicillin in the clinical environment, were present in the healthy community.

3.3.1. Source of Plasmids Employed in the Comparison Study

In December 1990, a survey of antibiotic resistance in urinary bacteria responsible for significant bacteriuria, isolated in central Scotland, was conducted (Thomson *et al.*, 1992a). Amongst the *E. coli* producing TEM-1 obtained from isolates in Edinburgh, 17 were able to transfer ampicillin resistance determinants to *E. coli* J62-2 (Thomson and Amyes, 1993a). These transconjugants were employed as representatives of

clinical plasmids and were compared with those plasmids (N = 14) obtained from the healthy community in the survey described previously (Section 3.1).

3.3.2. Examination of Plasmids Employed in the Comparison Study

A comparative investigation of plasmids encoding ampicillin resistance isolated from clinical *E. coli* with ampicillin resistant plasmids isolated from normal *E. coli* was carried out. All the plasmids examined had previously been transferred to *E. coli* J62-2 from the original *E. coli* isolate. The results (Figure 16) show that there were no common plasmids amongst those plasmids that had been isolated, on no more than two occasions, from both sources.

In contrast, however, the results also show (Figure 17) that two representatives of the epidemic plasmid isolated from the normal *E. coli* (Tracks B and D) were very similar to two representatives of plasmids isolated from the clinical source (Tracks A and C). Interestingly TRC-1, the novel TEM-derived plasmid-encoded β-lactamase, resistant to inhibition by clavulanic acid, was identified on the most common TEM-1 containing plasmid amongst the clinical urinary strains (Thomson and Amyes, 1992a). From Figure 18, it is appparent that the TRC-1 encoded plasmid was identical to three of the epidemic clinical plasmids (Tracks A, C and E) and to one of the normal *E. coli* plasmids (Track D) .

Figure 16. Agarose gel electrophoresis of endonuclease restriction digest of plasmid DNA extracted from normal faecal *E. coli* (Tracks A-I) and from clinical urinary *E. coli* (Tracks K-S)

Tracks:-A) ES 60; B) ES 62; C) ES 63; D) ES 64; E) ES 65; F) ES 67; G) ES 69; H) ES 72; I) ES 73; J) λ pre-cut with *Hin* dIII; K) CT 113; L) CT 114; M) CT 115; N) CT 116; O) CT 117; P) CT 118; Q) CT 119; R) CT 160; S) CT 120; T) λ pre-cut with *Hin* dIII

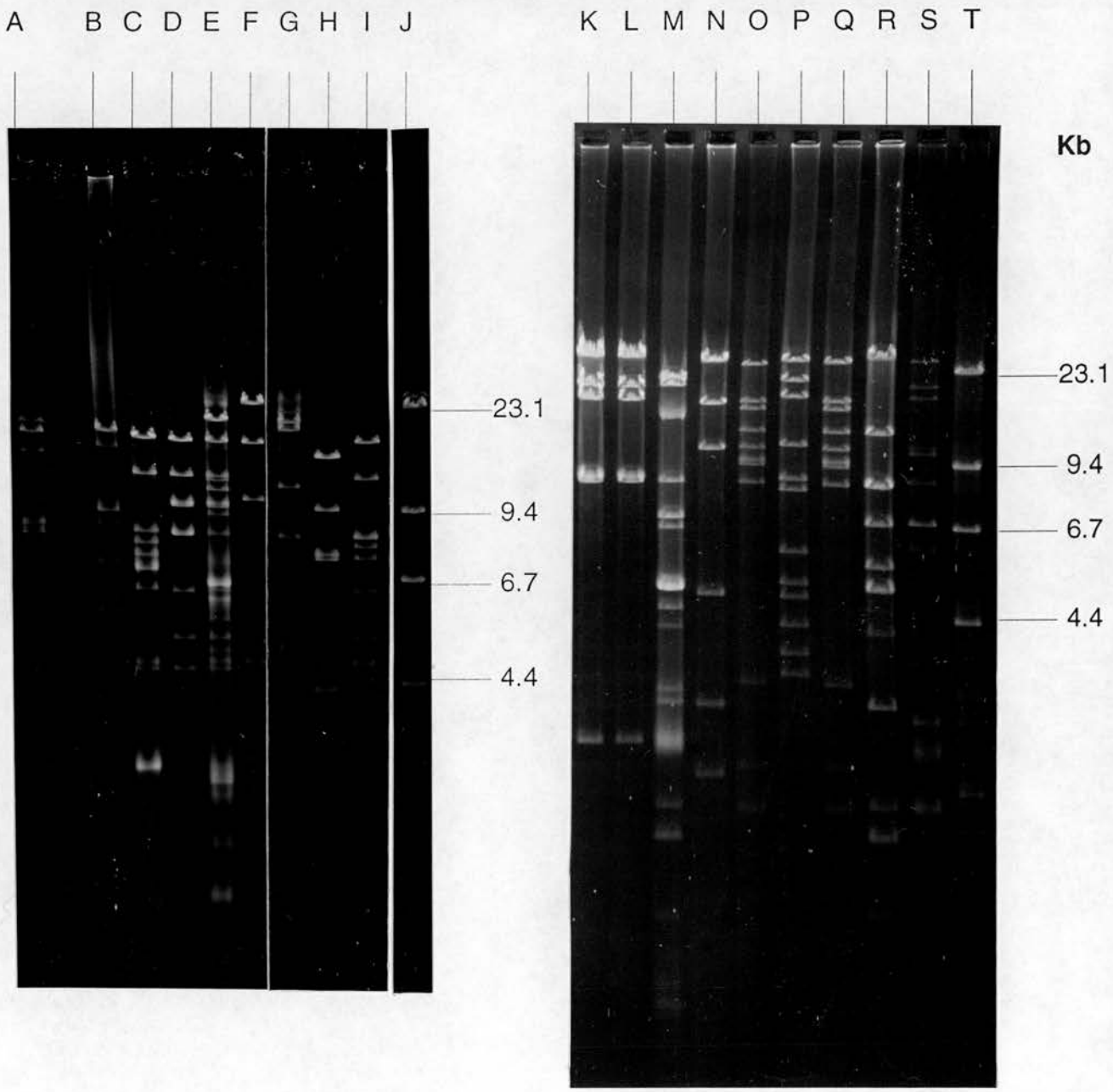


Figure 17. Agarose gel electrophoresis of endonuclease restriction digest of plasmid DNA extracted from normal faecal *E. coli* and from clinical urinary *E. coli*

Tracks:-

A) CT 111; B) ES 75; C) CT 112; D) ES 76; E) λ pre-cut with *Hin* dIII

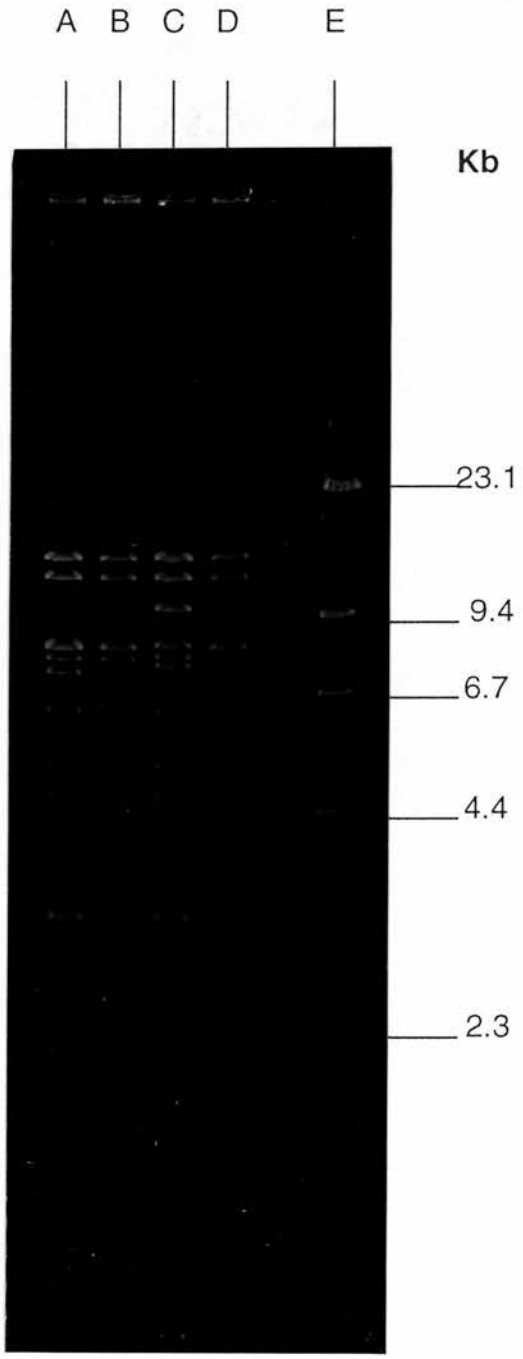
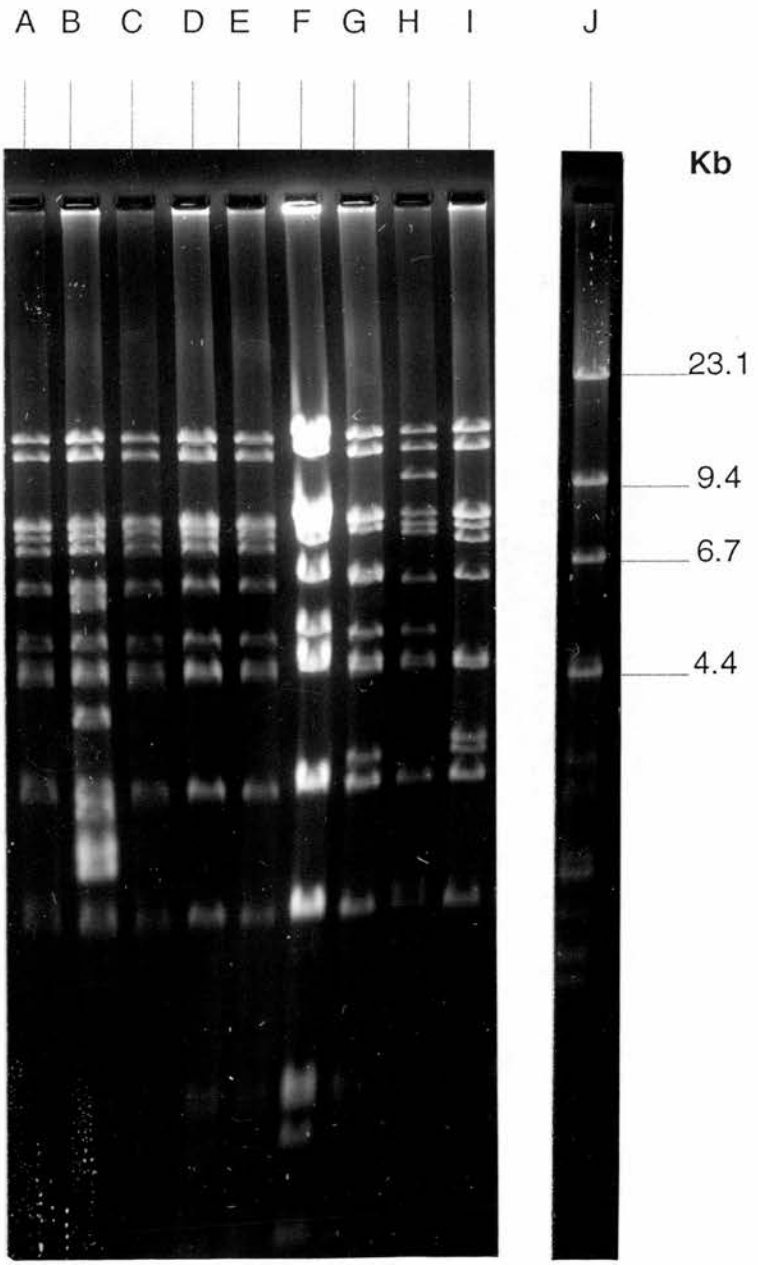


Figure 18. Agarose gel electrophoresis of endonuclease restriction digest of plasmid DNA extracted from normal faecal *E. coli*, from clinical urinary *E. coli* and from *E. coli* containing TRC-1 plasmid

Tracks:-

A) CT 109; B) ES 59; C) CT 110; D) ES 68; E) CT 111; F) CT 82; G) ES 75; H) CT 112; I) ES 76; J) λ pre-cut with *Hin* dIII



3.4. Survey of Antibiotic Resistance in the Aerobic Flora of Diagnostic Faecal Specimens in Edinburgh.

A number of surveys have investigated the carriage of antibiotic resistance amongst the commensal faecal flora of patients prior to their admission to hospital (Datta, 1969; Shaw *et al.*, 1973; Søgaard, 1975). Although clearly this population group may not be considered "healthy", pre-admission patients do, however, provide some indication of the carriage of antibiotic resistant bacteria in the non-clinical community. As a comparison with the survey of antibiotic resistance in the aerobic faecal flora of the healthy population in Edinburgh, a study of the carriage of antibiotic resistance in negative faecal specimens sent to the diagnostic laboratories of Edinburgh Royal Infirmary was performed. This population group, like pre-admission patients, provides an estimation of the carriage of antibiotic resistant bacteria in the non-clinical community.

3.4.1. Study Design

Between January and March 1991, 100 faecal specimens were obtained from the diagnostic laboratory of Edinburgh Royal Infirmary. As outlined in the Materials and Methods, these specimens had been submitted by general practitioners from their patients in the Edinburgh region in order that they may

be screened for the following bacterial pathogens: *Salmonella* spp., *Shigella* spp., *Campylobacter* spp. and *E. coli* 0157. All of the specimens employed in the discussed survey tested negative for each of these pathogens. The sex distribution of the donors of the specimens consisted of 26 males, 31 females and 43 unknown.

3.4.2. The Carriage of Antibiotic Resistance in the Aerobic Faecal Flora

The carriage of antibiotic resistant determinants amongst the bacteria isolated from the faecal specimens is shown in Table 18. The highest incidences of resistance were observed against cefuroxime and ampicillin; 65% and 60% of the faecal specimens contained bacteria resistant to these antibiotics respectively. Resistance to the later generation cephalosporin, ceftazidime, was in contrast, substantially lower at 6%. Bacterial resistance to another antimicrobial agent, tetracycline, was also high with 43% of the specimens containing resistant bacteria. A much lower proportion of specimens carried bacteria resistant to trimethoprim (8%). Similarly, very little bacterial resistance was observed against the quinolone agents tested; nalidixic acid resistant bacteria were identified in only 3% of specimens while there were no ciprofloxacin resistant bacteria. Gentamicin was the only other agent included in the study to which there was also no bacterial resistance.

Table 18. Proportion of specimens containing antibiotic resistant bacteria

Antimicrobial Agents	Concentration (mg/L)	Specimens with resistant bacteria (%)
Ampicillin	10	60
Cefuroxime	4	65
Ceftazidime	2	6
Gentamicin	4	0
Tetracycline	10	43
Nalidixic acid	10	3
Ciprofloxacin	10	0
Trimethoprim	10	8

3.4.3. Identification of the Ampicillin Resistant Strains

As in the previous survey, a single non-mucoid lactose-fermenting colony was isolated and purified from each antibiotic incorporated plate supporting growth. Each of the 60 isolated ampicillin resistant strains were identified employing either API 20E test strips or the biochemical tests outlined in the Materials and Methods. The identified strains were allocated to one of four groups as shown in Table 19. *E. coli* represented the largest proportion of ampicillin resistant bacteria (65%).

Table 19. Identification of the ampicillin resistant bacteria

Species	Number	Percentage
Escherichia coli	39	65
Klebsiella spp.	18	30
Citrobacter spp./ *Enterobacter* spp.	3	5
Other	0	0

All 60 of the ampicillin resistant strains were analysed further.

3.4.4. Sensitivity of Ampicillin Resistant Strains to a Range of β-Lactam Agents

As described previously, each ampicillin resistant strain was tested for its minimum inhibitory concentration of amoxycillin, amoxycillin plus clavulanic acid (2 :1 ratio), cefuroxime, ceftazidime and cefotaxime. The results are shown in Table 20.

A high level of resistance to amoxycillin (MIC > 512 mg/L) was observed in 29/60 (48%) of the ampicillin resistant isolates. These isolates were predominantly *E. coli* (65%). In contrast, the addition of clavulanic acid reduced the MIC of amoxycillin for these *E. coli* to below 32mg/L in all but five cases. Similarly, as in strains isolated from the healthy population, amongst the *E. coli* there was almost uniform sensitivity to cefotaxime and ceftazidime (MIC < 2mg/L). Cefuroxime resistance (MIC > 4mg/L) was, however, identified in 20.5% of the *E. coli*.

Table 20. Minimum Inhibitory Concentration (mg/L) of ampicillin resistant isolates to a range of antimicrobials

Strain No.	Antimicrobial (mg/L)					β-lactamase ✣	Species
	Amox	Amox + Clav	CTZ	CAZ	CXM		
* PS 78	>1024	8	2	2	8	TEM-1	*E. coli*
* PS 124	>1024	16	2	2	4	TEM-1	*E. coli*
* PS 145	>1024	16	2	2	2	TEM-1	*E. coli*
* PS 162	>1024	16	2	2	4	TEM-1	*E. coli*
* PS 165	>1024	16	2	2	2	TEM-1	*E. coli*
* PS 218	>1024	16	2	2	16	TEM-1	*E. coli*
PS 222	>1024	16	2	2	4	TEM-1	*E. coli*
PS 228	>1024	16	2	2	4	TEM-1	*E. coli*
* PS 249	>1024	8	2	2	4	TEM-1	*E. coli*
* PS 81	1024	4	2	2	4	TEM-1	*E. coli*
* PS 87	1024	8	2	2	4	TEM-1	*E. coli*
* PS 99	1024	8	2	2	2	TEM-1	*E. coli*
* PS 110	1024	8	2	2	2	TEM-1	*E. coli*
PS 62	1024	8	2	2	2	TEM-1	*E. coli*
* PS 133	1024	8	2	2	2	TEM-1	*E. coli*
* PS 149	1024	8	2	2	2	TEM-1	*E. coli*
* PS 151	1024	8	2	2	4	TEM-1	*E. coli*
* PS 155	1024	8	2	2	2	TEM-1	*E. coli*
* PS 158	1024	8	2	2	4	TEM-1	*E. coli*
* PS 170	1024	8	2	2	4	TEM-1	*E. coli*
* PS 174	1024	8	2	2	4	TEM-1	*E. coli*
* PS 191	1024	8	2	2	4	TEM-1	*E. coli*
* PS 194	1024	8	2	2	2	TEM-1	*E. coli*
* PS 231	1024	8	2	2	4	TEM-1	*E. coli*
* PS 239	1024	8	2	2	4	TEM-1	*E. coli*
PS 243	1024	8	2	2	4	TEM-1	*E. coli*
* PS 251	1024	8	2	2	2	TEM-1	*E. coli*
* PS 256	1024	8	2	2	2	TEM-1	*E. coli*
* PS 103	512	8	2	2	2	TEM-1	*E. coli*
PS 175	512	32	2	4	16	Chrom	*E. coli*
PS 185	256	32	2	2	16	Chrom	*E. coli*
PS 213	256	32	2	2	16	Chrom	*E. coli*
PS 56	128	32	2	2	8	Chrom	*E. coli*
PS 180	128	8	2	2	4	SHV	*E. coli*
* PS 51	32	32	2	2	16	TEM-1	*E. coli*
* PS 111	16	16	2	2	2	TEM-1	*E. coli*
PS 225	16	8	2	2	8	Chrom	*E. coli*
* PS 92	4	4	2	2	2	TEM-1	*E. coli*
* PS 138	4	4	2	2	2	TEM-1	*E. coli*
* PS 118	1024	32	2	2	64	TEM-1	*Klebsiella*
PS 254	128	2	2	2	2	SHV-1	*Klebsiella*
PS 246	128	2	2	2	4	SHV-1	*Klebsiella*
PS 253	64	2	2	2	2	SHV	*Klebsiella*
PS 237	64	2	2	2	2	SHV-1	*Klebsiella*
PS 209	64	2	2	2	2	SHV-1	*Klebsiella*
* PS 136	64	2	2	2	2	TEM-1	*Klebsiella*
PS 259	32	2	2	2	2	SHV	*Klebsiella*
PS 204	32	4	2	2	2	SHV	*Klebsiella*
PS 198	32	2	2	2	2	SHV	*Klebsiella*
* PS 142	32	2	2	2	2	TEM-1	*Klebsiella*
PS 140	32	2	2	2	2	SHV	*Klebsiella*
PS 129	32	4	2	2	2	K1	*Klebsiella*
* PS 106	32	2	2	2	2	TEM-1	*Klebsiella*
PS 83	32	2	2	2	2	SHV-1	*Klebsiella*
* PS 72	32	2	2	2	2	TEM-1	*Klebsiella*
* PS 66	32	2	2	2	2	TEM-1	*Klebsiella*
* PS 114	16	2	2	2	2	TEM-1	*Klebsiella*
PS 206	16	4	2	2	2	Chrom	*Ent/Cit*
PS 183	16	8	2	2	2	Chrom	*Ent/Cit*
* PS 74	16	8	2	2	8	TEM-1	*Ent/Cit*

* = Transferable strain - see section 3.4.5 ß- lactamase ✣ - see section 3.4.7. SHV = SHV derived enzyme

3.4.5. Transfer of Ampicillin Resistant Determinants

Following the procedure described in the Materials and Methods, each of the ampicillin resistant strains was tested for the ability to transfer the resistance determinants by conjugation to the standard bacterial strain *E. coli* J62-2. Amongst the ampicillin resistant strains, 62% were capable of transferring resistance; this is significantly higher than the transfer rate found amongst the ampicillin resistant strains in the healthy population (33%). From Table 21, it can be observed that the transferable strains were not all the same species. This is also in contrast to the transferable strains from the healthy population which were all identified as *E. coli*. *E. coli* did, however, exhibit the highest rate of transfer amongst the species isolated (78%).

Table 21. Speciation of the transferable strains

Transferable Species	Number	Percentage
Escherichia coli	29	78
Klebsiella spp.	7	19
Citrobacter spp./ *Enterobacter* spp.	1	3

3.4.6. Plasmid Analysis of Ampicillin Resistant Transconjugants

In order to characterise the plasmids in the transconjugants, the plasmids were extracted following the procedure outlined in the Materials and Methods. The similarity in profile and size of these plasmids was investigated by endonuclease restriction analysis with *Eco* R1. The plasmids were assigned to a number of groups according to the endonuclease digest pattern (Figures 19 - 22). As in section 3.1.7., plasmids with closely related endonuclease restriction profiles were assigned to the same category if they differed from one another by no more than three bands. Also, as before, if the profiles differed by three bands or more, the plasmids were assigned to a separate group.

Amongst the plasmids originating in this population group, a widespread heterogeneity was observed in the endonuclease restriction profiles; 21 different plasmid profiles were identified (Table 22). One single type (Group C), however, predominated and accounted for 19% of the plasmids in the isolates. When the banding pattern from the digest profile of this common plasmid was analysed in detail it was identical to the epidemic plasmid identified in the earlier studies. To confirm this finding restriction endonuclease digestions, of both the epidemic plasmid (ES 76) and this common plasmid, were separated side by side in an agarose gel. There was no difference in the pattern and plasmids were obviously of the same plasmid type. Although four other plasmid types (Groups A,B,O &P)

Figure 19. Agarose gel electrophoresis of restriction endonuclease digestion, with *Eco* R1, of plasmid DNA extracted from Edinburgh diagnostic transconjugants

Tracks:-

A) ES 18; B) ES 19; C) ES 21; D) λ pre-cut with *Hin* dIII; E) ES 26; F) ES 27; G) ES 28; H) λ pre-cut with *Hin* dIII

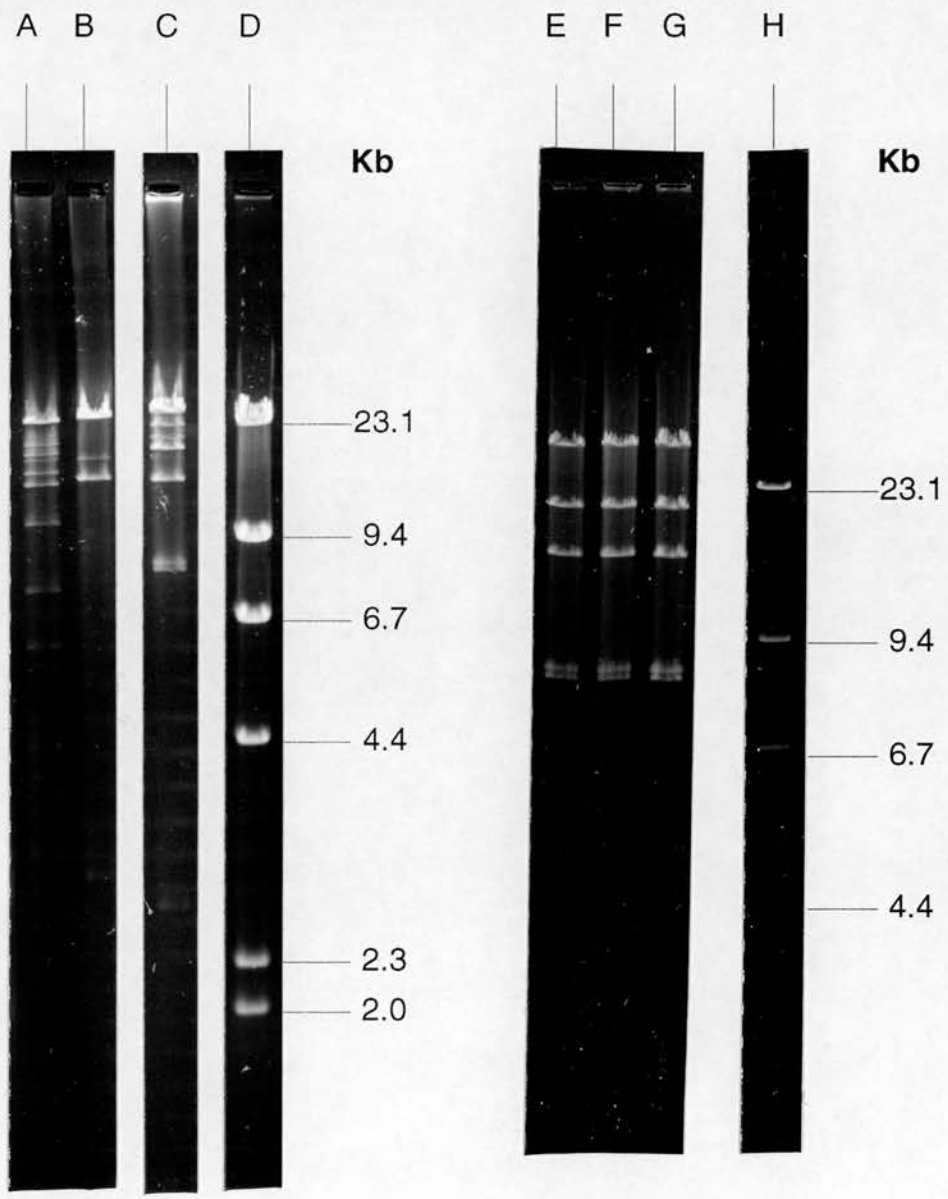


Figure 20. Agarose gel electrophoresis of restriction endonuclease digestion, with *Eco* R1, of plasmid DNA extracted from Edinburgh diagnostic transconjugants

Tracks:-

A) ES 39; B) ES 41; C) ES 42; D) ES 43; E) ES 44; F) ES 45; G) ES 46; H) ES 47; I) ES 40; J) λ pre-cut with *Hin* dIII

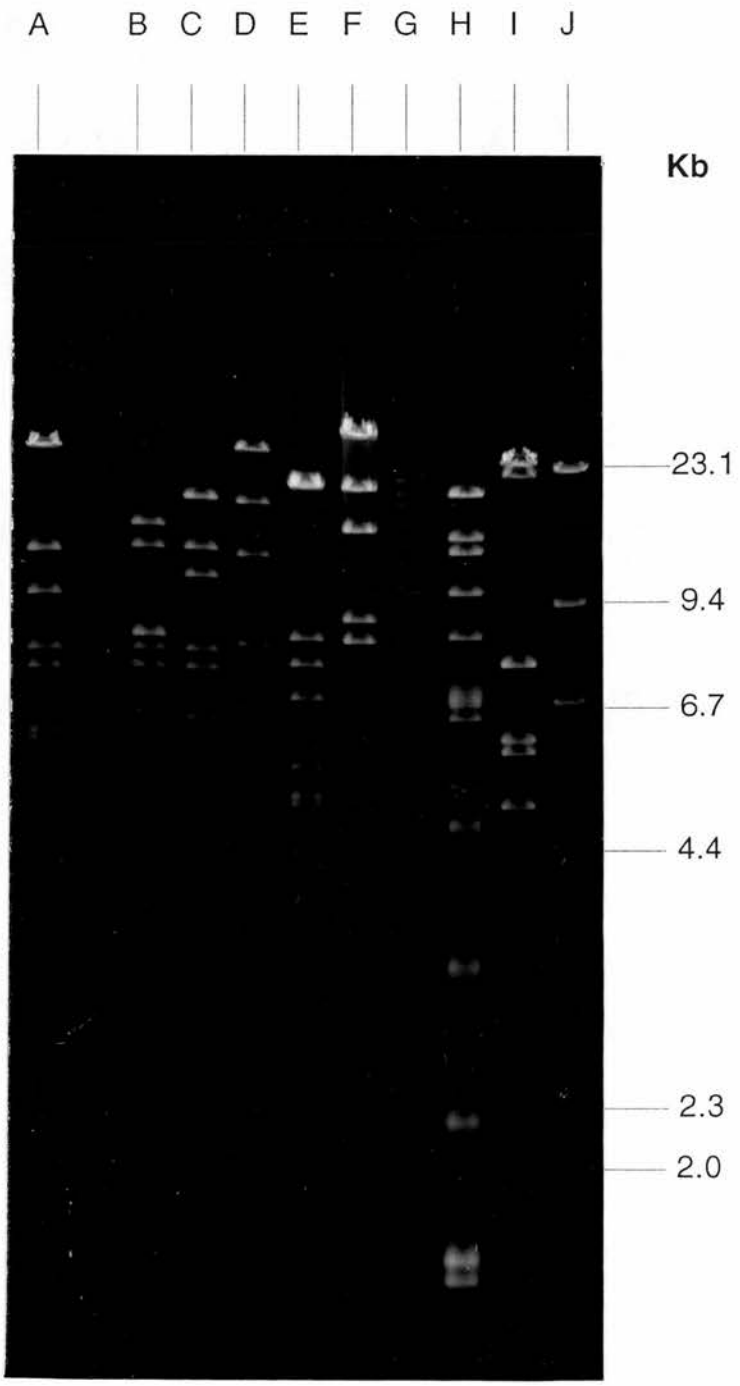


Figure 21. Agarose gel electrophoresis of restriction endonuclease digestion, with *Eco* R1, of plasmid DNA extracted from Edinburgh diagnostic transconjugants

Tracks:-

A) ES 49; B) ES 50; C) ES 51; D) ES 52; E) ES 66; F) ES 70; G) ES 71; H) ES 48; I) λ pre-cut with *Hin* dIII; J) ES 74; K) λ pre-cut with *Hin* dIII

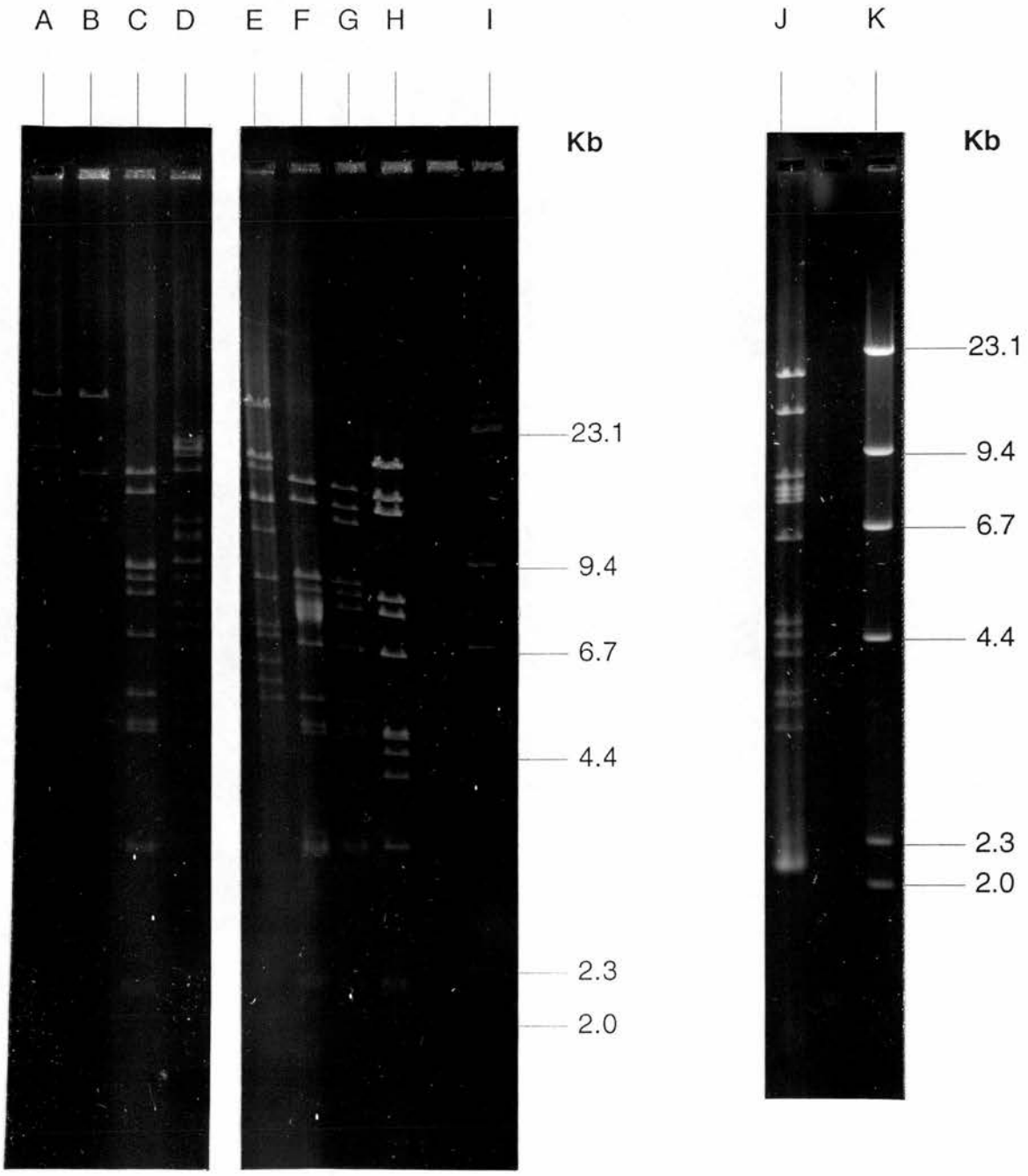


Figure 22. Agarose gel electrophoresis of restriction endonuclease digestion with *Eco* R1 of plasmid DNA extracted from Edinburgh diagnostic transconjugants

Tracks:-

A) ES 85; B) ES 87; C) ES 90; D) ES 94; E) ES 95; F) ES 96; G) ES 97; H) ES 99; I) ES 100; J) ES 101; K) ES 114; L) λ pre-cut with *Hin* dIII

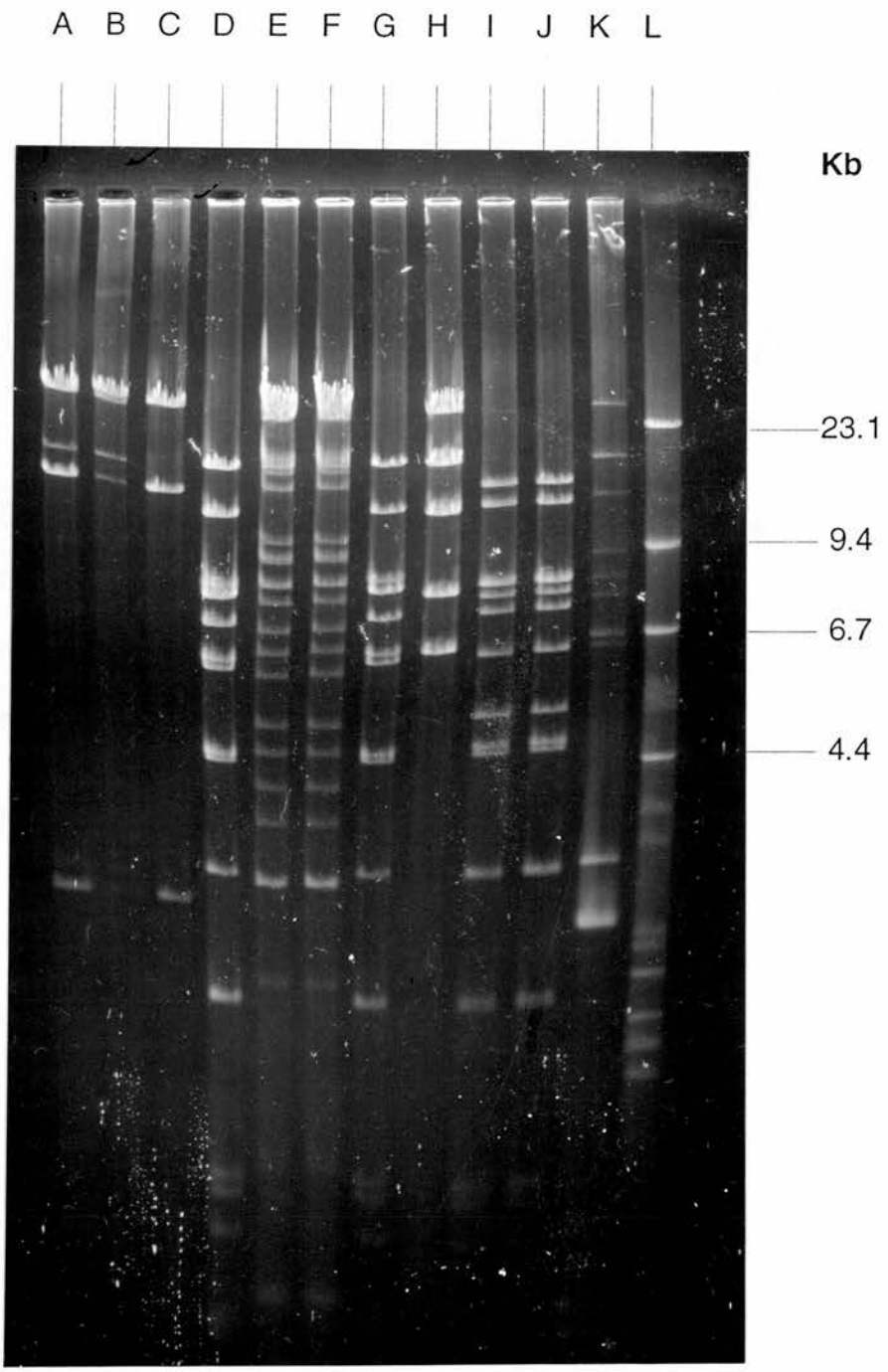


Table 22. Analysis of the diagnostic ampicillin resistant transconjugants

Strain Number	Plasmid Size (Kb)	R. Group	Antibiogram	
			Transconjugant	Wild type
ES 21 (PS 62)	78.0	A	Ap	ApSmTc
ES 26 (PS 72)	78.0	A	Ap	Ap
ES 27 (PS 74)	78.0	A	Ap	ApSpCf
ES 28 (PS 78)	78.0	A	Ap	ApSm
ES 19 (PS 99)	49.0	B	Ap	Ap
ES 85 (PS 51)	49.0	B	Ap	ApCfCm
ES 87 (PS 66)	49.0	B	Ap	Ap
ES 90 (PS 92)	49.0	B	Ap	Ap
ES 41 (PS 149)	85.0	C	ApSm	ApSmTc
ES 48 (PS 170)	85.0	C	ApSm	ApSm
ES 51 (PS 194)	85.0	C	ApSm	ApSmSp
ES 70 (PS 249)	85.0	C	ApSm	ApSm
ES 71 (PS 251)	85.0	C	ApSm	ApSm
ES 100 (PS 138)	85.0	C	ApSm	ApSm
ES 101 (PS 142)	85.0	C	ApSm	ApSm
ES 39 (PS 133)	81.7	D	ApSm	ApSm
ES 42 (PS 151)	98.0	E	ApSm	ApSmSp
ES 43 (PS 155)	66.9	F	Ap	Ap
ES 44 (PS 231)	85.0	G	Ap	ApTc
ES 45 (PS 239)	75.1	H	ApSmTc	ApSmTc
ES 46 (PS 158)	117.4	I	ApTc	ApSpTc
ES 47 (PS 162)	84.3	J	Ap	Ap
ES 49 (PS 174)	139.4	K	ApTpSmSpCmTc	ApTpSmSpCmTc
ES 50 (PS 191)	56.5	L	ApSp	ApSmSp
ES 52 (PS 103)	139.5	M	ApTpSmTc	ApTpSmSpTc
ES 66 (PS 256)	137.9	N	Ap	ApSm
ES 94 (PS 106)	74.8	O	Ap	Ap
ES 95 (PS 111)	59.9	P	ApTc	ApCf
ES 96 (PS 114)	59.9	P	Ap	Ap
ES 97 (PS 118)	74.8	O	Ap	ApCerCf
ES 99 (PS 136)	60.6	Q	Ap	ApSp
ES 114 (PS 218)	92.7	R	Ap	ApSpCer
ES 18 (PS 87)	129.0	S	ApSm	ApTpSmCmTc
ES 74 (PS 249)	92.0	T	ApSm	ApSm
ES 40 (PS 145)	52.6	U	Ap	ApTc
* ES 29 (PS 81)	-	-	Ap	ApSmSpTc
* ES 38 (PS 124)	-	-	Ap	ApTpSmSpCerTc

() = Original isolate number

R. Group = Restriction pattern group

* = No distinct profile obtained after three separate plasmid preparations and endonuclease restriction digestions

were each isolated on more than one occasion in different transconjugants (Figures 19-22), no other plasmid type was found as frequently.

3.4.7. Distribution of β-Lactamases in the Ampicillin Resistant Strains

The different β-lactamases responsible for mediating resistance to ampicillin amongst the strains isolated, were determined by iso-electric focusing. All of the transconjugants encoded the TEM-1 β-lactamase (Figure 23) regardless of the plasmid type on which the gene was carried. As in the survey of antibiotic resistance in the aerobic faecal flora of the healthy population in Edinburgh, the non-transferable strains which were also examined (Figures 24 - 25) encoded a variety of different β-lactamases.

Figure 23. Analytical iso-electric focusing of β-lactamases in the Edinburgh diagnostic transconjugants

Tracks:-

A) ES 18; B) ES 19; C) ES 21; D) ES 26; E) ES 27; F) R1 (TEM-1 producer); G) ES 28; H) ES 29; I) ES 39; J) ES 40

These selected TEM-1 producing transconjugants are representative of all of the transconjugants examined.

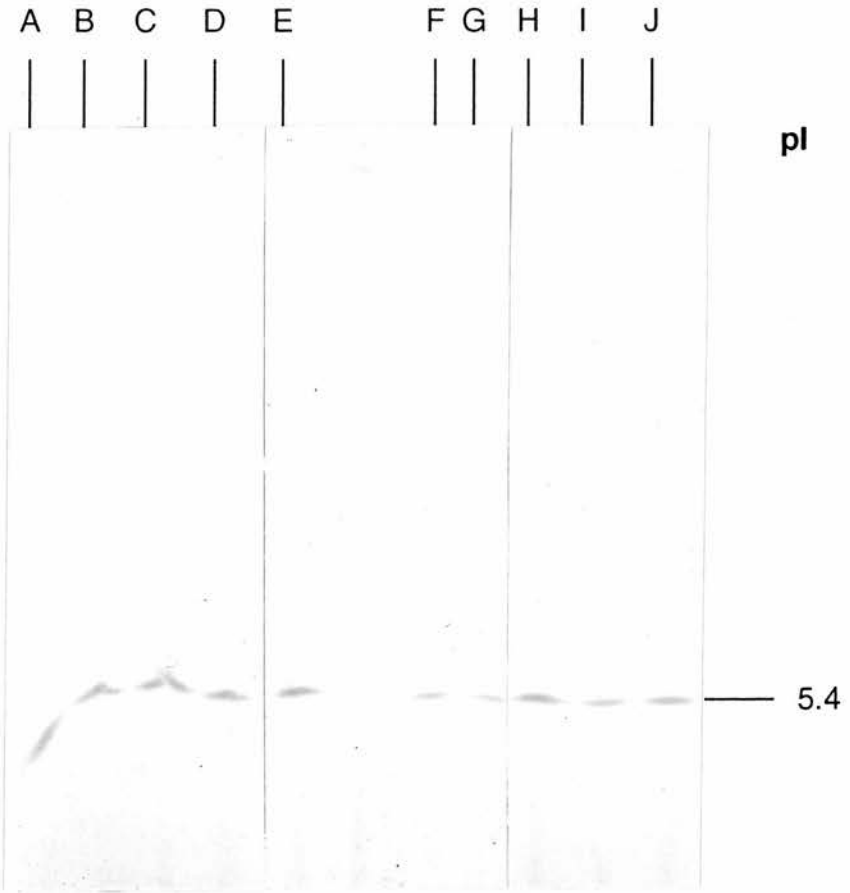


Figure 24. Analytical iso-electric focusing of β-lactamases isolated from wild type, non-transferable ampicillin resistant diagnostic strains in Edinburgh

Tracks:-

A) PS 204; B) PS 206; C) PS 209; D) PS 213; E) PS 222; F) R1 (TEM-1 producer); G) R1010 (SHV-1 producer); H) PS 225; I) PS 228; J) PS 237; K) PS 243; L) PS 246; M) PS 253; N) R1010; O) PS 254; P) PS 259; Q) R1

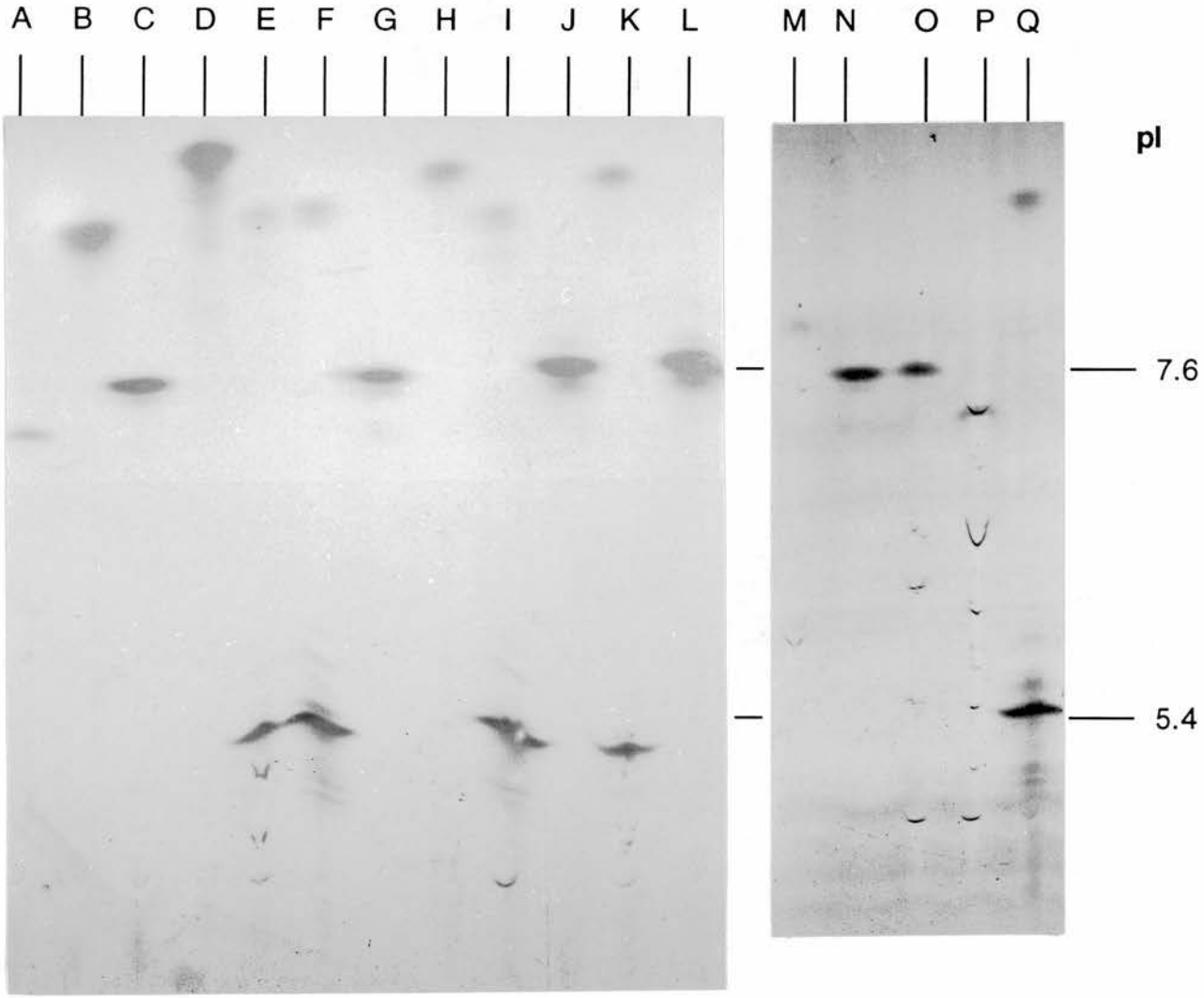
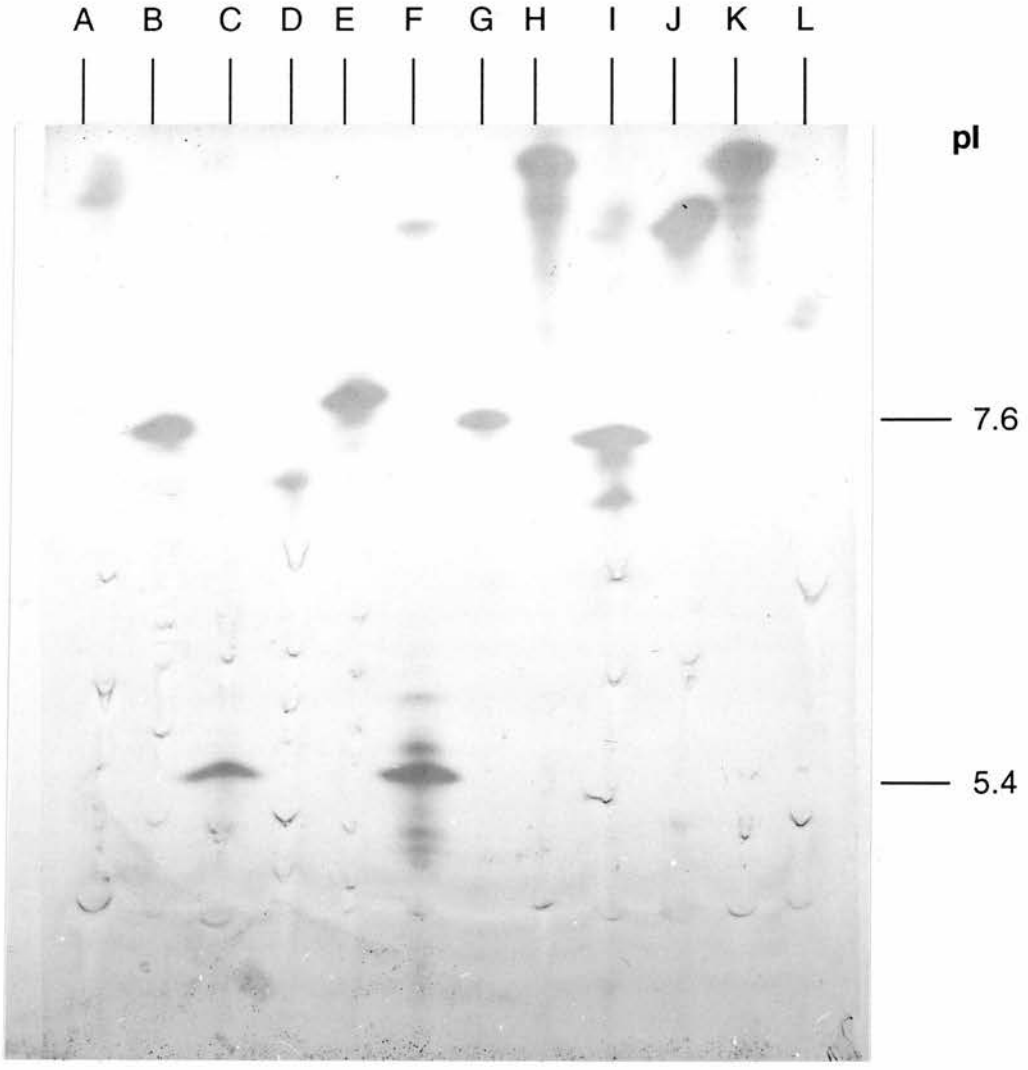


Figure 25. Analytical iso-electric focusing of β-lactamases isolated from wild type, non-transferable ampicillin resistant diagnostic strains in Edinburgh

Tracks:-

A) ES 56; B) ES 83; C) ES 110; D) ES 129; E) ES 140; F) R1 (TEM-1 producer); G) R1010 (SHV-1 producer); H) ES 175; I) ES 180; J) ES 183; K) ES 185; L) ES 198



3.5. Survey of Antibiotic Resistance in the Aerobic Faecal Flora of the Healthy Black Communities in South Africa.

From the limited studies conducted to determine the carriage of antibiotic resistance genes amongst the commensal faecal flora of healthy individuals, it appears that in this group, as in clinical isolates, there is a higher rate of resistance associated with the use of antibiotics in developing countries rather than developed countries.

3.5.1. Study Design

Between January and March 1992, a total of 361 faecal specimens were collected from healthy volunteers resident in the Transvaal, South Africa, as outlined in the Materials and Methods. Participants were included from both rural (183) and urban (178) areas. As in the Edinburgh study, no volunteer was allowed to participate in the survey if they had received any antimicrobial treatment in the three weeks prior to sampling. Each participant completed a questionnaire, the information from which has been collated and is represented in Table 23. On average, the male / female distribution was 1:1.4 in both rural and urban groups. Volunteers indicated a regular intake of meat (Table 23) although amongst the urban population, meat was included in the diet more frequently.

Table 23. Volunteers in the study

Location/ Study Group	Total Number Individuals	Number of Females	AGE Range (yrs)	AGE Mean (yrs)	AGE SD	Mode Meat/Week
Rural Study						
Infants	50	29	0-5	0.75	0.82	3
Children	47	26	6-11	9.1	1.42	3
Teenagers	36	19	12-19	14.0	1.24	3
Adults	50	35	>19	28.4	10.12	3
Total	183	109				
Urban Study						
Infants	45	23	0-5	2.4	1.28	4
Children	42	18	6-11	9.1	1.69	4
Teenagers	47	22	12-19	13.1	0.93	7
Adults	44	42	>19	33.0	6.06	3
Total	178	105				
Grand Total	361	214				

3.5.2. The Carriage of Antibiotic Resistance in the Aerobic Faecal Flora

The carriage of antibiotic resistant determinants amongst the commensal faecal flora of the healthy population examined in South Africa is shown in Table 24. The protocol employed in the isolation of resistant bacteria is described in the Materials and Methods. A high proportion of volunteers carried bacteria resistant to antibiotics, in particular to ampicillin (88.6%) and trimethoprim (74.2%). Five antibiotics were screened in the study and, for each, the corresponding level of resistance identified was slightly higher amongst the rural populations. There was no difference in the level of resistance to each antimicrobial agent and age group except that gentamicin resistance in the rural adults was much higher than it had been amongst their urban counterparts (38.6% versus 4%).

3.5.3. Recognition of Environmental Conditions

In an attempt to establish if environmental conditions exerted any influence over the carriage of antibiotic resistance in bacteria, various factors were examined.

Table 24. Percentage of specimens with organisms resistant to five antibiotics in South Africa

	Antibiotic									
	Urban					Rural				
Age (Years)	Ap	Tp	Cm	NA	Gm	Ap	Tp	Cm	NA	Gm
0 - 5	94.0	80.0	66.0	4.0	10.0	100	88.9	66.7	44.0	2.2
6 - 11	89.4	72.3	38.3	4.2	0	76.2	54.8	40.5	14.2	0
12 - 19	75.0	61.1	30.5	13.9	0	87.2	72.3	44.7	14.9	4.2
>19	84.0	68.0	44.0	10.0	4.0	100	93.2	86.4	18.2	38.6
Total	86.3	71.0	45.9	7.7	3.8	91.0	77.5	59.9	12.9	11.2

3.5.3.1. Water Supply

A representative water sample was taken in each study area except in Kagiso (Table 25). Screening in the urban locations revealed the water, which is municipally piped, to be free of any bacteria (< 1 organism / 50ml). In contrast, the school in Hekpoort is provided with water from a bore hole; this was contaminated with four different bacteria, *Bacillus cereus, Acinetobacter anitratus, Enterobacter cloacae* and *Pseudomonas aeruginosa* (> 1000 organisms / ml). In the Shongwe area, the rural adult population was not exposed to a contaminated water supply as the water utilized at Shongwe hospital was filtered and chlorinated river water. This water was completly bacteria-free (< 1 organism / 50ml). However, the water tested from Middleplaas clinic, stored rain water, contained *Pseudomonas aeruginosa, Serratia marcescens* and a Gram-positive coccus (>1000 organism/ml).

3.5.3.2. Living Conditions

The social conditions of the participants are reflected in Table 25. In both the rural and urban study, approximately 50% of the participants were associated with animals. Despite the possibility of animal to man transmission of bacteria, this was probably not a major contributory factor in the spread of resistance as resistant bacteria were as commonly observed in participants living in the absence of animals.

Table 25. Social conditions of volunteers in the survey

Location	Mean No./Family	Mean No./House	% with Animals	% Water Source = TAP	Water Contaminated
Rural Study					
Hekpoort	7.0	6.2	61	79	+
Shongwe	7.4	7.7	1	88	+
Urban Study					
SOWETO	8.6	8.7	68	99	-
Kagiso	6.0	6.3	50	100	NT

NT = Not tested

3.5.3.3. Population Density

The most influential environmental factors observed were the number of people per family and per house. Many of the rural homes were wattle and daub huts with only one room for sleeping. Similarly, it was not uncommon for many urban homes to have just one bedroom. As similar numbers of residents per house (mean = 6.0 people/house) were recorded in both the rural and urban areas, it would seem that this widespread over-crowding is a large contributor to the high levels of resistance.

3.5.4. Identification of the Ampicillin Resistant Strains

A total of 608 ampicillin resistant strains were collected from the survey. Between one and four morphologically distinct colonies were isolated from each ampicillin plate although all were lactose-fermenting strains. Each of the strains was identified (Table 26) employing the biochemical tests described in the Materials and Methods. *E. coli* represented the largest proportion of resistant bacteria when susceptibility to ampicillin was measured.

Table 26. The incidence of organisms isolated from the study

Species	Number	Percentage
E. coli	445	73
Klebsiella spp.	104	17
Enterobacter spp./ *Citrobacter* spp.	35	6
Other	24	4

The ampicillin resistant strains were analysed further.

3.5.5. Transfer of Ampicillin Resistant Determinants

The ability of the ampicillin resistant strains to transfer their resistance determinants to *E. coli* J62-2 was tested. Amongst the 608 strains, 158 (26%)

were shown to contain self-transmissible plasmids. There was a significant variation in the proportion of transferable strains in each population group. A particularly high carriage rate of transferable strains was found amongst the rural infants (74%) (Table 27). The transferable strains, however, were not restricted to one species (Table 28) although *E. coli* was found to predominate (67.7%).

Table 27. Proportion of transferable strains in the different population groups

Location/ Study Group	Total No. of Transconjugants	Total No. of Isolates	Percentage
Rural Study			
Infants	60	81	74
Children	17	54	31
Teenagers	9	82	11
Adults	12	112	10
Urban Study			
Infants	22	80	27
Children	16	66	24
Teenagers	11	53	20
Adults	11	80	14

Table 28. Proportion of transferable ampicillin resistant species

Species	Number	Percentage
E. coli	107	67.7
Klebsiella spp.	20	12.7
Enterobacter spp./ *Citrobacter* spp.	3	1.9
Other	28	17.7

3.5.6. Plasmid Analysis of Ampicillin Resistant Transconjugants

As outlined in the Materials and Methods, plasmids were extracted from the transconjugants and restricted with *Eco* R1. All of the restricted plasmid profiles investigated from each transconjugant are shown in Figures 26 to 40. As before, the plasmids were assigned to groups, categorised as described previously (see section 3.1.7.). Amongst the 158 transconjugants which had been obtained, a total of 92 different plasmid profiles were identified (Figures 26 - 40). The groups to which the different plasmid types were delegated are shown in Tables 29 to 36. In addition, the calculated size (Kb) for each plasmid identified, the donor number from which the wild type strains were isolated and the antibiogram of both the transferable wild type strains and the transconjugants are also shown in these

tables. In determining the antibiogram profile, the ability of the strains to confer resistance to a range of antimicrobials including amoxycillin (10mg/L), amoxycillin plus clavulanic acid (10:5mg/L), trimethoprim (10mg/L), gentamicin (4mg/L), cephaloridine (10mg/L), cefuroxime (4mg/L), cefotaxime (1mg/L), nalidixic acid (10mg/L), ciprofloxacin (1mg/L), tetracycline (10mg/L), chloramphenicol (10mg/L), spectinomycin (10mg/L) and streptomycin (10mg/L) were all tested. Whilst a wide variety of antibiogram profiles were identified amongst the transferable wild type strains, between the transconjugants the most frequently recognised resistant determinants were those of trimethoprim and ampicillin.

From the tables, it is apparent that in the vast majority of cases, different plasmids were identified from within each of the different donors. However, on two occasions, the same plasmid was isolated from two separate strains from the same donor. Amongst the rural infants, a plasmid of 65.1Kb was carried by two separate strains in donor 11 (Table 29). As both of the donor strains share the same antibiogram, it may be concluded that the same strain had been isolated twice from the same donor. Similarly, this situation occurred amongst the urban infants where a plasmid of 89.3Kb was carried by two separate strains in donor 43 (Table 33). On a further seven occasions the same plasmid was also isolated from two separate strains from the same donor. However, in contrast to the preceding situation, the antibiogram of the wild type strains differed from each other indicating the presence of the same plasmid in different

strains in the same donor. This phenomenon was restricted to rural infants in donor numbers 1, 9, 24, 28, and 29 (Table 29), and again in urban adults in donor numbers 4 and 13 (Table 36).

The distribution of plasmids in each of the population groups will be considered in turn.

3.5.6.1. Rural Infants

The characteristics of the plasmids extracted from each of the rural infant isolates are summarised in Table 29 while the endonuclease restriction profiles of these plasmids are shown in Figures 26 - 30. Amongst this particular group of transconjugants, three distinct plasmid types were each identified on more than three occasions. The most frequently isolated plasmid belonged to plasmid group 16 and was calculated to be 65.1Kb. It represented 14 of the 58 plasmids analysed within this population. Amongst those antimicrobials tested, this plasmid conferred resistance only to ampicillin and trimethoprim. The second such plasmid, from group 26, was found in 9 transconjugants. This plasmid mediated resistance to ampicillin and tetracycline and was 61.6Kb. The final plasmid to be discovered on multiple occasions (7) was 49.8Kb and belonged to plasmid group 18. As with the first, frequently isolated plasmid, this plasmid carried ampicillin and trimethoprim resistant determinants. The remainder of the plasmids in this group were identified in either one, two or at most, in three transconjugants.

Table 29. Antibiogram and restriction profiles of transconjugants obtained from rural infants

Strain Number	Donor Number	Plasmid Group	Plasmid Size (Kb)	Antibiogram	
				Transconjugant	Wildtype
PS 1236	1	16	65.1	ApTp	ApSmTcTp
PS 1237	1	16	65.1	ApTp	ApTp
PS 1244	6	16	65.1	ApTp	ApTp
PS 1245	7	16	65.1	ApTp	ApSpSmAcTcTp
PS 1247	8	16	65.1	ApTp	ApSmTpCm
PS 1249	9	16	65.1	ApTp	ApSmAcCerTcTpCm
PS 1251	9	16	65.1	ApTp	ApSmTp
PS 1252	10	16	65.1	ApTp	ApSpSmAcTcTp
PS 1254	11	16	65.1	ApTp	ApSpSmTp
PS 1255	11	16	65.1	ApTp	ApSpSmTp
PS 1262	16	16	65.1	ApTp	ApSmCtxTcTpCm
PS 1263	18	16	65.1	ApTp	ApSpSmTp
PS 1265	19	16	65.1	ApTp	ApSpAcTp
PS 1268	21	16	65.1	ApTp	ApSmAcCerTcTpCm
PS 1239	2	17	65.9	ApTp	ApSmTp
PS 1240	3	17	65.9	ApTp	ApTp
PS 1283	37	17	65.9	Ap	ApSmTp
PS 1238	1	18	49.8	ApTp	ApSpSmTp
PS 1250	9	18	49.8	ApTp	ApCerTp
PS 1253	10	18	49.8	ApTp	ApTp
PS 1259	14	18	49.8	ApTp	ApTp
PS 1260	15	18	49.8	ApTp	ApSmTp
PS 1261	16	18	49.8	ApTp	ApTp
PS 1264	18	18	49.8	ApTp	ApSpSmTp
PS 1241	3	19	82.5	ApTp	ApTcTp
PS 1242	4	19	82.5	ApTp	ApSmTcTp
PS 1243	6	19	82.5	ApTp	ApSmTp
PS 1246	8	20	116.7	ApTp	ApSpSmTp
PS 1248	8	21	45.3	Ap	ApSpTcTp
PS 1256	13	22	97.3	ApTp	ApSmTp

Table 29. Continued

Strain Number	Donor Number	Plasmid Group	Plasmid Size (Kb)	Antibiogram	
				Transconjugant	Wildtype
PS 1257	13	23	58.8	Ap	ApSmTcTp
PS 1258	14	24	89.4	ApSm	ApSmTp
PS 1269	22	25	134.5	ApTc	ApSmTcCm
PS 1270	23	25	134.5	ApTc	ApSmTcTpCm
PS 1266	20	26	61.6	ApTc	ApSmTcTpCm
PS 1271	23	26	61.6	ApTc	ApSmAcTcTp
PS 1272	24	26	61.6	ApTc	ApSmAcCerTcTp
PS 1273	24	26	61.6	ApTc	ApSpSmAcTc
PS 1274	25	26	61.6	ApTc	ApSmTpTc
PS 1276	28	26	61.6	ApTc	ApSmTcCm
PS 1277	28	26	61.6	ApTc	ApSmAcCerCtxTcTpCm
PS 1278	29	26	61.6	ApTc	ApAcCerTc
PS 1279	29	26	61.6	ApTc	ApSmAcCerCtxTcTpCm
PS 1267	20	27	63.3	ApTp	ApSmTp
PS 1282	35	27	63.3	ApTp	ApTp
PS 1275	27	28	42.1	ApTpSm	ApSmTp
PS 1281	35	28	42.1	Ap	ApSmTp
PS 1280	33	29	68.5	ApSpSmTc	ApSpSmTcTp
PS 1284	37	30	60.0	ApSmTcTpCm	ApSmTcTpCm
PS 1288	41	30	60.0	ApSmTcCm	ApSmTcTpCm
PS 1285	39	31	78.7	Ap	ApSmTp
PS 1286	40	32	30.5	Ap	Ap
PS 1287	40	33	119.5	Ap	Ap
PS 1289	42	34	64.4	ApTp	ApSmTp
PS 1290	42	35	48.3	ApTp	ApSmTp
PS 1291	42	36	51.9	ApTp	ApSmTp
PS 1292	43	37	19.5	Ap	ApSpSmAcTcTpCm
PS 1294	31	38	77.7	ApTp	ApSmAcTcTp
PS 1293	31	88	50.1	ApTp	ApSmAcTcTp

Figure 26. Agarose gel electrophoresis of an endonuclease restriction with *Eco* R1 of plasmid DNA extracted from South African rural infant transconjugants

Tracks:-

A) PS 1236; B) PS 1237; C) PS 1238; D) PS 1239; E) PS 1240; F) PS 1241; G) PS 1242; H) PS 1243; I) PS 1244; J) PS 1245; K) λ DNA pre-cut with *Hin* dIII

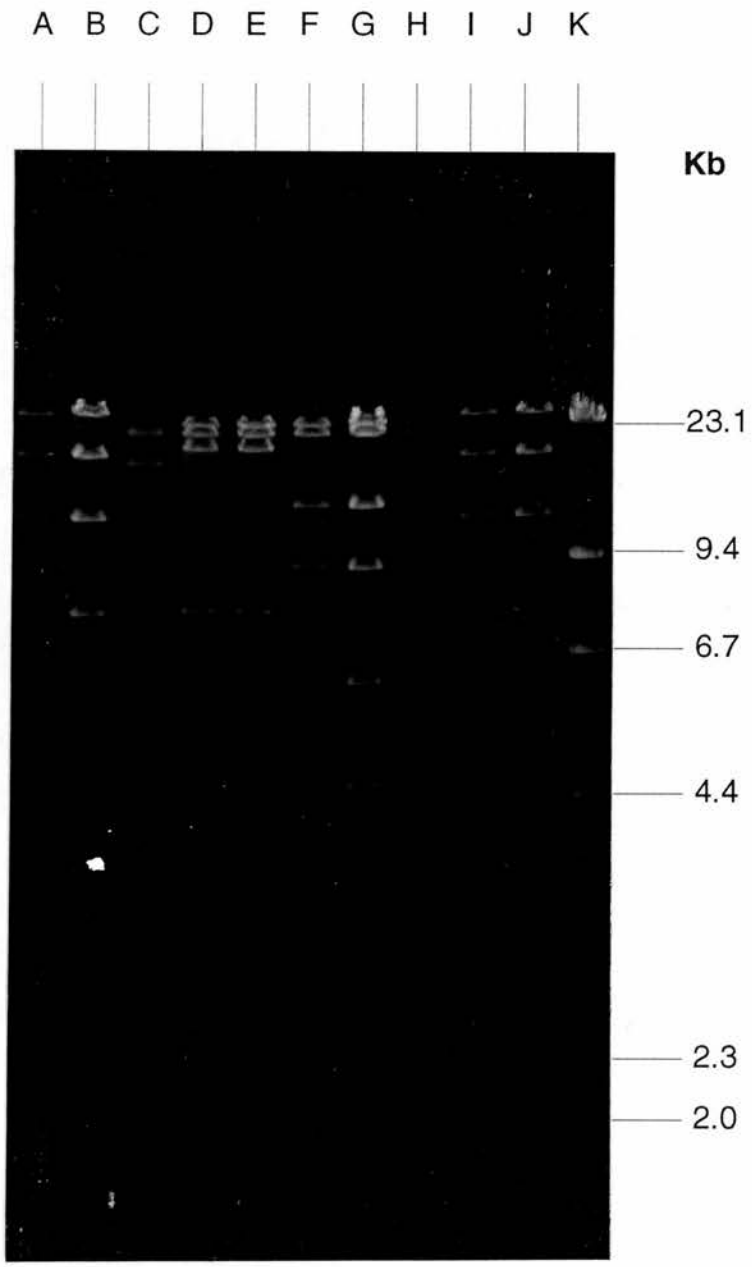


Figure 27. Agarose gel electrophoresis of an endonuclease restriction with *Eco* R1 of plasmid DNA extracted from South African rural infant transconjugants

Tracks:-

A) PS 1246; B) PS 1247; C) PS 1248; D) PS 1249; E) PS 1250; F) PS 1252; G) PS 1253; H) PS 1254; I) PS 1255; J) PS 1256; K) PS 1257; L) λ DNA pre-cut with *Hin* dIII; M) PS 1251; N) λ DNA pre-cut with *Hin* dIII

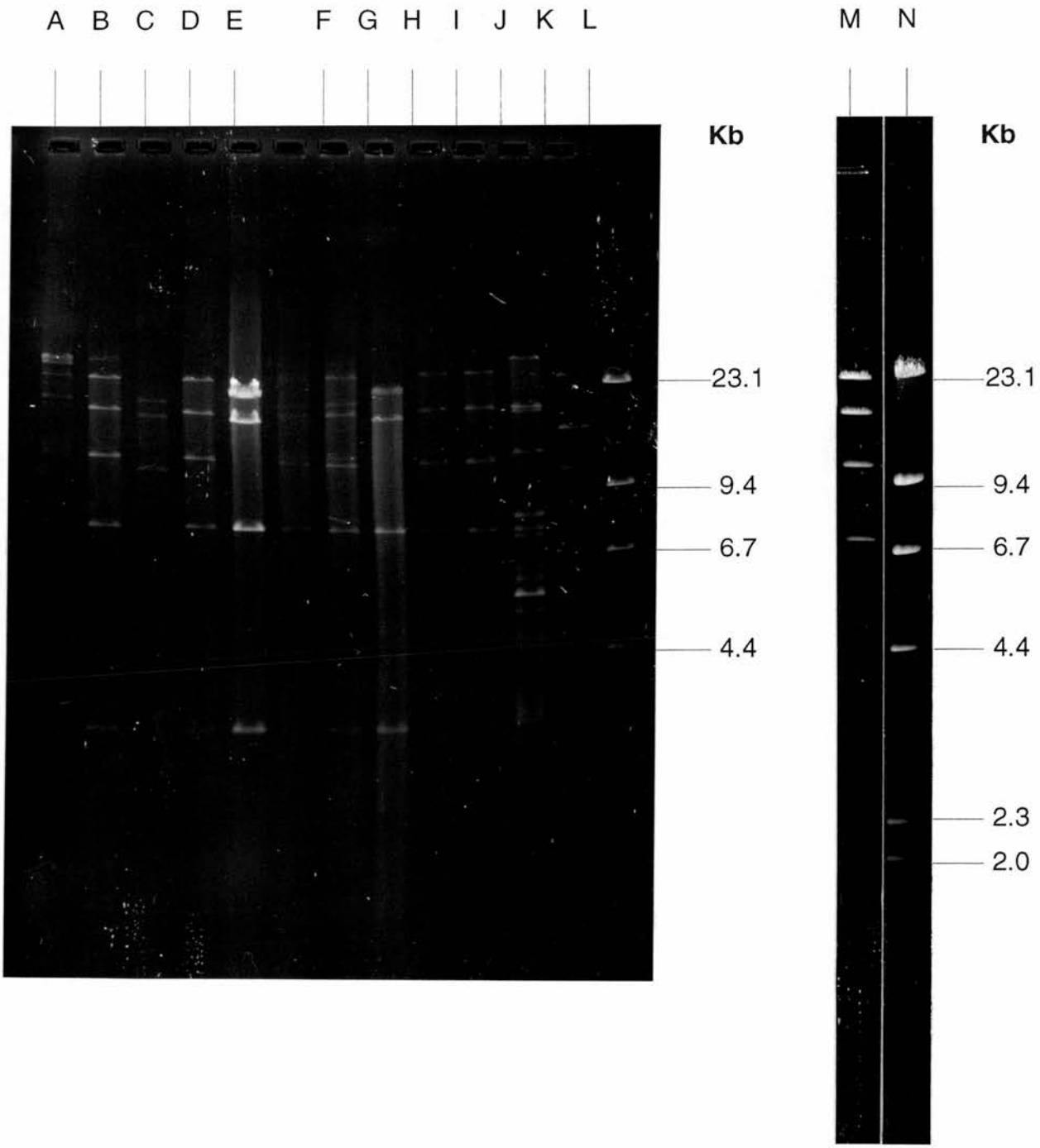


Figure 28. Agarose gel electrophoresis of an endonuclease restriction with *Eco* R1 of plasmid DNA extracted from South African rural infant transconjugants

Tracks:

A) PS 1258; B) PS 1259; C) PS 1260; D) PS 1261; E) PS 1262; F) PS 1263; G) PS 1264; H) PS 1265; I) PS 1266; J) PS 1267; K) PS 1268; L) PS 1269; M) λ DNA pre-cut with *Hin* dIII

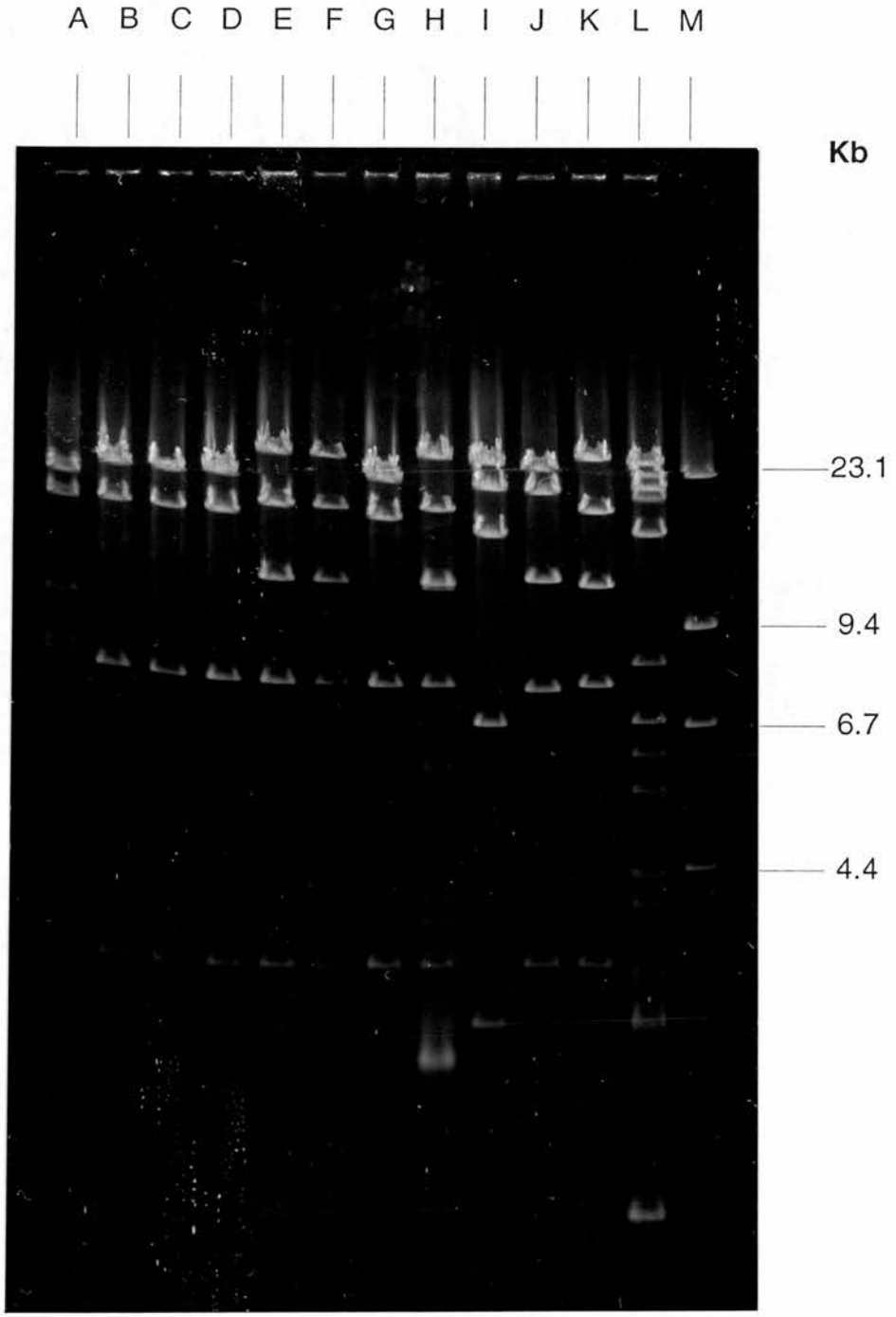


Figure 29. Agarose gel electrophoresis of an endonuclease restriction with *Eco* R1 of plasmid DNA extracted from South African rural infant transconjugants

Tracks:-

A) PS 1270; B) PS 1271; C) PS 1272; D) PS 1273; E) PS 1274; F) PS 1275; G) PS 1276; H) PS 1277; I) PS 1278; J) PS 1279; K) λ DNA pre-cut with *Hin* dIII; L) PS 1294; M) λ DNA pre-cut with *Hin* dIII

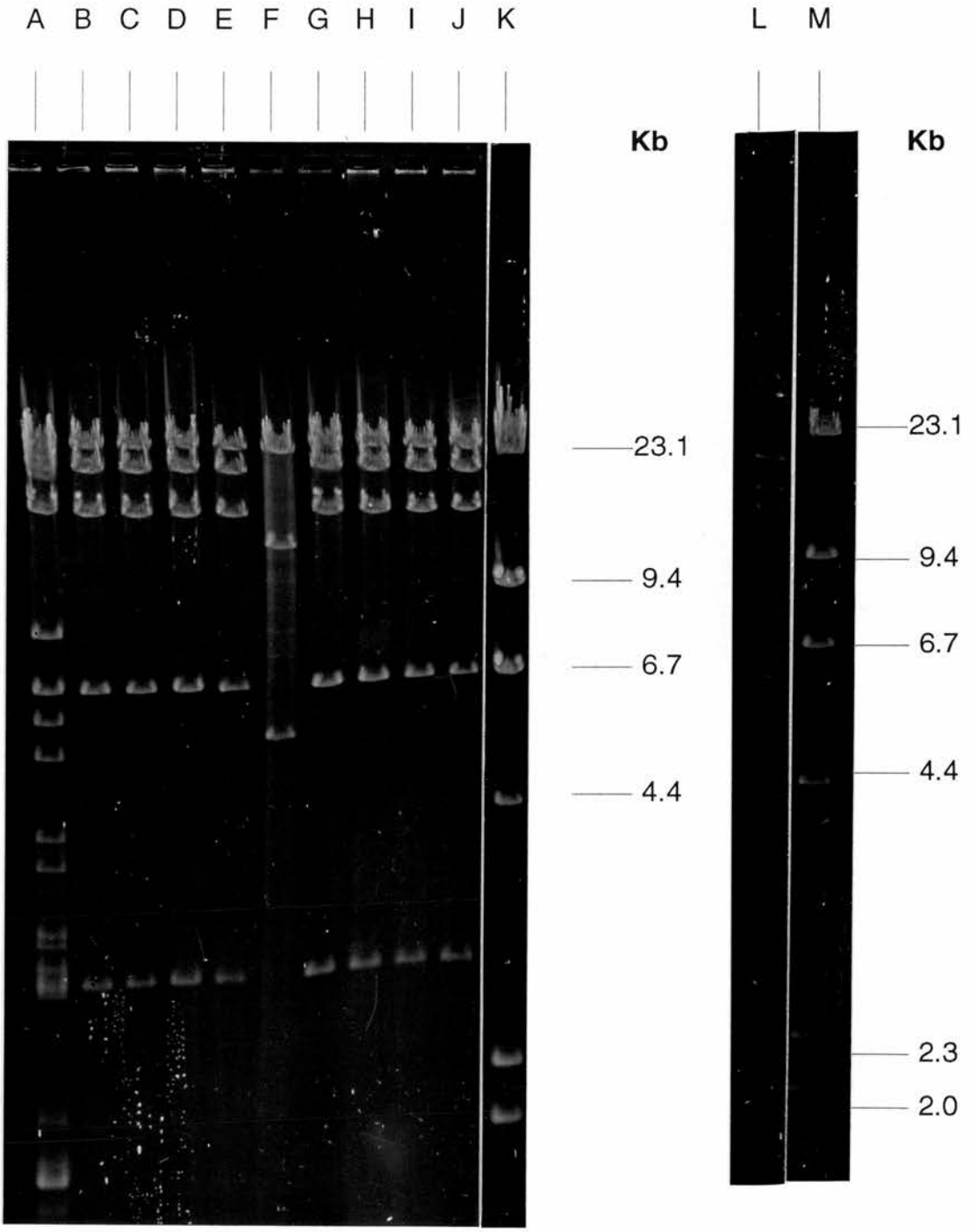
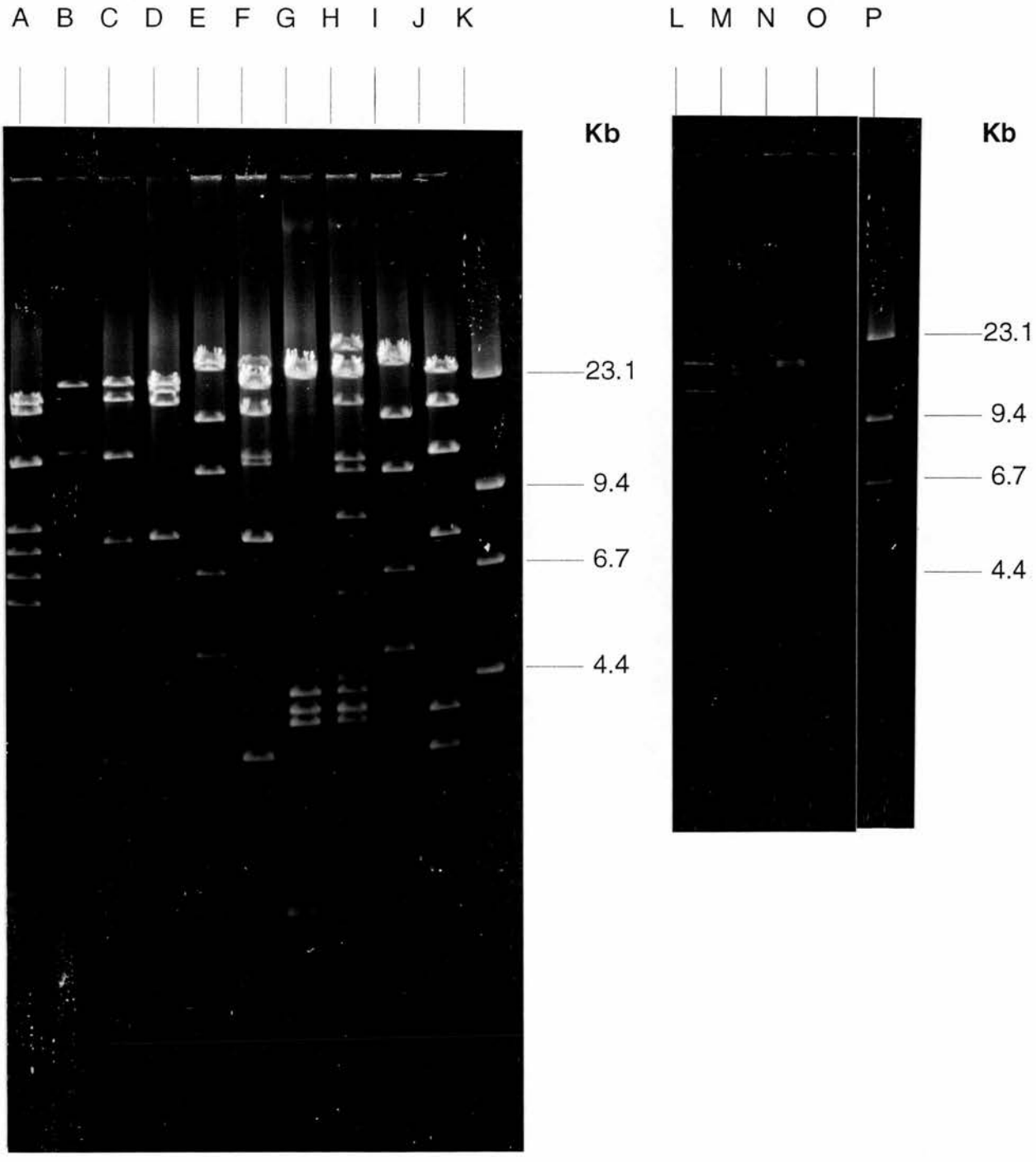


Figure 30. Agarose gel electrophoresis of an endonuclease restriction with *Eco* R1 of plasmid DNA extracted from South African rural infant transconjugants

Tracks:-

A) PS 1280; B) PS 1281; C) PS 1282; D) PS 1283; E) PS 1284; F) PS 1285; G) PS 1286; H) PS 1287; I) PS 1288; J) PS 1289; K) λ DNA pre-cut with *Hin* dIII; L) PS 1290; M) PS 1291; N) PS 1292; O) PS 1293; P) λ DNA pre-cut with *Hin* dIII



3.5.6.2. Rural Children

The characteristics of the plasmids extracted from each of the rural children isolates are summarised in Table 30 while their endonuclease restriction profiles are shown in Figures 31 - 32. The maximum number of occasions on which a single plasmid type was isolated in this group, was four. The plasmid, named group 5, was 71.4Kb and conferred resistance to ampicillin alone.

Table 30. Antibiogram and restriction profiles of transconjugants obtained from rural children

Strain Number	Donor Number	Plasmid Group	Plasmid Size (Kb)	Antibiogram	
				Transconjugant	Wildtype
PS 1210	3	1	59.0	Ap	Ap
PS 1220	28	1	59.0	Ap	Ap Ac Cer
PS 1225	3	1	59.0	Ap	Ap Ac
PS 1211	5	2	82.5	ApSpTcTp	ApSpTcTp
PS 1213	8	3	59.1	ApTc	ApSmTc
PS 1212	7	4	62.2	Ap	Ap
PS 1214	12	4	62.2	Ap	Ap
PS 1215	12	5	71.4	Ap	Ap
PS 1217	15	5	71.4	Ap	Ap
PS 1219	25	5	71.4	Ap	Ap
PS 1226	10	5	71.4	Ap	ApSpTc
PS 1216	14	6	69.2	ApTp	ApAcTp
PS 1218	19	6	69.2	ApTp	ApTp
PS 1221	29	7	54.6	ApSpSm	ApSpSmTp
PS 1222	41	8	97.4	Ap	ApTcTp
PS 1224	48	8	97.4	ApTp	ApSmAcTp
PS 1223	42	91	23.5	ApSmTc	ApSmTc

Figure 31. Agarose gel electrophoresis of an endonuclease restriction with *Eco* R1 of plasmid DNA extracted from South African rural children transconjugants

Tracks:-

A) PS 1210; B) PS 1214; C) PS 1215; D) λ DNA pre-cut with *Hin* dIII; E) PS 1222; F) PS 1223; G) PS 1224; H) PS 1225; I) PS 1226; J) λ DNA pre-cut with *Hin* dIII

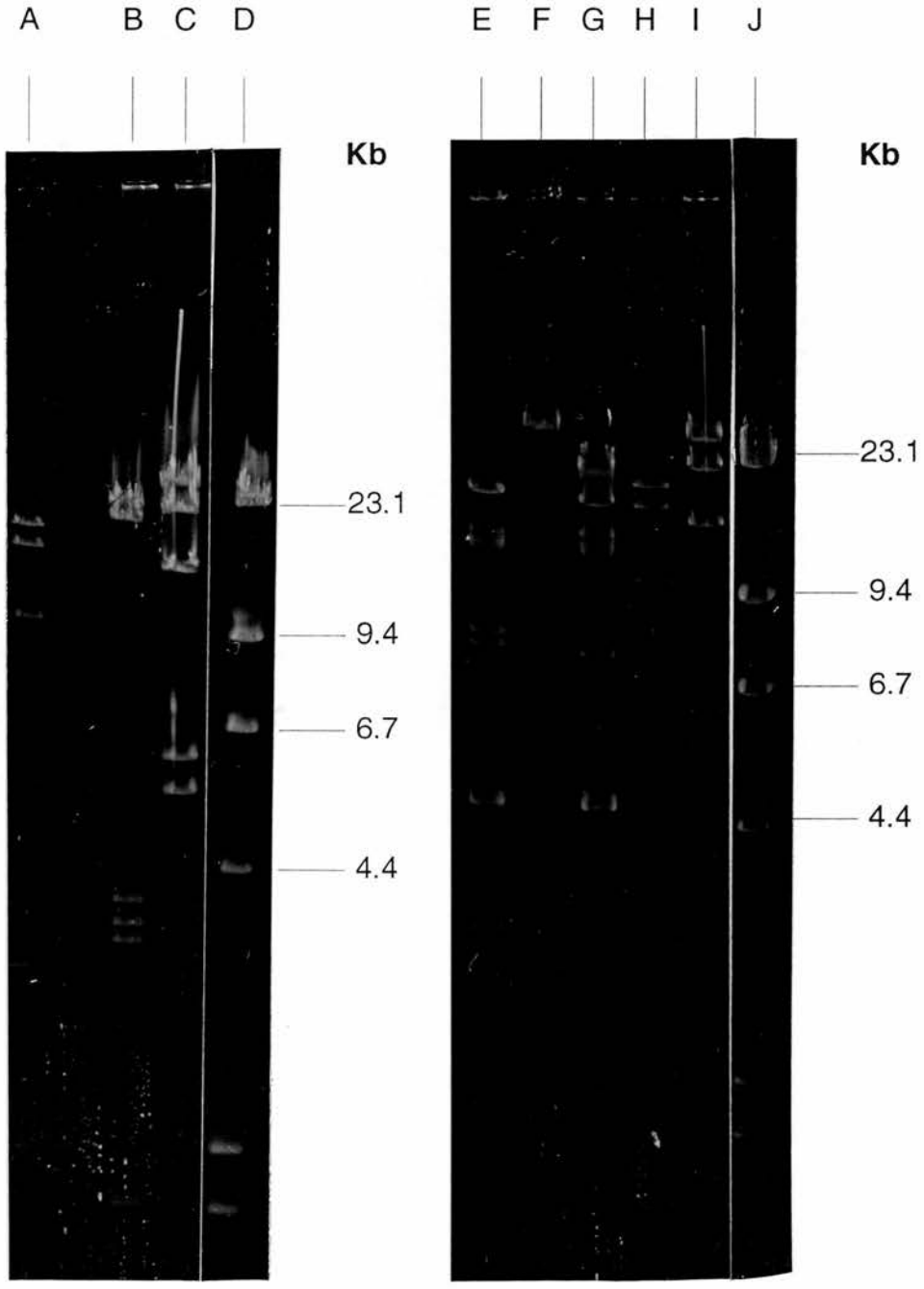
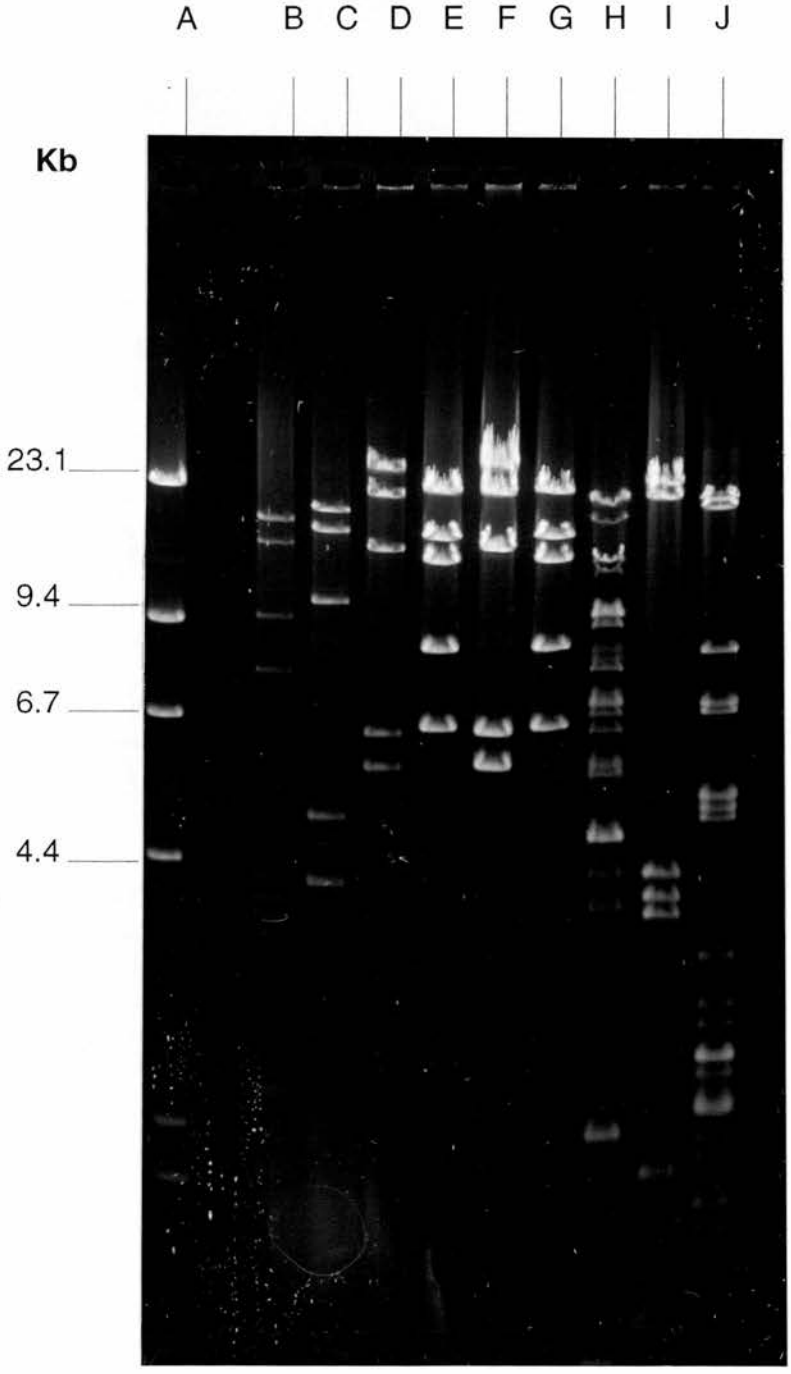


Figure 32. Agarose gel electrophoresis of an endonuclease restriction with *Eco* R1 of plasmid DNA extracted from South African rural children transconjugants

Tracks:-

A) λ DNA pre-cut with *Hin* dIII; B) PS 1221; C) PS 1220; D) PS 1219; E) PS 1218; F) PS 1217; G) PS 1216; H) PS 1213; I) PS 1212; J) PS 1211



3.5.6.3. Rural Teenagers

The characteristics of the plasmids extracted from each of the rural teenagers isolates are summarised in Table 31 while the endonuclease restriction profiles of these plasmids are shown in Figure 33. Although the group of plasmids isolated from rural teenagers was small, it possessed the greatest heterogeneity of both carriage of resistance determinants and size of plasmids amongst all the population groups. The largest plasmid to be identified amongst all the transconjugants was present in this group. Belonging to group 12, this plasmid was calculated as being 178.2Kb in size. Of interest, the wild type strain carried the highest number of resistance determinants recognised.

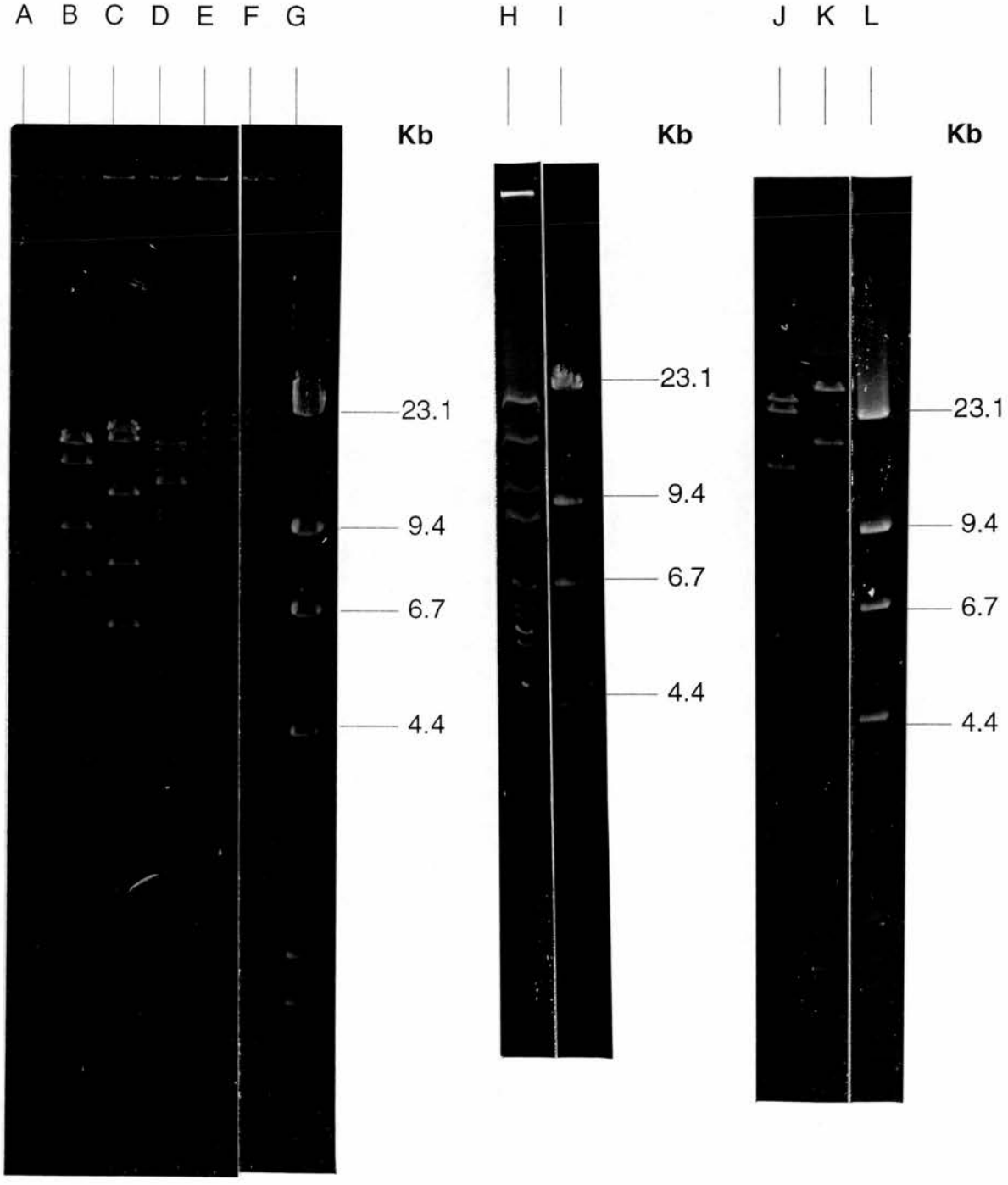
Table 31. Antibiogram and restriction profiles of transconjugants obtained from rural teenagers

Strain Number	Donor Number	Plasmid Group	Plasmid Size (Kb)	Antibiogram	
				Transconjugant	Wildtype
PS 1228	19	7	54.6	ApSpTcTp	ApSpTcTp
PS 1227	15	9	87.2	ApSpSmTc	ApSpSmTcTp
PS 1229	25	10	78.3	ApSpTc	ApSpAcCerTc
PS 1230	29	11	119.4	ApTc	ApTc
PS 1231	36	12	178.2	ApTcTpGmCm	ApSpSmAcTcTpGmCm
PS 1233	45	12	178.2	ApTcTpGmCm	ApSmAcCerCxmCtxTc GmCmTp
PS 1232	44	13	109.7	ApSmTc	ApSmTc
PS 1234	48	14	62.0	ApTp	ApTp
PS 1235	52	15	52.8	Ap	ApSmAcTcTp

Figure 33. Agarose gel electrophoresis of an endonuclease restriction with *Eco* R1 of plasmid DNA extracted from South African rural teenager transconjugants

Tracks:-

A) PS 1227; B) PS 1228; C) PS 1229; D) PS 1230; E) PS 1231; F) PS 1233; G) λ DNA pre-cut with *Hin* dIII; H) PS 1232; I) λ DNA pre-cut with *Hin* d 111; J) PS 1234; K) PS 1235; L) λ DNA pre-cut with *Hin* dIII



3.5.6.4. Rural Adults

The characteristics of the plasmids extracted from each of the rural adults isolates are summarised in Table 32 while the endonuclease restriction profiles of these plasmids are shown in Figure 34. Of the 12 transconjugants, all the plasmids were shown to be distinct by endonuclease restriction digestion with *Eco* R1.

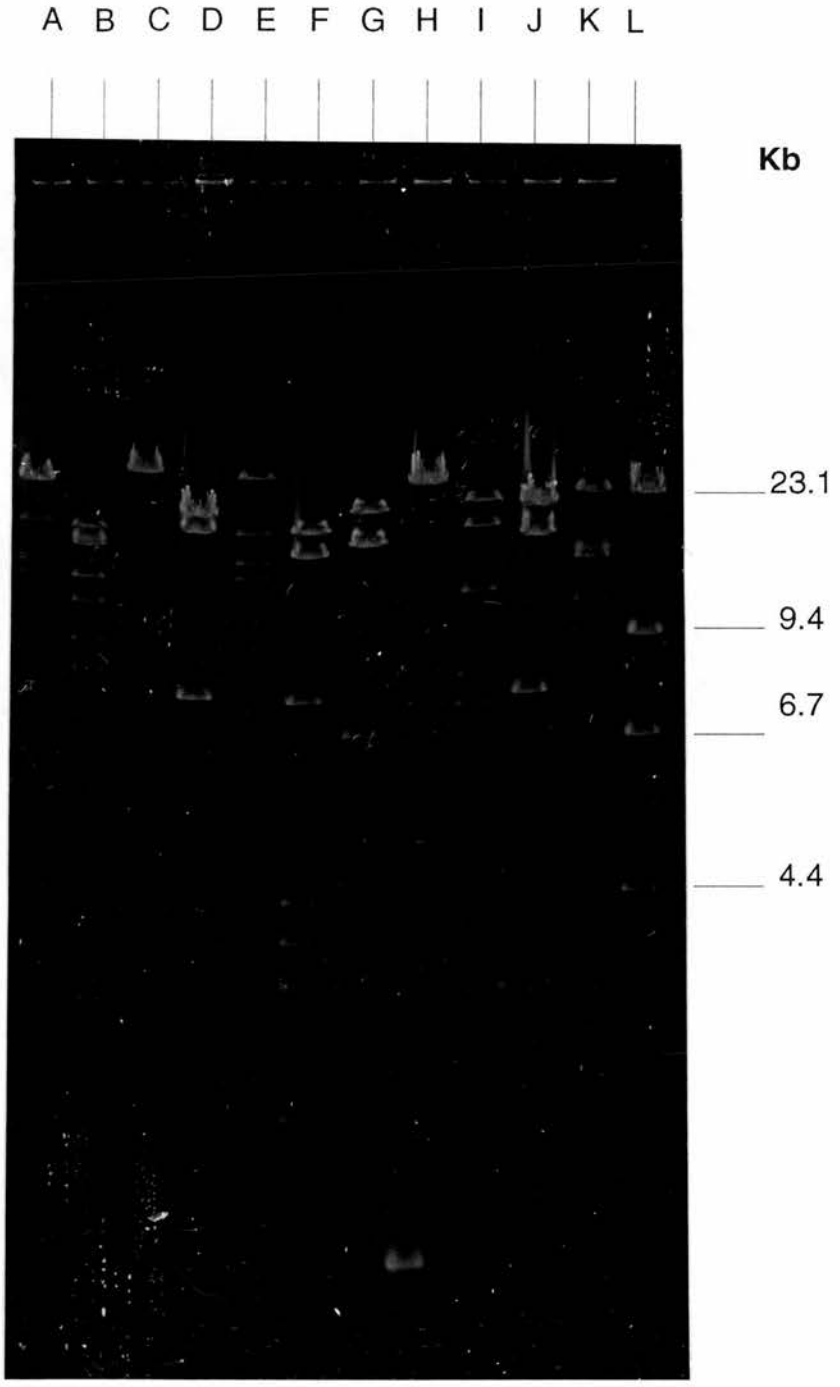
Table 32. Antibiogram and restriction profiles of transconjugants obtained from rural adults

Strain Number	Donor Number	Plasmid Group	Plasmid Size (Kb)	Antibiogram	
				Transconjugant	Wildtype
PS 1304	23	17	65.9	Ap	ApSmTp
PS 1310	49	18	49.8	ApTp	ApSpSmTp
PS 1301	1	39	95.0	ApSmTc	ApSmTcTp
PS 1302	2	40	126.3	ApSpTc	ApSpTc
PS 1305	24	41	94.8	ApSpSmTcTp	ApSpSmTcTpCm
PS 1306	24	42	60.1	ApSmTp	ApSmTp
PS 1307	36	43	55.3	Ap	ApSmTcTp
PS 1308	38	44	26.4	ApSmTcTp	ApSmTcTp
PS 1309	47	45	87.3	ApSpSmTcTp	ApSpSmTcTpGm
PS 1311	50	46	102.5	ApSpSm	ApSpSmAcTcTpCm
PS 1312	48	47	77.0	Ap	Ap
PS 1303	14	89	23.9	ApSpTp	ApSpTcTp

Figure 34. Agarose gel electrophoresis of an endonuclease restriction with *Eco* R1 of plasmid DNA extracted from South African rural adult transconjugants

Tracks:-

A) PS 1301; B) PS 1302; C) PS 1303; D) PS 1304; E) PS 1305; F) PS 1306; G) PS 1307; H) PS 1308; I) PS 1309; J) PS 1310; K) PS 1311; L) λ DNA pre-cut with *Hin* dIII



3.5.6.5. Urban Infants

The characteristics of the plasmids extracted from each of the urban infant isolates are summarised in Table 33 while the endonuclease restriction profiles of these plasmids are shown in Figures 35 - 36. Three of the plasmids isolated from this group of transconjugants shared exactly the same restriction profile as plasmid group number 18, identified in the rural infants. However, unlike plasmid group number 18, resistance to streptomycin was also mediated by these plasmids and they were therefore classified as plasmid group number 18a. The antibiogram profile, streptomycin, ampicillin and trimethoprim was recognised in 10 of the 22 transconjugants in this group.

3.5.6.6. Urban Children

The characteristics of the plasmids extracted from each of the urban children isolates are summarised in Table 34 while the endonuclease restriction profiles of these plasmids are shown in Figures 37-38. Plasmid number 18a which was present in the urban infants was identified on a single occasion in transconjugant number PS 1346. All the other plasmids identified were unique to this group.

Table 33. Antibiogram and restriction profiles of transconjugants obtained from urban infants

Strain Number	Donor Number	Plasmid Group	Plasmid Size (Kb)	Antibiogram	
				Transconjugant	Wildtype
PS 1329	40	4	57.3	Ap	Ap
PS 1315	3	18	49.8	ApSmTp	ApSpSmTpTc
PS 1322	21	18	49.8	ApSmTp	ApSmTp
PS 1334	49	18	49.8	ApSmTp	ApSmTp
PS 1313	1	48	71.8	ApSmTp	ApSmTp
PS 1320	14	48	71.8	ApSmTp	ApSmTp
PS 1324	25	48	71.8	ApSmTp	ApSmTp
PS 1325	28	48	71.8	ApSmTp	ApSmTp
PS 1326	29	48	71.8	ApSmTp	ApSmTp
PS 1314	2	49	89.2	ApSmTp	ApSmTp
PS 1316	5	50	11.5	ApSpTc	ApSpTc
PS 1317	7	51	68.4	Ap	ApSmTpCm
PS 1318	7	52	96.3	Ap	ApSpSmTcTpCm
PS 1327	29	52	95.8	Ap	ApSpSmTcTpCm
PS 1319	8	53	58.7	ApTp	ApTp
PS 1321	20	54	108.0	ApSmTp	ApSmTcTpCm
PS 1323	22	55	90.1	ApSm	ApSmTp
PS 1328	36	56	47.2	ApTp	ApSmTcTp
PS 1330	43	57	89.3	ApSmTc	ApSmAcCerTc
PS 1331	43	57	89.3	ApSmTc	ApSmAcCerTc
PS 1332	46	58	77.9	ApSm	ApSmTp
PS 1333	46	59	75.3	ApSmTcTp	ApSmTcTp

Figure 35. Agarose gel electrophoresis of an endonuclease restriction with *Eco* R1 of plasmid DNA extracted from South African urban infant transconjugants

Tracks:-

A) PS 1313; B) PS 1314; C) PS 1317; D) PS 1318; E) PS 1319; F) PS 1320; G) PS 1312; H) PS 1322; I) PS 1323; J) PS 1324; K) λ DNA pre-cut with *Hin* dIII

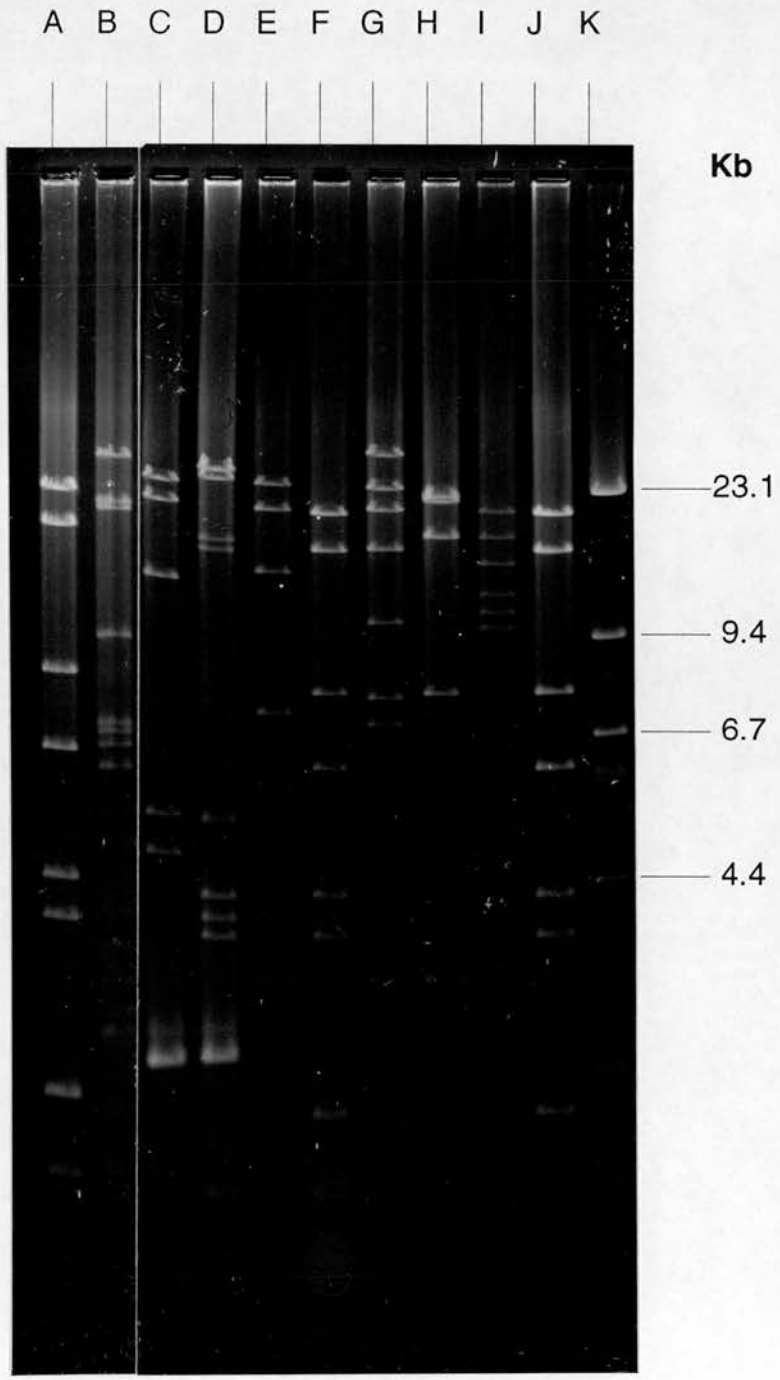


Figure 36. Agarose gel electrophoresis of an endonuclease restriction with *Eco* R1 of plasmid DNA extracted from South African urban infant transconjugants

Tracks:-

A) PS 1315; B) PS 1316; C) λ DNA pre-cut with *Hin* dIII; D) PS 1325; E) PS 1326; F) PS 1327; G) PS 1328; H) PS 1329; I) PS 1330; J) PS 1331; K) PS 1332; L) PS 1333; M) PS 1334; N) λ DNA pre-cut with *Hin* dIII

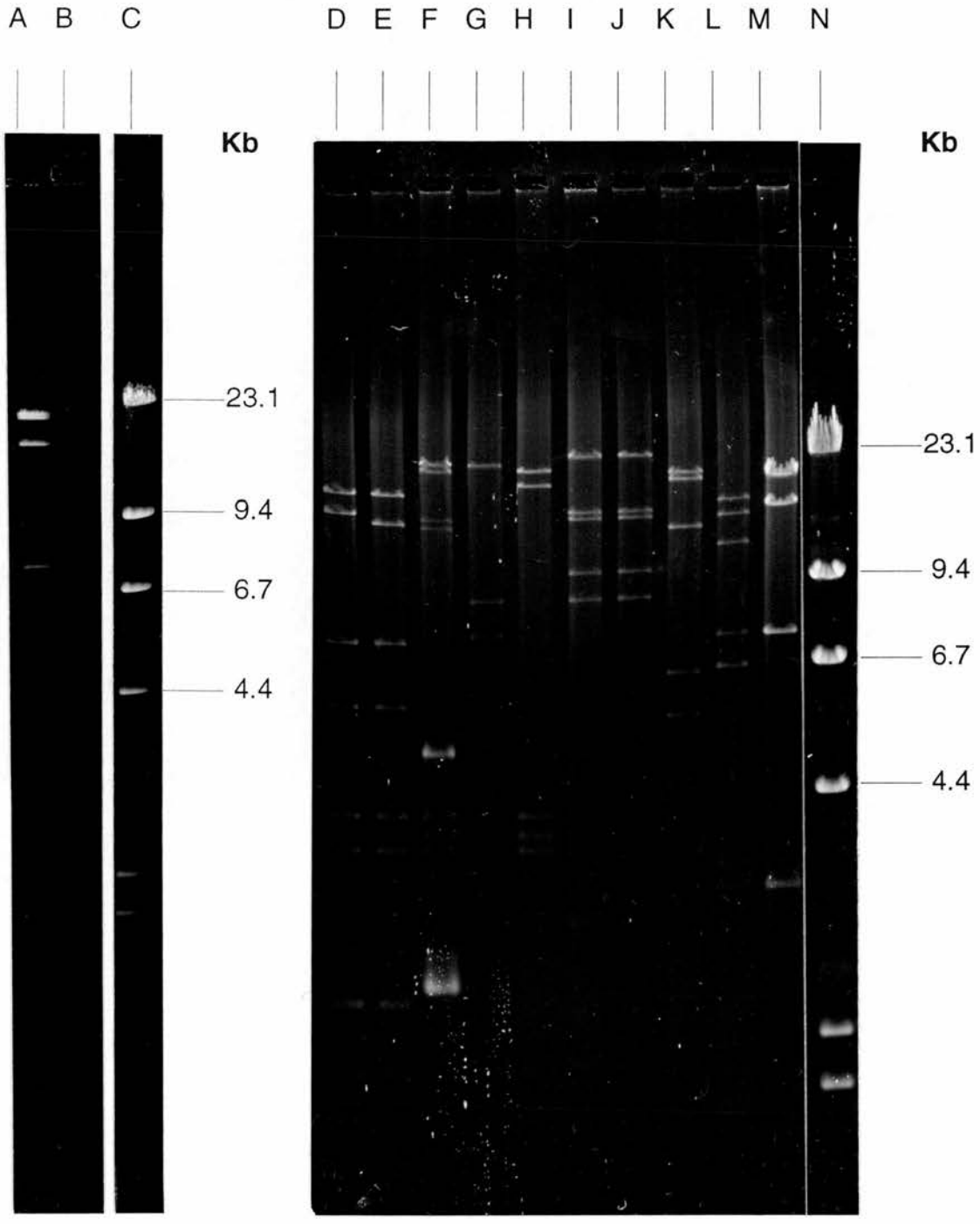


Table 34. Antibiogram and restriction profiles of transconjugants obtained from urban children

Strain Number	Donor Number	Plasmid Group	Plasmid Size (Kb)	Antibiogram	
				Transconjugant	Wildtype
PS 1346	31	18a	49.8	ApSmTp	ApSmTp
PS 1337	7	28	42.1	ApSm	ApSpSmAcTcTpCm
PS 1338	8	28	42.1	ApSm	ApSm
PS 1339	9	28	42.1	Ap	ApAcCerCtx
PS 1341	14	28	42.1	ApSm	ApSmTpCm
PS 1335	3	60	78.0	ApTp	ApTp
PS 1336	5	61	58.0	Ap	ApSmTp
PS 1340	12	62	125.5	Ap	ApTp
PS 1342	16	63	42.0	ApSmTp	ApSmTp
PS 1347	39	63	42.0	ApTp	ApTp
PS 1343	28	64	116.6	ApSpTcTp	ApSpTcTp
PS 1344	30	65	89.5	ApSpSmTcCm	ApSpSmTcTpCm
PS 1345	31	66	39.3	ApSpTc	ApSpTc
PS 1349	45	67	94.8	ApSpSmTc	ApSpSmCerTcTp
PS 1350	48	68	65.7	ApSmTp	ApSmTp
PS 1351	53	69	55.4	Ap	ApAcCerCtx
PS 1348	45	90	61.5	ApSpTcTp	ApSpTcTp

Figure 37. Agarose gel electrophoresis of an endonuclease restriction with *Eco* R1 of plasmid DNA extracted from South African urban children transconjugants

Tracks:-

A) PS 1337; B) PS 1338; C) PS 1339; D) PS 1340; E) PS 1341; F) PS 1342; G) PS 1343; H) PS 1344; I) PS 1345; J) PS 1346; K) PS 1347; L) PS 1348; M) λ DNA pre-cut with *Hin* dIII

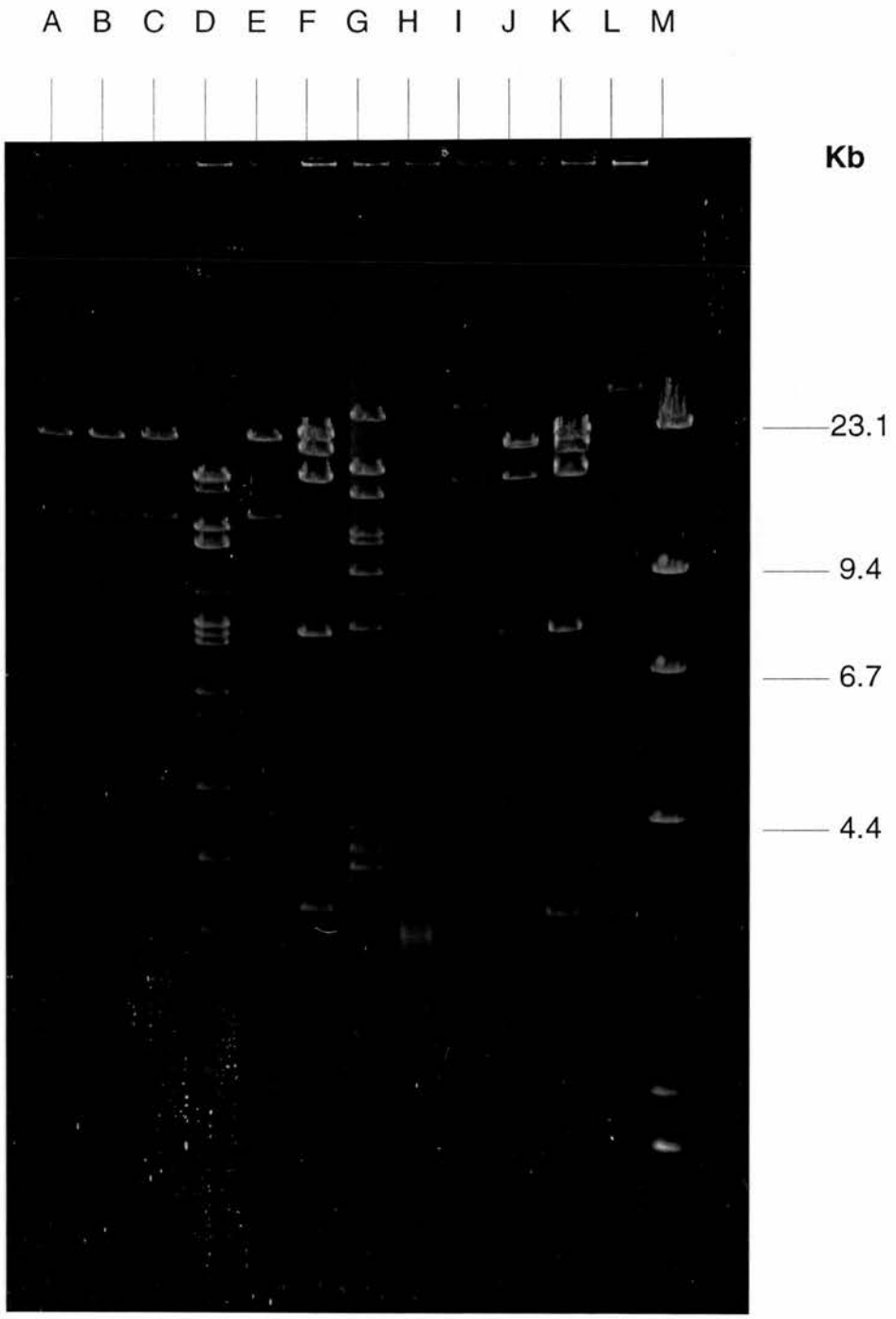
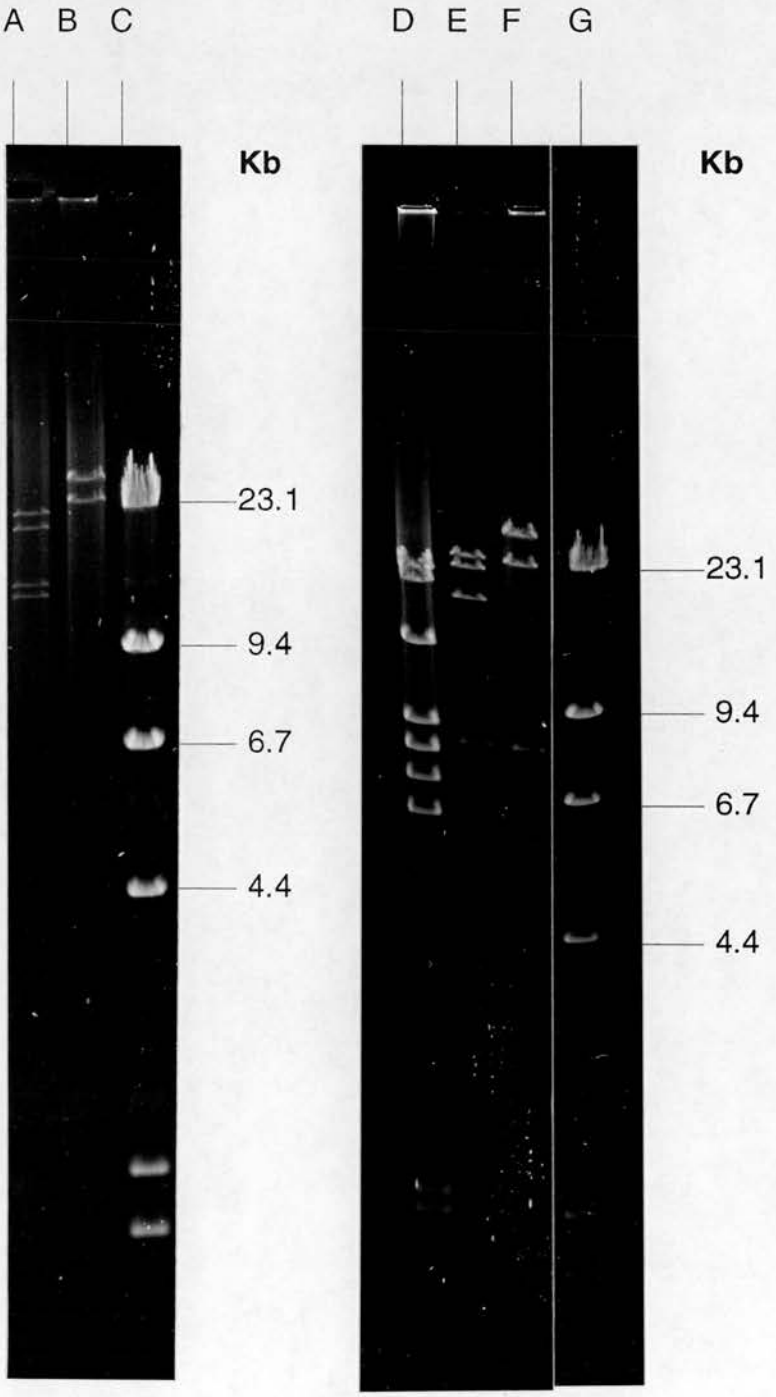


Figure 38. Agarose gel electrophoresis of an endonuclease restriction with *Eco* R1 of plasmid DNA extracted from South African urban children transconjugants

Tracks:-

A) PS 1335; B) PS 1336; C) λ DNA pre-cut with *Hin* dIII; D) PS 1349; E) PS 1350; F) PS 1351; G) λ DNA pre-cut with *Hin* dIII



3.5.6.7. Urban Teenagers

The characteristics of the plasmids extracted from each of the urban teenagers isolates are summarised in Table 35. Each of the plasmids in this group were shown to be different from each other in restriction profile (Figure 39) even though there was some similarity in antibiogram profile.

3.5.6.8. Urban Adults

The characteristics of the plasmids extracted from each of the urban adults isolates are summarised in Table 36. This plasmid population group was heterogeneous with a variety of endonuclease restriction profiles (Figure 40).

There was no correlation observed between the size of the plasmid and the number of resistance determinants that it carried; 20 plasmids were larger than 100Kb, yet the number of resistance determinants carried by them ranged from one to five. Table 37 provides an indication of the number of resistant determinants found on the plasmids.

Table 37. Number of resistance determinants identified in the plasmids

No. of Resistance Determinants	Number of Plasmids
1	35
2	74
3	30
>3	19

Table 35. Antibiogram and restriction profiles of transconjugants obtained from urban teenagers

Strain Number	Donor Number	Plasmid Group	Plasmid Size (Kb)	Antibiogram	
				Transconjugant	Wildtype
PS 1353	8	70	110.4	ApSmTcTp	ApSmTcTp
PS 1354	14	71	113.5	Ap	ApTp
PS 1355	15	72	42.8	ApSm	ApSpSmTp
PS 1356	17	73	43.6	Ap	ApSpSmTc
PS 1357	21	74	70.4	ApSm	ApSmTp
PS 1358	27	75	38.3	Ap	ApSmTc
PS 1359	32	76	110.1	Ap	Ap
PS 1361	45	77	60.7	Ap	Ap
PS 1362	45	78	43.2	ApSm	ApSmTp
PS 1352	2	86	22.0	ApSm	ApSpSm
PS 1360	44	87	22.0	ApSpTp	ApSpTp

Table 36. Antibiogram and restriction profiles of transconjugants obtained from urban adults

Strain Number	Donor Number	Plasmid Group	Plasmid Size (Kb)	Antibiogram	
				Transconjugant	Wildtype
PS 1365	13	78	43.2	ApSm	ApSmAcCerTpCm
PS 1366	13	78	43.2	ApSm	ApSmTp
PS 1367	15	79	82.0	ApTp	ApTp
PS 1368	15	80	116.0	ApSmTc	ApSmTc
PS 1369	24	81	129.2	ApSpTcTp	ApSpSmTcTp
PS 1370	28	82	134.5	ApSpSmTcTp	ApSpSmTcTpCm
PS 1371	29	83	85.0	ApTc	ApTc
PS 1372	41	84	40.9	ApSmTp	ApSmTp
PS 1373	47	85	39.5	ApSm	ApSmTcTp
PS 1363	4	92	99.7	ApSm	ApSmTp
PS 1364	4	92	99.7	ApSm	ApSm

Figure 39. Agarose gel electrophoresis of an endonuclease restriction with *Eco* R1 of plasmid DNA extracted from South African urban teenager transconjugants

Tracks:-

A) PS 1353; B) PS 1354; C) PS 1355; D) PS 1356; E) PS 1357; F) PS 1358; G) PS 1359; H) PS 1360; I) λ DNA pre-cut with *Hin* dIII; J) PS 1352; K) PS 1361; L) PS 1362; M) λ DNA pre-cut with *Hin* dIII

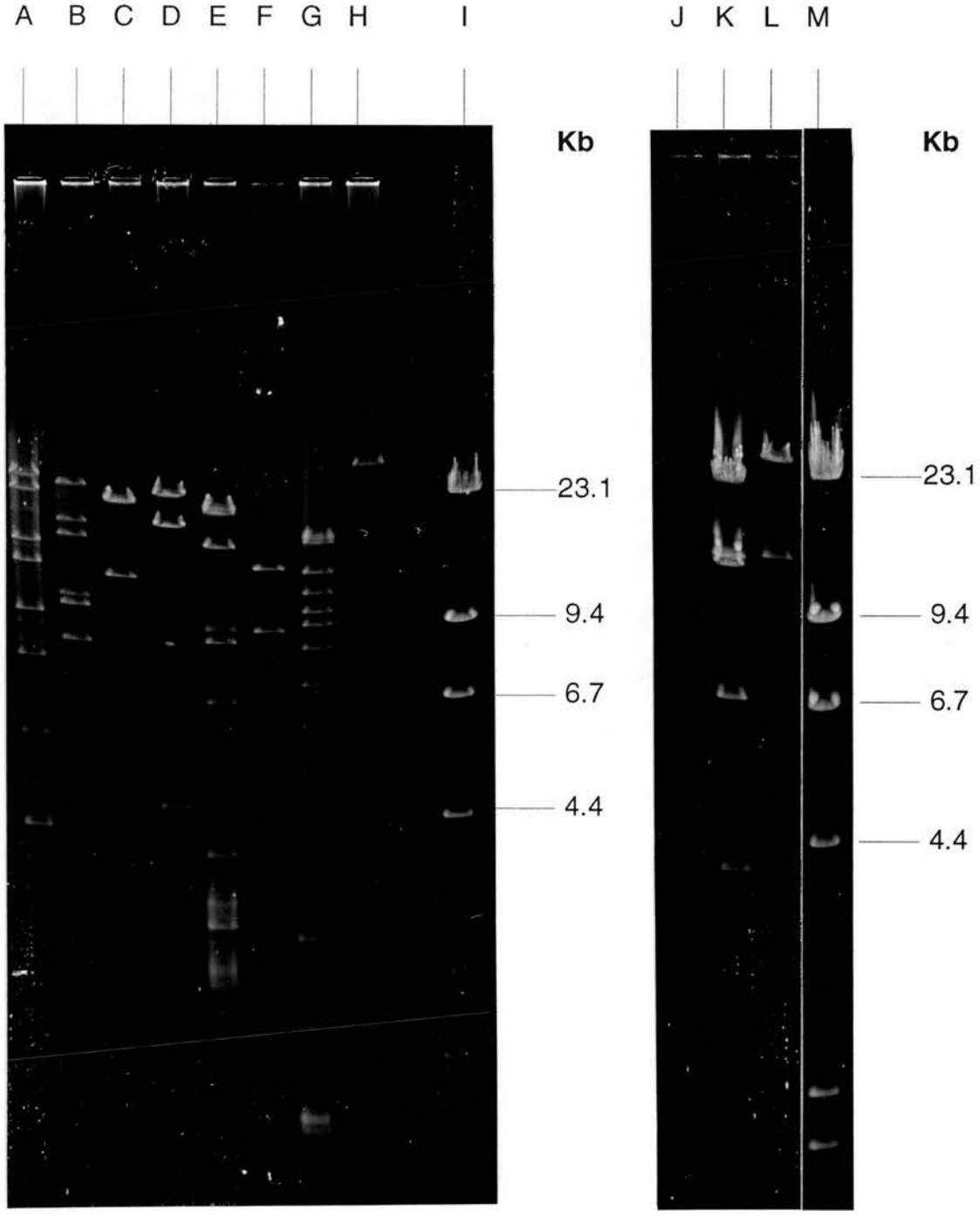
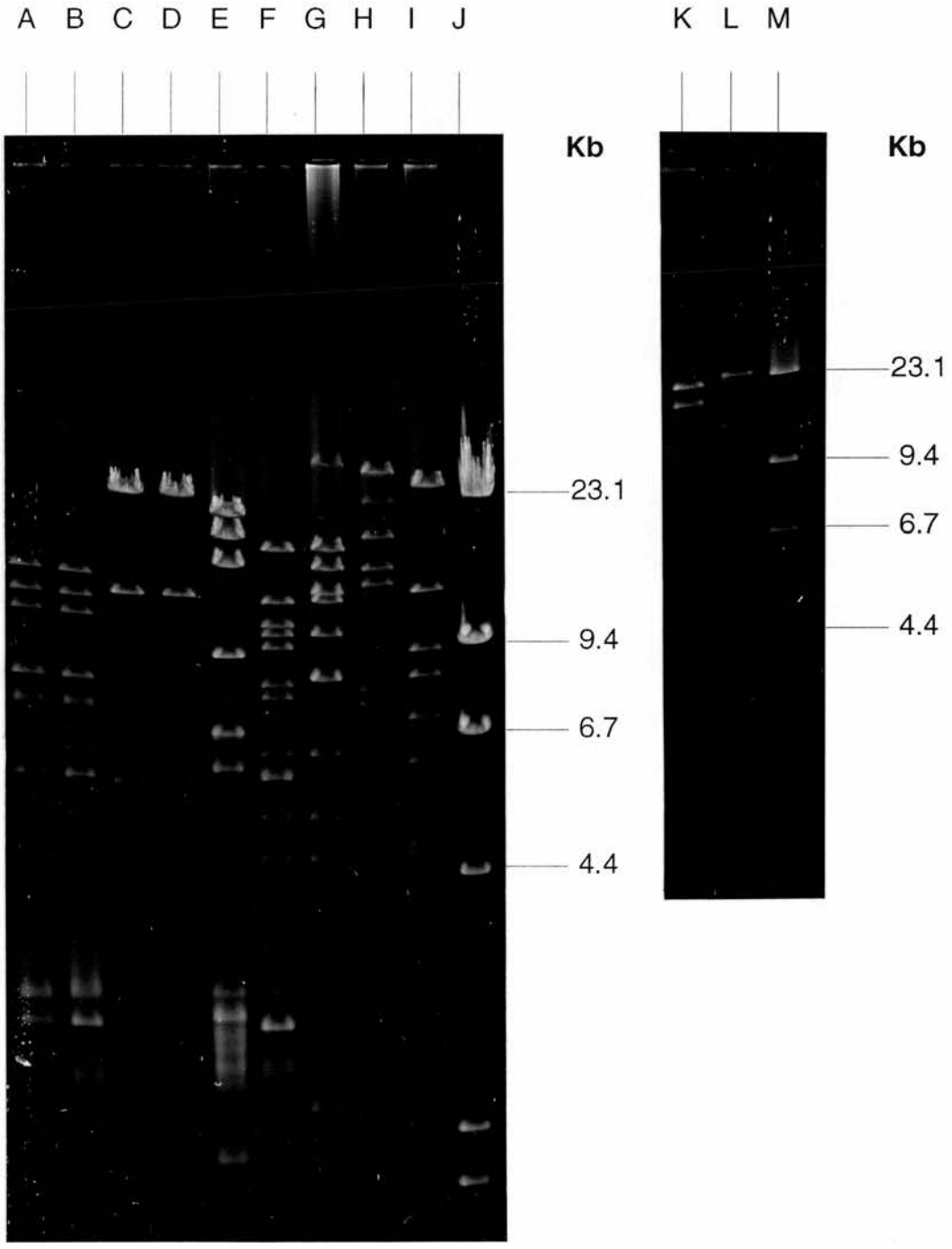


Figure 40. Agarose gel electrophoresis of an endonuclease restriction with *Eco* R1 of plasmid DNA extracted from South African adult transconjugants

Tracks:-

A) PS 1363; B) PS 1364; C) PS 1365; D) PS 1366; E) PS 1367; F) PS 1368; G) PS 1369; H) PS 1370; I) PS 1371; J) λ DNA pre-cut with *Hin* dIII; K) PS 1372; L) PS 1373; M) λ DNA pre-cut with *Hin* dIII



3.5.7. Distribution of β-Lactamases in the Ampicillin Resistant Transconjugants

The different β-lactamases responsible for mediating resistance to ampicillin amongst the transconjugants were determined by iso-electric focusing. The TEM-1 β-lactamase was the most frequently isolated β-lactamase accounting for 91.8% of the plasmid-encoded β-lactamases in the rural transconjugants and 98.4% of β-lactamases in the urban transconjugants (Figure 41). TEM-2 β-lactamase was only found in four (4.1%) of the rural transconjugants (Figure 42), while SHV-1 was identified in four (4.1%) of rural transconjugants and, in addition, in one (1.6%) of the urban transconjugants (Figure 43). Interestingly, all four transconjugants containing plasmids encoding the TEM-2 β-lactamase, had the same antibiogram, that is, they conferred resistance to ampicillin alone (Table 29 and 30). Furthermore two of these plasmids, PS 1210 and PS 1225 were identical in endonulease restriction profile whereas the other two (PS 1212 and PS 1248) were very different from these and each other. In contrast, the five transconjugants containing plasmids encoding the SHV-1 β-lactamase exhibited a diversity of antibiogram and restriction profiles.

3.5.8. Specific Activity of the TEM-1 β-Lactamase and Resistance to Clavulanic Acid

As before, the specific activities of the TEM-1 β-lactamases, isolated from the transconjugants, were determined. The MICs of amoxycillin and amoxycillin plus

Figure 41. Analytical iso-electric focusing of β-lactamases in the South African transconjugants

Tracks:-

A0 PS 1222; B) PS 1223; C) PS 1224; D) RP4 (TEM-2 producer); E) PS 1226; F) R1 (TEM -1 producer); G) PS 1227; H) PS 1228; I) PS 1229; J) PS 1230; K) PS 1231; L) R1010 (SHV-1 producer)

These selected TEM-1 producing tranconjugants are representative of all the TEM-1 producing tranconjugants examined.

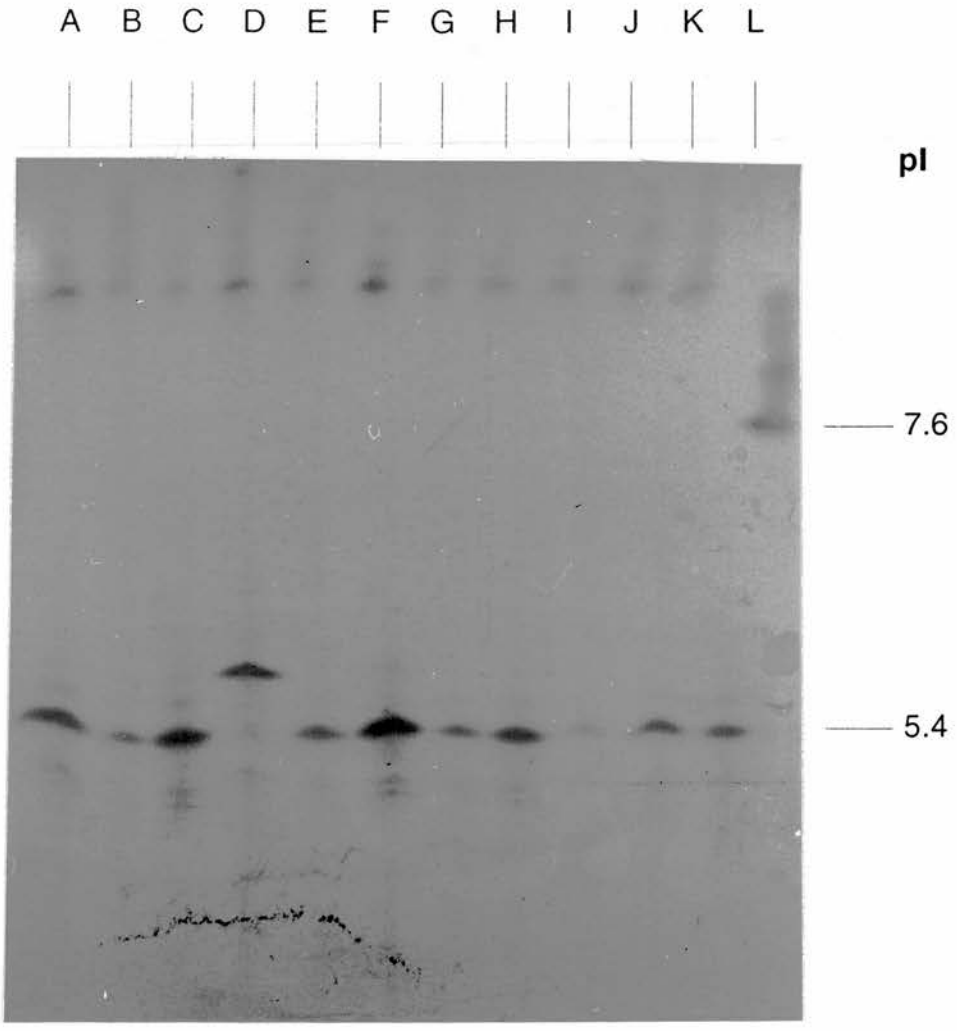


Figure 42. Analytical iso-electric focusing of TEM-2 β-lactamases in the South African transconjugants

Tracks:-

A) PS 1210; B) PS 1212; C) R1 (TEM-1 producer); D) RP4 (TEM-2 producer); E) PS 1225; F) PS 1248

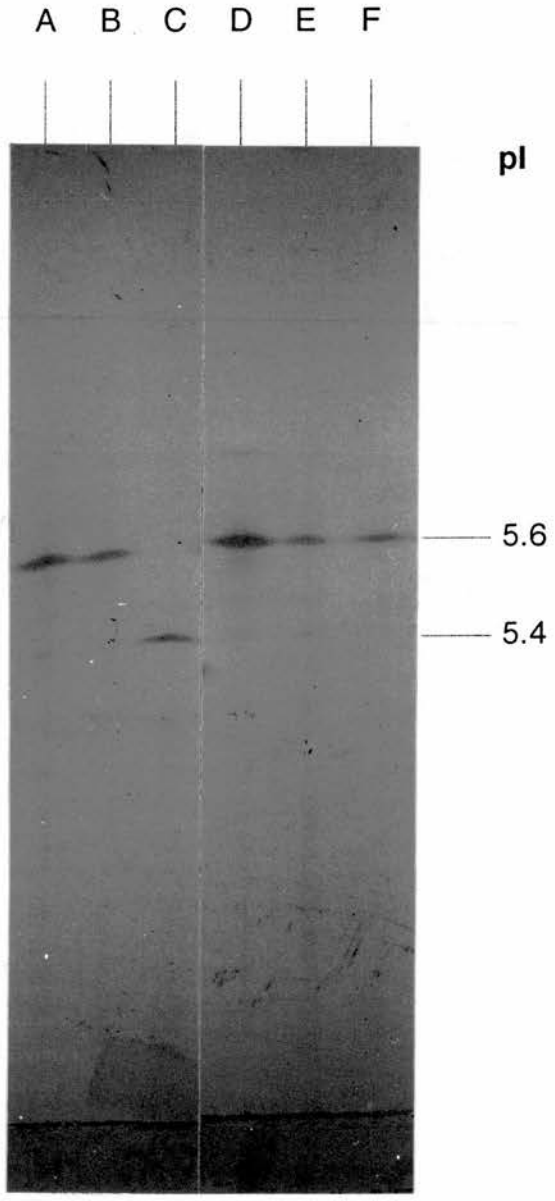
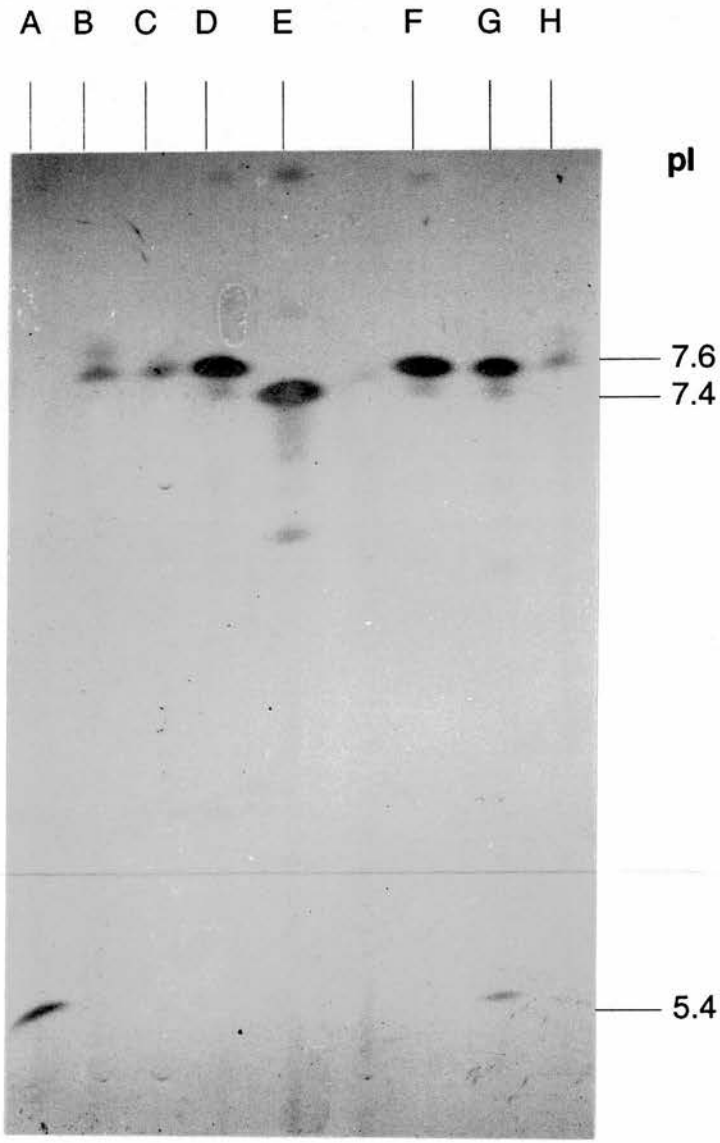


Figure 43. Analytical iso-electric focusing of SHV-1 β-lactamases in the South African transconjugants

Tracks:-

A) R1 (TEM-1 producer); B) PS 1219; C) PS 1232; D) R1010 (SHV-1 producer); E) R455 (OXA-1 producer); F) PS 1233; G) PS 1312; H) PS 1316



clavulanic acid in the transconjugants were also ascertained (Tables 38 - 45). As with the transconjugants obtained from the Edinburgh commensal faecal survey, no significant difference could be identified between the MIC values and the specific activity of the β-lactamase.

Table 38. The different specific activities and the sensitivities to amoxycillin and amoxycillin plus clavulanic acid of the ampicillin resistant transconjugants from rural infants

Transconjugant No.	MIC amox*		MIC amox/clav*		Specific Activity ✣
	WT	Trans	WT	Trans	
PS 1236 (PS 712)	1024	512	8	8	0.188
PS 1237 (PS 713)	128	512	4	8	0.070
PS 1244 (PS 722)	>1024	512	16	8	0.001
PS 1245 (PS 723)	>1024	512	16	4	0.007
PS 1247 (PS 725)	1024	512	8	8	0.004
PS 1252 (PS 731)	1024	512	16	8	0.017
PS 1254 (PS 733)	1024	512	8	8	0.033
PS 1255 (PS 734)	1024	512	8	8	0.001
PS 1262 (PS 742)	1024	512	8	8	0.0004
PS 1265 (PS 746)	>1024	512	16	8	0.0002
PS 1268 (PS 749)	>1024	512	16	8	0.004
PS 1239 (PS 715)	1024	512	8	8	0.008
PS 1240 (PS 716)	64	512	2	8	0.006
PS 1283 (PS 778)	1024	512	8	8	0.0005
PS 1238 (PS 714)	256	512	4	8	0.039
PS 1250 (PS 729)	32	512	4	8	0.003
PS 1253 (PS 732)	64	512	2	8	0.009
PS 1259 (PS 739)	1024	512	8	8	0.055
PS 1260 (PS 740)	1024	512	8	8	0.0002
PS 1261 (PS 741)	128	512	2	8	0.002
PS 1264 (PS 745)	1024	512	8	8	0.004
PS 1241 (PS 717)	>1024	512	16	8	0.011
PS 1242 (PS 719)	>1024	512	16	8	0.047
PS 1243 (PS 721)	512	512	8	8	0.098
PS 1246 (PS 724)	128	512	8	8	0.001
PS 1256 (PS 736)	>1024	518	2	8	0.011
PS 1257 (PS 737)	>1024	512	16	4	0.017
PS 1258 (PS 738)	>1024	256	8	4	0.268
PS 1269 (PS 753)	512	512	8	4	0.001

Table 38. Continued

Transconjugant No.	MIC amox*		MIC amox/clav*		Specific Activity
	WT	Trans	WT	Trans	✣
PS 1270 (PS 754)	1024	256	8	8	0.002
PS 1266 (PS 747)	>1024	512	16	8	0.002
PS 1271 (PS 755)	>1024	512	16	8	0.006
PS 1272 (PS 756)	>1024	512	16	4	0.0004
PS 1273 (PS 757)	>1024	512	16	4	0.002
PS 1274 (PS 758)	1024	256	16	4	0.001
PS 1276 (PS 762)	256	512	4	4	0.001
PS 1277 (PS 763)	128	512	16	8	0.0005
PS 1278 (PS 764)	32	256	16	4	0.0004
PS 1279 (PS 765)	256	256	16	4	0.0006
PS 1267 (PS 748)	1024	512	8	8	0.001
PS 1282 (PS 776)	512	512	8	8	0.004
PS 1275 (PS 761)	128	512	4	8	0.005
PS 1281 (PS 775)	1024	512	8	8	0.0005
PS 1280 (PS 771)	1024	512	8	8	0.001
PS 1284 (PS 779)	1024	512	8	8	0.003
PS 1288 (PS 786)	1024	512	8	8	0.0005
PS 1285 (PS 782)	1024	512	8	8	0.0001
PS 1286 (PS 784)	1024	512	8	8	0.009
PS 1287 (PS 785)	1024	512	8	8	0.009
PS 1289 (PS 787)	1024	512	8	8	0.03
PS 1290 (PS 788)	1024	512	8	4	0.001
PS 1291 (PS 789)	1024	512	8	8	0.001
PS 1292 (PS 790)	1024	512	16	8	0.0077
PS 1294 (PS 769)	>1024	512	16	8	0.003
PS 1293 (PS 768)	>1024	512	16	8	0.001

Table 39. The different specific activities and the sensitivities to amoxycillin and amoxycillin plus clavulanic acid of the ampicillin resistant transconjugants from rural children

Transconjugant No.	MIC amox*		MIC amox/clav*		Specific Activity
	WT	Trans	WT	Trans	⁜
PS 1220 (PS 905)	64	512	16	8	0.065
PS 1211 (PS 879)	1024	512	8	8	0.027
PS 1213 (PS 884)	1024	512	8	8	0.003
PS 1214 (PS 890)	1024	512	8	8	0.177
PS 1215 (PS 891)	128	512	2	8	0.150
PS 1217 (PS 896)	256	512	4	8	0.002
PS 1226 (PS 886)	64	512	2	8	0.04
PS 1216 (PS 894)	>1024	512	16	8	0.028
PS 1218 (PS 900)	128	512	2	8	0.029
PS 1221 (PS 908)	1024	512	8	8	0.007
PS 1222 (PS 919)	>1024	>1024	16	8	0.300
PS 1224 (PS 926)	>1024	>1024	16	8	0.041
PS 1223 (PS 920)	>1024	1024	8	8	0.045

Table 40. The different specific activities and the sensitivities to amoxycillin and amoxycillin plus clavulanic acid of the ampicillin resistant transconjugants from rural teenagers

Transconjugant No.	MIC amox*		MIC amox/clav*		Specific Activity
	WT	Trans	WT	Trans	⊹
PS 1228 (PS 816)	1024	512	8	8	0.3268
PS 1227 (PS 808)	1024	512	8	8	0.729
PS 1229 (PS824)	1024	512	16	8	0.120
PS 1230 (PS 831)	1024	512	8	8	0.003
PS 1231 (PS 842)	>1024	>1024	16	8	0.1106
PS 1233 (PS 860)	>1024	>1024	16	8	0.098
PS 1234 (PS 863)	>1024	512	16	8	0.393
PS 1235 (PS 867)	>1024	512	16	8	0.156

Table 41. The different specific activities and the sensitivities to amoxycillin and amoxycillin plus clavulanic acid of the ampicillin resistant transconjugants from rural adults

Transconjugant No.	MIC amox*		MIC amox/clav*		Specific Activity
	WT	Trans	WT	Trans	⊹
PS 1304 (PS 645)	1024	512	8	8	0.009
PS 1310 (PS 708)	512	512	8	8	0.017
PS 1301 (PS 600)	1024	256	4	4	0.0004
PS 1302 (PS 603)	1024	512	8	8	0.001
PS 1305 (PS 646)	1024	512	8	8	0.0008
PS 1306 (PS 649)	512	512	8	8	0.004
PS 1307 (PS 679)	>1024	512	8	8	0.002
PS 1308 (PS 682)	1024	1024	8	8	0.014
PS 1311 (PS 711)	>1042	512	8	8	0.0004
PS 1312 (PS 706)	256	256	2	2	0.001
PS 1303 (PS 626)	1024	1024	8	8	0.001

Table 42. The different specific activities and the sensitivities to amoxycillin and amoxycillin plus clavulanic acid of the ampicillin resistant transconjugants from urban infants

Transconjugant No.	MIC amox*		MIC amox/clav*		Specific Activity
	WT	Trans	WT	Trans	⊹
PS 1329.(PS 987)	>1024	512	16	8	0.091
PS 1315.(PS 934)	>1024	512	16	8	0.049
PS 1322 (PS 962)	>1024	512	16	8	0.107
PS 1334 (PS 1006)	1024	512	8	8	0.001
PS 1313 (PS 929)	1024	512	8	8	0.12
PS 1320 (PS 953)	1024	512	16	8	0.128
PS 1324 (PS 966)	1024	512	16	8	0.033
PS 1325 (PS 972)	1024	512	16	8	0.131
PS 1326 (PS 973)	1024	512	16	8	0.137
PS 1314 (PS 931)	1024	512	16	8	0.003
PS 1316 (PS 936)	>1024	1024	8	8	0.102
PS 1317 (PS 938)	>1024	512	16	8	0.029
PS 1318 (PS 939)	>1024	512	16	8	0.053
PS 1327 (PS 974)	>1024	512	16	8	0.049
PS 1319 (PS 940)	1024	512	8	8	0.073
PS 1321 (PS 960)	>1024	512	16	8	0.024
PS 1323 (PS 963)	>1024	512	16	8	0.026
PS 1328 (PS 982)	512	512	8	8	0.112
PS 1330 (PS 992)	1024	512	16	8	0.188
PS 1331 (PS 994)	1024	1024	16	8	0.068
PS 1332 (PS 999)	>1024	512	16	8	0.098
PS 1333 (PS 1000)	512	512	8	8	0.024

Table 43. The different specific activities and the sensitivities to amoxycillin and amoxycillin plus clavulanic acid of the ampicillin resistant transconjugants from urban children

Transconjugant No.	MIC amox*		MIC amox/clav*		Specific Activity
	WT	Trans	WT	Trans	✣
PS 1346 (PS 1045)	1024	512	8	8	0.087
PS 1337 (PS 1015)	>1024	512	16	8	0.048
PS 1338 (PS1016)	64	512	2	8	0.116
PS 1339 (PS1018)	256	512	16	8	0.025
PS 1341 (PS 1024)	1024	512	16	8	0.146
PS 1335 (PS 1009)	1024	512	16	8	0.527
PS 1336 (PS 1012)	>1024	1024	16	8	0.138
PS 1340 (PS 1022)	1024	512	16	8	0.100
PS 1342 (PS 1025)	1024	512	8	8	0.076
PS 1347 (PS 1054)	1024	512	8	8	0.117
PS 1343 (PS 1039)	1024	512	8	8	0.019
PS 1344 (PS 1043)	1024	1024	8	8	0.117
PS 1345 (PS 1044)	512	512	8	8	0.026
PS 1349 (PS 1062)	>1024	512	16	8	0.054
PS 1350 (PS 1066)	1024	512	8	8	0.146
PS 1351 (PS 1074)	256	1024	16	8	0.081
PS 1348 (PS 1061)	1042	512	8	8	0.131

Table 44. The different specific activities and the sensitivities to amoxycillin and amoxycillin plus clavulanic acid of the ampicillin resistant transconjugants from urban teenagers

Transconjugant No.	MIC amox*		MIC amox/clav*		Specific Activity
	WT	Trans	WT	Trans	⁜
PS 1353 (PS 1087)	1024	512	8	8	0.466
PS 1354 (PS 1095)	1024	512	8	8	0.039
PS 1355 (PS 1096)	1024	512	16	8	0.029
PS 1356 (PS 1099)	1024	512	8	8	0.051
PS 1357 (PS 1104)	1024	1024	8	8	0.017
PS 1358 (PS 1111)	>1024	1024	16	8	0.0003
PS 1359 (PS 1117)	>1024	1024	16	8	0.070
PS 1361 (PS 1122)	1024	512	16	8	0.064
PS 1362 (PS 1123)	>1024	512	16	8	0.293
PS 1352 (PS 1080)	1024	1024	8	8	0.065
PS 1360 (PS 1121)	>1024	1024	16	8	0.062

Table 45. The different specific activities and the sensitivities to amoxycillin and amoxycillin plus clavulanic acid of the ampicillin resistant transconjugants from urban adults

Transconjugant No.	MIC amox*		MIC amox/clav*		Specific Activity
	WT	Trans	WT	Trans	⁜
PS 1365 (PS 1142)	>1024	512	16	8	0.024
PS 1366 (PS 1144)	1024	512	8	8	0.139
PS 1367 (PS 1145)	512	512	8	8	0.073
PS 1368 (PS 1146)	>1024	512	16	8	0.046
PS 1369 (PS 1162)	>1024	512	16	8	0.088
PS 1370 (PS 1171)	1024	256	8	8	0.078
PS 1371 (PS 1173)	1024	512	8	8	0.033
PS 1372 (PS 1190)	1024	512	8	8	0.058
PS 1373 (PS 1200)	>1024	512	16	8	0.087
PS 1363 (PS 1130)	>1024	512	16	8	0.031
PS 1364 (PS 1132)	128	512	2	8	0.044

* = mg/L ⁜= μ moles nitrcephin hydolysed / minute / mg protein
WT = wild type Trans = transconjugant

3.6. Biochemical Investigation of TEM-1 β-Lactamase from Disparate Plasmids

Newer generation cephalosporins and β-lactam/β-lactamase inhibitor combinations have the potential to select for mutations in the common plasmid-mediated β-lactamases, TEM-1, TEM-2 and SHV-1 (Payne and Amyes, 1991). Such mutations occur in a step-wise manner and consequently, resistance may be either conferred to these agents directly or after a number of sequential mutations (Amyes and Gemmell, 1992). Some extended-spectrum β-lactamases eg. TEM-9, have arisen as a result of a silent mutation. While such a mutation is recognised in nucleotide sequencing, there is no detectable alteration in the pI value of the new β-lactamase; analytical iso-electric focusing is established as the main β-lactamase definitive criterion. Similarly, it has been demonstrated that a mutation at position 69 in the TEM-1 β-lactamase, resulting in a leucine substitution for methionine, promotes resistance to β-lactam /β-lactamase inhibitor combinations although this change is indistinguishable by analytical iso-electric focusing. Furthermore, as with TRC-1 (Thomson and Amyes, 1992a), these new adapted β-lactamases may not be recognised by an altered MIC value. Such alterations raise the question as to whether all β-lactamases described as TEM-1 (pI 5.4) are indeed identical to one another.

Ten TEM-1 producing strains isolated from the surveys were investigated biochemically in order to ascertain whether any biochemical variation existed

between the TEM-1 β-lactamase in each of the strains.

3.6.1. Biochemical Analysis

Cell free extracts of each strain were prepared from 1 litre of bacterial culture (Materials and Methods). The β-lactamase was partially purified and isolated in a calibrated sephadex G75 column (Materials and Methods). As described previously, spectrophotometric assays were conducted in order to determine the relative rates of hydrolysis of benzylpenicillin, ampicillin, cephaloridine and nitrocephin exhibited by each of the strains. The relative efficiency of hydrolysis was also normalised with respect to benzylpenicillin. In addition, the concentration of clavulanic acid required to inhibit, by 50%, the hydrolysis of nitrocephin substrate by the β-lactamase (ID_{50}) was determined by spectrophotometric assay. The results are shown in Table 46.

From Table 46, it is clear that there is little variation in the biochemistry of the enzymes extracted from the strains listed. Each isolate had a similar sensitivity to amoxycillin when combined with clavulanic acid as is indicated by both the MIC to the combination and the clavulanic acid ID_{50} value. Although the values for ES 64 and ES 67 were slightly higher, the spectrophotometric assays identified a general lack of variation in the rates of hydrolysis of ampicillin, cephaloridine and nitrocephin.

Table 46. Profile of each of the 10 strains included in the investigation

No.	MIC mg/L		Rates of Hydrolysis				Clav
	Amox	Amox/Clav	Pen G	Amp‡	Ceph‡	Nit‡	ID_{50}*
ES 43	1024	8	100	3.5	2.4	16	0.08
ES 59	256	8	100	5.1	2	17	0.1
ES64	512	8	100	25	29	7.1	0.18
ES 67	512	8	100	21	30	12.4	0.05
ES 72	512	8	100	34	2.3	9	0.03
PS 1236	512	8	100	<1	<1	5.8	0.03
PS 1261	512	8	100	5.9	1.5	13.6	0.08
PS 1271	256	8	100	9.8	5.5	22.6	0.06
PS 1323	256	4	100	<1	4.7	15.8	0.077
PS 1337	512	8	100	32.9	2.8	17.0	0.062

‡ = Rates of hydrolysis expressed as a percentage of hydrolysis rate of benzyl penicillin

* = µmole clavulanic acid

3.6.2. Selection for Mutants of the TEM-1β-Lactamase

TEM-1, TEM-2 and SHV-1 are encoded by a variety of plasmid types. As the distribution of extended-spectrum β-lactamases is limited, it has been speculated that certain plasmid types may be either more successful in a

nosocomial environment or may have properties that promote resistance gene evolution (Jacoby and Sutton, 1991). Investigations were conducted to determine whether some plasmids isolated from the commensal faecal flora of healthy populations would adapt more successfully than others in a new antibiotic environment.

The TEM derived β-lactamase TRC-1 arose in an epidemic plasmid which has been shown to be widespread in both hospital and community isolates that contained TEM-1 β-lactamase. The implication that TRC-1 arose as a result of clinical therapy was demonstrated in an *in vitro* study which continuously subcultured *E. coli* J62-2 containing R1, a TEM-1 producing plasmid, in the presence of a range of sub-inhibitory concentrations of amoxycillin plus clavulanic acid (Thomson and Amyes, 1993b). The result was the isolation of a mutant enzyme with increased resistance to β-lactamase inhibitors and biochemical properties comparable with TRC-1. In order to ascertain whether TRC-1 or a comparable laboratory mutant would be selected for *in vitro* from the TEM-1 containing commensal plasmids, each of the 10 strains were subjected to a similar challenge with amoxycillin plus clavulanic acid. The treated strains resulting from this were investigated. Of interest, strain ES 59, contains the epidemic plasmid identical to the epidemic plasmid on which TRC-1 was located.

As described in the Materials and Methods, each of the 10 strains were

challenged for five days in 100ml Isosensitest broth containing different concentrations (2 - 32mg/L) of amoxycillin in combination with clavulanic acid in a 2:1 ratio. Table 47 shows the concentrations of amoxycillin plus clavulanic acid in which growth of each strain persisted throughout the five day testing. All of the 10 parent strains were able to grow throughout the complete challenge in the presence of both 2 and 4mg/L amoxycillin/clavulanate (Table 47). However, only two parent strains tolerated increased concentrations of this combination. While ES 43 grew at 8mg/L, growth was suppressed at 16mg/L of the combination. PS 1261 was able to grow in the presence of 16mg/L amoxycillin/clavulanate but no growth was observed at 32mg/L amoxycillin/clavulanate.

As indicated in the Materials and Methods, bacteria from each flask were isolated on Isosensitest agar plates containing the same concentration of antibiotic in which the bacteria had been grown. Ten colonies were selected from each agar plate. On MacConkey agar plates, these colonies were identified as lactose non-fermenting strains. When the auxotrophic requirements were checked on plates lacking histidine, the integrity of each colony was confirmed as *E. coli* J62-2.

Table 47. Growth of isolates treated with amoxycillin plus clavulanic acid at a range of concentrations

	Amoxycillin / Clavulanic Acid Concentration (mg/L) Ratio 2:1 - expressed as amoxycillin				
Strain No.	2	4	8	16	32
ES 43	++	++	++	-	-
ES 59	++	++	-	-	-
ES 64	++	++	-	-	-
ES 67	++	++	-	-	-
ES 72	++	++	-	-	-
PS 1236	++	++	-	-	-
PS 1261	++	++	++	++	-
PS 1271	++	++	-	-	-
PS 1323	++	++	-	-	-
PS 1337	++	++	-	-	-

++ = Growth
\- = No growth

3.6.3. β-Lactamase Analysis of the Treated Strains

The type of β-lactamase produced by each of the checked strains obtained from each concentration was ascertained by iso-electric focusing, as already outlined, but the polyacrylamide gel contained combined ampholines, ranges pH 3.5 -10 and pH 4 - 6, in a 1:1 mixture. A total of 230 isolates were examined. All of the isolates still produced the TEM-1 β-lactamase irrespective of the concentration of amoxycillin plus clavulanic acid with which they had been challenged. Figures 44 and 45 show a single representative for each of the 10 TEM-1 producing strains isolated from each challenge concentration of amoxycillin plus clavulanic acid.

3.6.4. Sensitivity of the Treated Strains to Amoxycillin and Amoxycillin plus Clavulanic Acid

Following the procedure outlined previously, the MIC of amoxycillin and amoxycillin plus clavulanic acid was measured for the representative from each of the 23 groups of 10 treated strains. The results (Table 48) indicate that challenge with different sub-inhibitory concentrations of amoxycillin/ clavulanate induces different responses in the strains. A variety of phenotypes were observed in the challenged isolates. In general, after challenge with only 2mg/L of the combination, either no alteration or a slight reduction in the MIC to amoxycillin alone and amoxycillin/clavulanate, was observed. However, when

Figure 44. Analytical iso-electric focusing of single representatives of TEM-1 producing strains after challenge with amoxycillin/clavulanate

Tracks:-

A) ES 43 (2); B) ES 43 (4); C) ES 43 (8); D) ES 59 (2); E) ES 59 (4); F) ES 64 (2); G) ES 64 (4); H) R1 (TEM-1 producer); I) ES 67 (2); J) ES 67 (4); K) ES 72 (2); L) ES 72 (4); M) PS 136 (2); N) PS 1236 (4); O) R1

Figure in parenthesis equals challenge concentration (mg/L) amoxycillin/clavulanate.

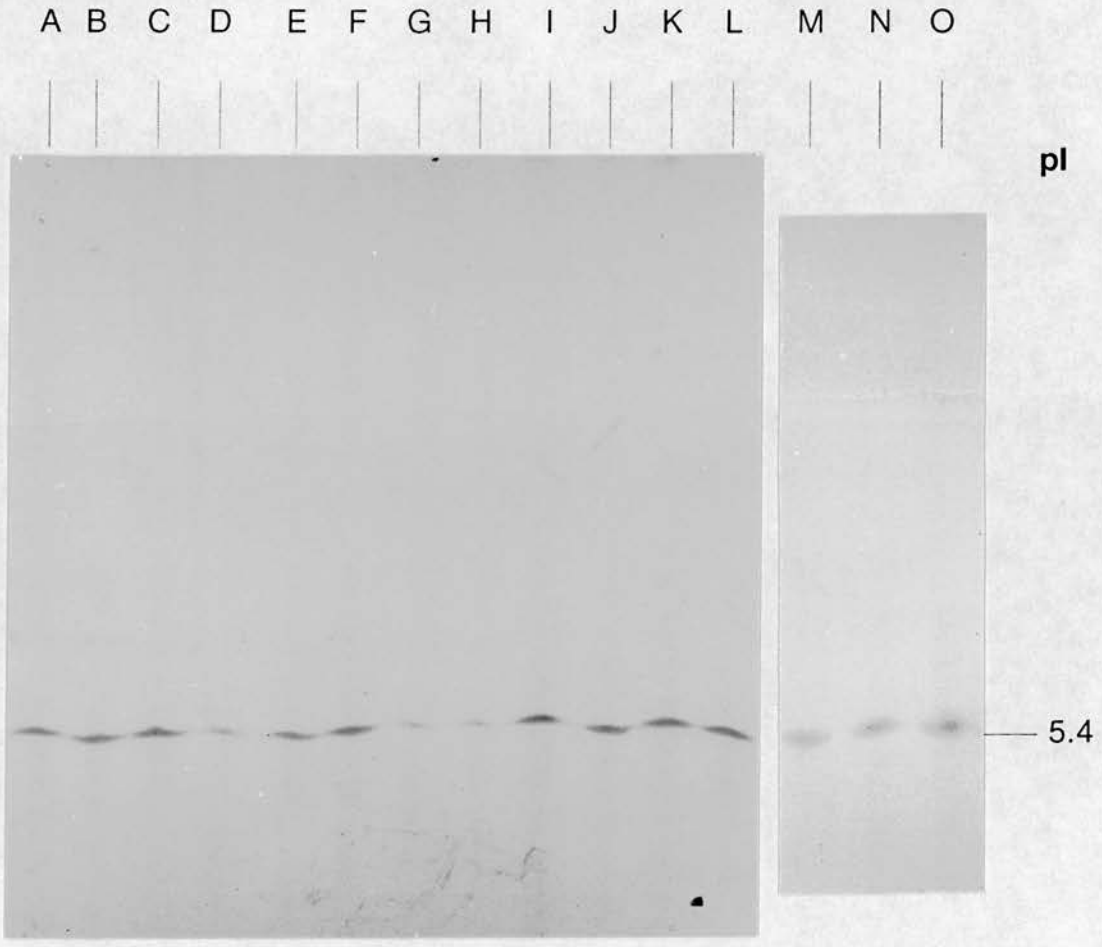
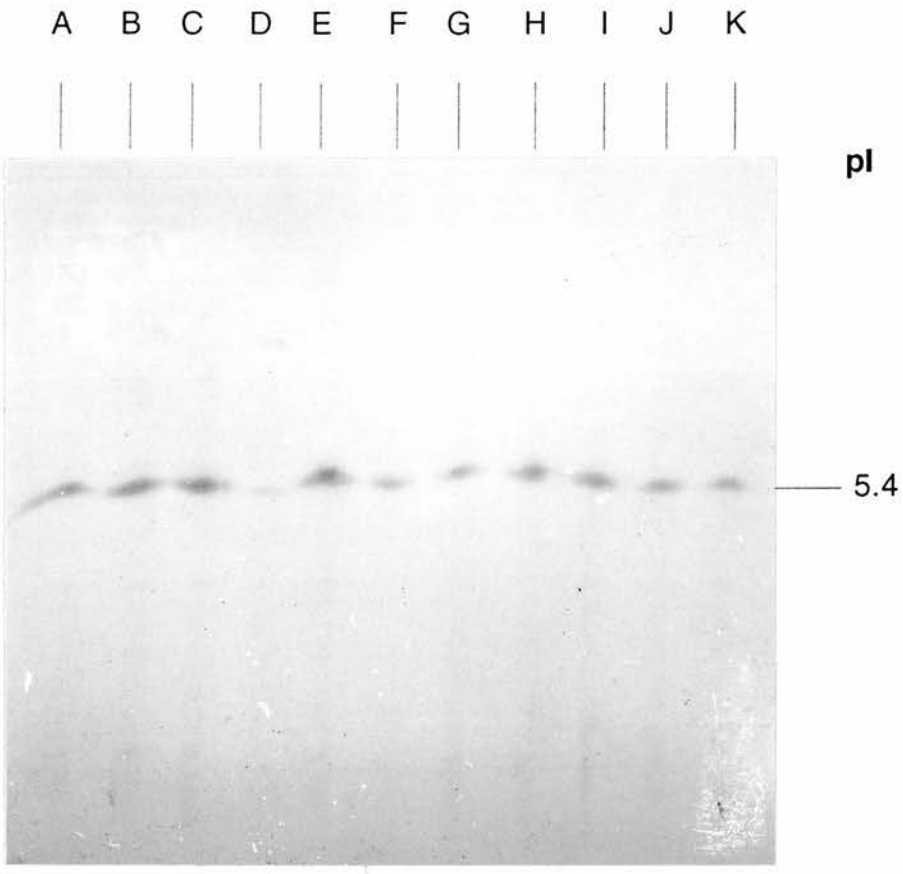


Figure 45. Analytical iso-electric focusing of single representatives of TEM-1 producing strains after challenge with amoxycillin/clavulanate

Tracks:-

A) PS 1261 (2); B) PS 1261 (4); C) PS 1261 (8); D) PS 1261 (16); E) PS 1271 (2); F) PS 1271 (4); G) PS 1323 (2); H) PS 1323 (4); I) PS 1337 (2); J) PS 1337 (4); K) R1 (TEM-1 producer)

Figure in parenthesis equals challenge (mg/L) amoxycillin/clavulanate.



the challenge concentration was increased, the MIC of both amoxycillin alone and amoxycillin/clavulanate was seen either not to change or to increase slightly in the strains. These phenotypic variations are in spite of no detectable variation in the iso-electric focusing point as indicated in Section 3.6.3. In addition, the specific activity of the β-lactamase produced by this isolate is also included in Table 48.

3.6.5 Plasmid Analysis of the Challenged and Unchallenged Strains

Variation in the challenged strains from the parent strains was investigated. The plasmids from both the unchallenged parent strains and the representative challenged strains were extracted and restricted by endonuclease digestion as described previously. Figures 46 and 47 both show the plasmid profiles of each unchallenged parent strain together with the plasmid profiles of their associated challenged strains at the various concentrations. As there is no difference in the profiles originating from challenged strains and the profiles of the unchallenged strains, it may be inferred that there has been no plasmid rearrangement.

Table 48. Comparison of isolates challenged with amoxycillin / clavulanate

Strain Number	Challenge Conc (mg/L)	MIC amox mg/L		MIC amox/clav mg/L		β-lactamase specific activity✣
		UC	CH	UC	CH	
ES 43	2	1024	1024	8	8	0.008
ES 43	4	1024	1024	8	8	0.036
ES 43	8	1024	>1024	8	16	0.014
ES 59	2	256	256	8	4	0.018
ES 59	4	256	512	8	8	0.017
ES 64	2	512	256	8	4	0.003
ES 64	4	512	512	8	8	0.004
ES 67	2	512	512	8	8	0.057
ES 67	4	512	1024	8	16	0.618
ES 72	2	512	512	8	8	0.010
ES 72	4	512	1024	8	16	0.009
PS 1236	2	512	256	8	4	0.035
PS 1236	4	512	1024	8	8	0.010
PS 1261	2	512	256	8	4	0.026
PS 1261	4	512	>1024	8	8	0.195
PS 1261	8	512	>1024	8	8	0.013
PS 1261	16	512	>1024	8	32	0.014
PS 1271	2	256	512	8	4	0.017
PS 1271	4	256	512	8	8	0.005
PS 1323	2	256	256	4	4	0.005
PS 1323	4	256	>1024	8	8	0.038
PS 1337	2	512	512	8	4	0.196
PS 1337	4	512	512	8	8	0.177

✣ = Δ μmoles nitrocephin hydrolysed / minute / mg protein

UC = Unchallenged strain CH = Challenged strain

Figure 46. Agarose gel electrophoresis of an endonuclease restriction digest of plasmid DNA extracted from both amoxycillin / clavulanate unchallenged and challenged strains

Tracks:-

A) ES 43 (0); B) ES 43 (2); C) ES 43 (4); D) ES 43 (8); E) ES 59 (0); F) ES 59 (2); G) ES 59 (4); H) ES 64 (0); I) ES 64 (2); J) ES 64 (4); K) ES 67 (0); L) ES 67 (4); M) ES 72; N) ES 72 (4)

Figure in parenthesis equals challenge concentration (mg/L) amoxycillin / clavulanate.

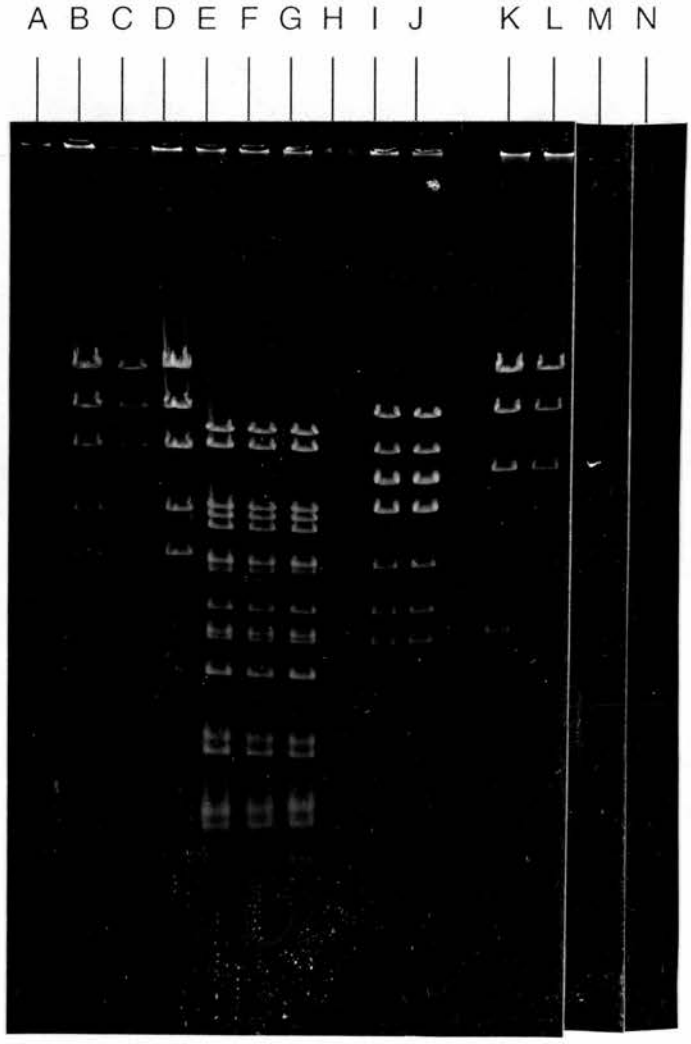
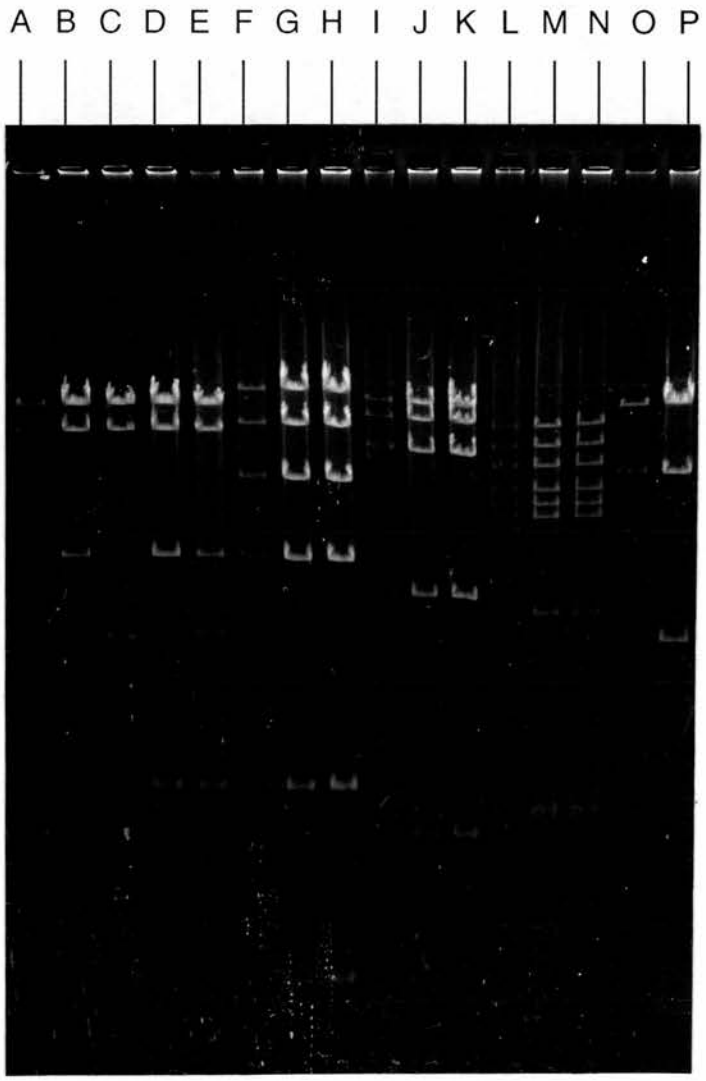


Figure 47. Agarose gel electrophoresis of an endonuclease restriction digest of plasmid DNA extracted from both amoxycillin / clavulanate unchallenged and challenged strains

Tracks:-

A) PS 1261 (0); B) PS 1261 (2); C) PS 1261 (4); D) PS 1261 (8); E)PS 1261 (16); F) PS 1236 (0); G) PS 1236 (2); H) PS 1236 (4); I) PS 1271 (0); J) PS 1271 (2); K) PS 1271 (4); L) PS 1323 (0); M) PS 1323 (2); N) PS 1323 (4); O) PS 1337(0); P) PS 1337 (4)

Figure in parenthesis equals challenge concentraion (mg/L) amoxycillin / clavulanate.



From the results, it is clear that there is no significant difference between the strains in the rates of hydrolysis for each of the substrates tested. In addition, the ID_{50} to clavulanic acid observed amongst the strains was very similar. It may be concluded therefore, that there is little variation in the TEM-1 β-lactamase which is produced in each strain.

Inspite of employing the published procedure to select variants of the TEM-1 β-lactamase (Thomson and Amyes, 1993b), no mutants were obtained from the series of challenge experiments. The fact that no mutations were observed could result from a number of reasons. First, as discussed previously, the genetic background, that is plasmid type, may have a bearing on the ease of selection of the various TEM β-lactamases (Jacoby and Sutton, 1991). Interestingly, strain ES 59 contained the same plasmid on which TRC-1 had arisen from TEM-1. As no mutations were obtained from this strain, it is clear that the genetic background is not the only factor in the selection of TEM variant enzymes. The fact that TRC-1 emerged on this plasmid in the clinical environment, presumably results from different challenge conditions *in vivo*.

Chapter 4

Discussion

Bacteria have responded to the development and clinical use of antibiotics with a bewildering array of resistance mechanisms (Amyes and Gemmell, 1992). The incidence of antibiotic resistance amongst clinical isolates is of worldwide concern and has been extensively studied (Atkinson and Lorian, 1984; Shears *et al.*, 1987; McGowan *et al.*, 1989; Møller, 1989; Dornbusch *et al.*, 1990; Senerwa *et al.*, 1991; Singh(b) *et al.*, 1992; MacGowan *et al.*, 1993). In particular, the development of antibiotic resistance to the β-lactam agents has been closely monitored (Sanders and Sanders, 1992). Amongst this group of antimicrobials, there has been an especial interest in antibiotic resistance mediated to ampicillin, as this is the most widely used antimicrobial throughout the world (Amyes, 1989; Young *et al.*, 1989; Sanders and Sanders, 1992).

Much less attention has, however, been paid to the carriage of antibiotic resistance genes in the normal non-pathogenic aerobic gut flora of healthy

individuals. As indicated in a number of recent studies, this bacterial population is of importance as the normal flora of healthy individuals could represent a constant pool of resistance genes (Levy *et al.*, 1988; Lester *et al.*, 1990). Such bacteria have the potential to complicate therapy, either by causing an endogenous infection, or by transferring their resistance determinants to invading pathogens (Datta, 1969; Moorhouse, 1969; Shaw *et al.*, 1973; Shears *et al.*, 1988a).

The purpose of the commensal faecal flora survey, conducted in Edinburgh, was to ascertain the carriage rates of antibiotic resistance amongst the healthy population. As a minimum requirement for participation, donors were requested not to have received antibiotic treatment in the three weeks prior to sampling. This time period was selected because previous studies have shown that changes in faecal flora subjected to antimicrobials were usually reversed by 10 - 14 days after stopping the drug (Levy, 1986).

In this study, the identified carriage rate of antibiotic resistance amongst 100 healthy volunteers was 42%, 39%, 27% and 12% for ampicillin, cefuroxime, tetracycline and trimethoprim respectively. The high carriage rate of cefuroxime resistant bacteria was unexpected. This is a result of the cefuroxime concentration (4mg/L) incorporated into the MacConkey agar plates; this breakpoint value, chosen for use with clinical therapy (BSAC guidelines, 1991), does, however, not reflect changes in the development of bacterial resistance. In addition, the low carriage rate of trimethoprim resistant bacteria was also

unexpected given the fact that this antimicrobial has been available in general practice for over 20 years (Huovinen, 1987; Elwell and Fling, 1989). No resistance was detected to the third generation cephalosporin, ceftazidime and only a low level of resistance was found to gentamicin, perhaps reflecting the restricted use of these compounds to hospitals. Similarly, there was only a low level of resistance to the quinolone antibacterials. This has been noted before in surveys of resistance in commensal faecal flora (Amyes *et al.,* 1992) and may result from the lack of plasmid-mediated resistance to these agents (Lewin *et al.*, 1990; Courvalin, 1990; Amyes and Gemmell, 1992).

Amongst the other commensal faecal flora surveys of the healthy population which have been conducted in the developed world, comparable carriage rates of antibiotic resistance have been reported. In a survey conducted in Boston, of 289 volunteers, 34.9%, 30.1% and 33.6% of the healthy population carried bacteria resistant to ampicillin, tetracycline and sulphamethoxazole respectively (Levy *et al.,* 1988). Trimethoprim resistance was not studied. An examination of *E. coli* from faecal samples of 310 healthy persons from different areas of the Netherlands revealed the prevalence of antibiotic resistant *E. coli* to be 75.5% for ampicillin, 58.5% for tetracycline, 60.5% for sulphamethoxazole, 43.5% for trimethoprim and 38.0% for nitrofurantoin (Bonten *et al.*, 1992). These are higher rates of resistance than those reported from an earlier study undertaken in the Netherlands, which revealed the overall carriage rate of resistant *E. coli* as 26% for ampicillin, 42% for tetracycline

and 46% for sulphamethoxazole (Degener *et al.*, 1983). The most recent investigation of this kind examined 85 healthy urban and rural dwellers in Jeddah and reported the carriage rate of ampicillin, streptomycin, tetracycline, kanamycin, and gentamicin resistant determinants to be 40.4%, 29.7%, 34.0%, 23.25% and 11.75% respectively (Ismaeel, 1993). The results from each of the various surveys of the carriage of antibiotic resistance amongst the commensal faecal flora of the healthy population are shown in Table 49.

In common with other surveys of the carriage of resistance in commensal faecal flora, the highest carriage rate of resistance in the Edinburgh population was to ampicillin. Ampicillin has been the most widely used antibiotic worldwide (Amyes, 1987; Young *et al.*, 1989; Sanders and Sanders, 1992) and this may partly explain the high levels of resistance encountered. In addition, ampicillin is poorly absorbed from the gut facilitating the selection of resistant gut flora (Neu, 1979; Amyes, 1989).

The renewed interest in the carriage of resistance genes in the normal faecal flora has been made with a view to predicting the importance of this bacterial population as a threat to the use of antibiotics. However, determining the carriage of antibiotic resistance in this bacterial population is, in common with clinical isolates, associated with a number of complicating factors. In particular, amongst clinical isolates, problems have arisen in relation to the choice of resistance breakpoints, inoculum size and the correct medium (Hamilton-Miller, 1984).

Table 49. The incidence of antibiotic resistance in commensal faecal flora of the healthy population; a comparison of surveys

Survey	Antimicrobial									
	Ap	Tp	NA	Cm	Tc	Su	Nit	Sm	Kn	Gm
Developed Countries										
1) Ireland	62	-	0	16	76	-	-	-	63	-
2) U.K.	25	-	-	5	22	17	-	21	-	-
3) The Netherlands	26	-	-	-	42	46	-	-	-	-
4) The Netherlands	76	25	-	-	47	86	29	-	-	
5) The Netherlands	76	44	-	-	59	61	38	-	-	-
6) U.S.A.	35	-	1	4	31	-	-	34	21	5
7) U.S.A	23	3	-	15	33	36	-	38	8	0
8) Scotland	42	12	2	-	27	-	-	-	-	1
Developing Countries										
9) Venezuela	85	61	-	56	93	93	-	95	56	0
10) China	47	64	-	42	92	87	-	79	26	32
11) India	98	98	23	97	-	-	-	-	-	-
12) Saudia Arabia	41	-	-	-	34	-	-	30	23	12
13) South Africa	87	74	10	53	-	-	-	-	-	8

1) Moorhouse, 1969; 2) Linton *et al.*, 1972 (urban study only); 3) Degener *et al.*, 1983; 4) Bonten *et al.*, 1990; 5) Bonten *et al.*, 1992; 6) Levy *et al.*, 1988; 7) Lester *et al.*, 1990; 8) Ph.D Edinburgh survey, 1991; 9) Lester *et al.*, 1990; 10) Lester *et al.*, 1990; 11) Amyes *et al.*, 1992; 12) Ismaeel, 1993; 13) Ph.D South Africa survey, 1992.

Furthermore, the selection of specimens and patients may influence the findings. Therefore, it has been concluded that the most significant assessment of changes in resistance are made from long-term follow-up surveys in a single centre (Amyes and Gemmell, 1992). These factors are all applicable to the faecal flora surveys of the healthy population. In particular, it is difficult to draw comparative conclusions between these different surveys as different protocols were employed in each. The initial screening procedure varies not only in the amount of faecal sample examined (Bonten *et al.*, 1990; 1992) but also in the concentration of antibiotic used to determine resistance (Levy *et al.*, 1988; Lester *et al.*, 1990). Consequently, this variability may affect the overall figures for the carriage rates of resistant organisms. This point is demonstrated firstly, in a study in the Netherlands where 75.5% of the healthy population examined contained ampicillin resistant faecal flora. The screening procedure was given as a partial explanation for this high carriage rate (Bonten *et al.*, 1992). Secondly, in a study of the carriage of *E. coli* resistant to antimicrobial agents from healthy children in three cities, two methods of colony selection were employed, namely, random colony selection and disk diffusion colony selection. The latter procedure was described as having 1000-fold greater sensitivity than the former (Lester *et al.*, 1990).

The genetic and biochemical basis for the development and spread of β-lactam resistance is well documented in clinical settings (Simpson *et al.*, 1980; Huovinen *et al.*, 1988b; Mabilat and Courvalin, 1990; Cooksey *et al.*, 1990;

Burman *et al.*, 1992; Thomson and Amyes, 1993a). Unfortunately, only a few of the studies on normal *E. coli* have proceeded to examine the mechanisms of resistance in the strains isolated (Simpson *et al.*, 1986; Singh(a) *et al.*, 1992; Thomson *et al.*, 1993b). The value of such studies is obvious to establish whether the resistance genes and plasmids in the normal flora are indeed the progenitors of those encountered in clinical isolates.

In this study, the β-lactamases responsible for transferable and non-transferable ampicillin resistance and the genetic carriers for the self-transmissable resistance determinants have been identified. Restriction endonuclease fingerprinting of the transconjugant plasmids identified nine different plasmid types. The fact that one plasmid type not only occurred on five occasions but was also isolated from three of the four different sample population groups and another, very similar plasmid type occurred on two occasions, suggests the presence of an epidemic plasmid in the community. Although the spread of epidemic plasmids in hospitals is well reported (Amyes *et al.*, 1987; Sirot *et al.*, 1988; Bauernfeind *et al.*, 1993), this study represents the first indication that this may also happen in bacteria carried by the healthy community.

The maintenance and stability of plasmids within bacterial populations is uncertain (Hawkey, 1986). The Edinburgh commensal faecal flora follow-up survey was, therefore, of considerable importance in that it provided some indication of plasmid maintenance. This study examined five faecal specimens

which had been obtained from those volunteers in the original survey whose faecal specimen contained bacteria capable of transferring ampicillin resistance. Interestingly, three specimens contained bacteria which were resistant to ampicillin suggesting that, in these volunteers, the presence of ampicillin resistant bacteria amongst the commensal faecal flora may either have been continuous, or alternatively, intermittent throughout the two year period. Of further interest, only in one strain was the ampicillin resistance determinant located on a self-transmissable plasmid. When analysed by endonuclease restriction fingerprinting, the plasmid was found to be identical in profile to the plasmid which had been extracted from ampicillin resistant bacteria obtained from the same volunteer two years previously. Although this newly isolated plasmid differed in antibiogram profile from the same plasmid identified in the first survey, the loss of the spectinomycin and cefuroxime resistance determinants had clearly not affected the plasmid arrangement. It is likely that this difference has occurred as a result of the problems associated with antibiogram sensitivity testing; variations in sensitivity may be recorded in strains when the MIC of an antibiotic is very close to the breakpoint concentration (BSAC guidelines, 1991). The fact that a plasmid should persist in the commensal faecal flora in the absence of any antibiotic selective pressure was of some surprise.

The identification and dissemination of epidemic plasmids within the clinical community has been widely reported (Amyes *et al.*, 1987; Sirot *et al.*, 1988;

Bauernfeind *et al.*, 1993). However, to date, there have been no comparative investigations between plasmids isolated from normal flora with those plasmids isolated from clinical flora. The study conducted to compare plasmids isolated from urinary isolates in Edinburgh with those plasmids isolated in the faecal flora survey was, therefore, of great interest. This was particularly so as the results from this research have shown that the epidemic plasmid identified amongst the commensal faecal flora of the healthy population was identical to an epidemic plasmid identified in urinary isolates in Edinburgh. It should be remembered that the vast majority of urinary tract infections registered in general practice are endogenous infections (Brumfitt *et al.*, 1971; Degener *et al.*, 1983). Therefore, from these findings, it may be concluded that, in the absence of antibiotic selection pressure, the resistance genes and plasmids in the normal flora are evidently the progenitors of those encountered in clinical isolates.

It was of especial interest, that the epidemic plasmid present in the Edinburgh community, common to both urinary isolates and isolates from faecal flora of healthy individuals, was shown to be identical to the plasmid on which the TEM derived β-lactamase, TRC-1, was encoded when it was initially isolated (Thomson and Amyes, 1992a). Although there was no increased resistance to amoxycillin plus clavulanate detected amongst isolates from the healthy community that contained the epidemic plasmid, the potential clearly exists for TRC-1 type and TRI type (Vedel *et al.*, 1992) enzymes to emerge in the community

given firstly, the presence of the exact plasmid arrangement which encoded TRC-1 and secondly, the widespread use of β-lactamase inhibitor combinations amongst general practitioners which encourages the selection of such enzymes (Thomson and Amyes, 1992b). Unlike extended-spectrum β-lactamases, inhibitor-resistant enzymes do, at present, seem to be rare (Vedel *et al.*, 1992; Thomson and Amyes, 1993b). However, it is vital not to become complacent. It has been reported that increased resistance to β-lactamase inhibitor combinations may be the result of a silent mutation which is not detectable by isoelectric focusing; that is, there is no alteration in the pI value (Delaire *et al.*, 1992). Such initial subtle changes that occur in the development of resistance may be masked through the inadequacies of routine sensitivity testing (Thomson and Amyes, 1993b). In this research, ten disparate plasmids, all carrying genes encoding the TEM-1 β-lactamase, were challenged with various concentrations of amoxycillin plus clavulanic acid. No mutants with increased resistance to this β-lactam/β-lactamase inhibitor combination were obtained suggesting that amongst this plasmid population, there has been no predisposition for the selection of these TEM derived β-lactamases.

Amongst clinical isolates, the TEM-1 β-lactamase is the most frequently isolated β-lactamase (Matthew, 1979; Medeiros, 1984; Medeiros *et al.*, 1988; Amyes, 1989; Young *et al.*, 1989; Wiedemann *et al.*, 1989; Sanders and Sanders, 1992). Examination of the β-lactamases produced by the transconjugants of this study revealed that plasmids encoding the TEM-1 β-lactamase were responsible for

mediating resistance to ampicillin in every case. Although a variety of different plasmid types were identified amongst the transconjugants, an epidemic plasmid also prevailed. It may therefore be concluded that the high incidence of resistance to ampicillin in Edinburgh is not only a result of the independent spread of the TEM-1 β-lactamase gene alone but also the spread of an epidemic plasmid encoding this β-lactamase.

The widespread presence of the TEM-1 β-lactamase amongst isolates from the healthy population is of concern. The TEM-1 β-lactamase is recognised as the progenitor to many extended-spectrum β-lactamases (Medeiros *et al.*, 1988; Philippon *et al.*, 1989; Payne and Amyes, 1991), which from clinical experience have been shown to be selected for in the presence of the newer cephalosporins (Sirot *et al.*, 1992; Bauernfeind *et al.*, 1993). The potential exists, therefore, for extended-spectrum β-lactamases to appear amongst the healthy community as the introduction of the oral later generation cephalosporins into general practice is imminent. Although, it has been argued that oral cephalosporins are no more likely to select out extended-spectrum β-lactamases than their parenteral counterparts (Cullman, 1992), it should be remembered that the threat of these new compounds is in their increased usage.

Examination of the wild type ampicillin resistant isolates for their resistance to other β-lactam agents revealed that amongst the *E. coli* there was complete sensitivity to the newer cephalosporins, cefotaxime and ceftazidime. In

addition, clavulanic acid was effective in restoring the sensitivity to amoxycillin (MIC < 32mg/L) in 96% of cases. This resistance pattern results from the predominance of the TEM-1 β-lactamase as the mediator of ampicillin resistance in these strains. Amongst those strains identified as *Klebsiella* spp., there was also a universal sensitivity to the newer cephalosporins and clavulanic acid in combination with amoxycillin. Not surprisingly, SHV-1 predominated as the responsible β-lactamase mediating resistance in these strains as others have noted elsewhere (Matthew, 1979; Shannon *et al.*, 1990b).

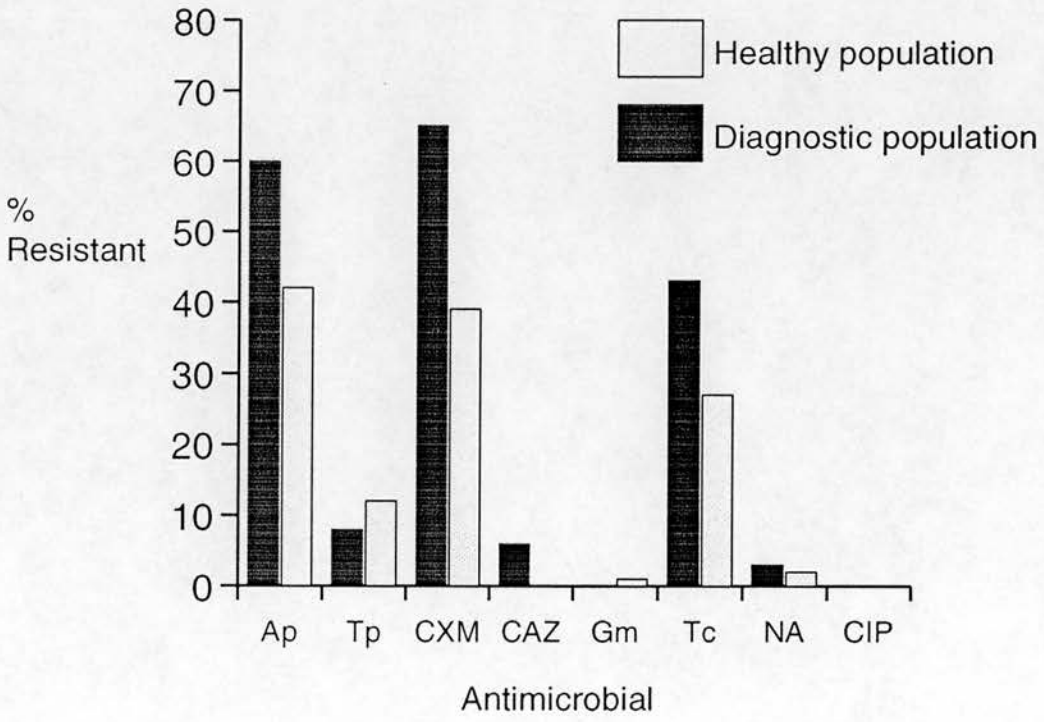
Comparisons between commensal faecal flora surveys has been complicated by variations in the selection criteria for volunteer participation. In order to make an accurate assessment of the extent of the carriage of resistance genes in the healthy population, the study groups should be as representative of the general community as possible. Unfortunately, this may not always be possible as encouraging participation in such studies is difficult. It is perhaps for this reason that many of the earlier studies undertaken have looked at specific populations and their relevance to the general community is therefore questionable. Specific population groups examined for the carriage of antibiotic resistance determinants in faecal flora include children (Moorhouse, 1969; Lidin-Janson *et al.*, 1977; Lester *et al.*, 1990), particularly those attending day care centres (Reves *et al.*, 1987; 1990), and students (Bonten *et al.*, 1990; 1992; Lamikanra *et al.*, 1989). In addition, the commensal faecal flora of patients prior

to their admission to hospital has been well investigated (Datta, 1969; Shaw *et al.*, 1973; Søgaard, 1975). This population group may not be considered "healthy" but none-the-less, it does provide some indication of the carriage of antibiotic resistant bacteria in the nonclinical community.

For comparison with the commensal faecal flora survey of the healthy population in Edinburgh, the survey of negative faecal specimens obtained from the diagnostic laboratories in Edinburgh Royal Infirmary was conducted. Although the donors of the specimens in this group, as with pre-admission patients, may not be considered healthy, no obvious association exists with the clinical community. It is not clear what is the impact of illness or debility on the commensal faecal flora. In this study, amongst the 100 faecal specimens obtained, a higher carriage of antibiotic resistant bacteria were identified than in the commensal faecal flora of the healthy population in Edinburgh. This is clearly represented in the histogram (Figure 48). The most noticeable differences are for ampicillin, cefuroxime and tetracycline. In the diagnostic survey, the identified carriage rates of resistance were found to be 60%, 65% and 43% respectively, but in the healthy faecal flora survey were 42%, 39% and 27% respectively. It may be speculated that although not clinically related, donors of specimens from the diagnostic survey may have been receiving antimicrobials for treatment of a gastrointestinal related condition. Under such circumstances, increased levels of antibiotic resistant bacteria would be anticipated on account

of the selection pressure. Of interest, a lower carriage of trimethoprim resistant bacteria was observed (8%). As before, the proportion of strains carrying gentamicin or ceftazidime resistant determinants was low which again reflects the restricted use of these antimicrobials to the hospital environment. Similarly nalidixic acid and ciprofloxacin resistance was low amongst these bacterial strains. Again this is probably a consequence of the non-transferable mechanism of resistance associated with the quinolones (Lewin *et al.*, 1990; Courvalin, 1990; Amyes and Gemmell, 1992).

Figure 48. Comparison of the carriage of antibiotic resistant bacteria in the "healthy" population with the "diagnostic" population in Edinburgh



As in the analysis of the wild type ampicillin resistant strains obtained from the commensal faecal flora of the healthy population in Edinburgh, the wild type ampicillin resistant strains obtained from the diagnostic survey were tested for their sensitivity to a range of β-lactam agents. The results obtained closely resembled the results of the first survey. Amongst the *E. coli*, there was a universal sensitivity to the newer cephalosporins, cefotaxime and ceftazidime and in addition clavulanic acid was effective in restoring the sensitivity to amoxycillin (MIC < 32mg/L) in all but five cases. Significantly, in each of the five cases, a chromosomal β-lactamase was identified as the mediator of resistance. The *Klebsiella* spp. also displayed a similar sensitivity profile with universal susceptibility to amoxycillin plus clavulanic acid, ceftazidime, cefotaxime and cefuroxime. Notably, lower MIC values of amoxycillin (MIC < 128 mg/L) were recorded amongst the *Klebsiella* spp. except in one isolate which had an MIC of 1024mg/L. This strain was identified as being a TEM-1 β-lactamase producer.

The genetic and biochemical basis for ampicillin resistance in these isolates were also ascertained for comparison with the initial survey. The diagnostic strains demonstrated a much higher frequency of transfer of resistant determinants (65%) into *E. coli* J62-2 than the ampicillin resistant strains from the first survey (33%). Given the higher carriage of ampicillin resistant strains identified in this population, this finding is perhaps not surprising. When the transconjugants were analysed, the antibiogram profiles were found to be very

similar to those antibiogram profiles detected in the transconjugants of the healthy population. This presumably results from the fact that both populations are exposed to the same selecting agents.

It was anticipated that a wide variety of plasmid profiles, as determined by restriction endonuclease digestion, would exist; 21 different profiles amongst 37 transferable strains was consequently not surprising. One plasmid type (Group C) did, however, predominate as it was found in 19% of the isolates. Interestingly, endonuclease restriction analysis showed that this plasmid was identical to the epidemic plasmid encoding the TEM-1 gene that had been identified in the commensal faecal flora survey of the healthy Edinburgh population, the associated follow-up survey which was conducted two years later and in addition, in the Edinburgh urinary isolates. It is of concern that the same plasmid should persist in each of the different population groups examined.

The incidence of antibacterial drug resistance amongst clinical isolates has been shown to be higher in the developing world rather than the developed world (Farrar, 1985). The limited number of conducted surveys indicate that this is also the case with the carriage of antibiotic resistant strains within the normal gut flora (Lester *et al.*, 1990; Amyes *et al.*, 1992). For comparison, therefore, the study which ascertained the carriage of antibiotic resistance amongst the commensal faecal flora of the black populations in South Africa was conducted.

South Africa is representative of a unique situation whereby both the developed and developing world are represented. The black communities experience third world living conditions with two important exceptions; there is generally no unprescribed use of antibiotics and the water supply is often bacteria-free.

Such variations in living conditions are reflected in the vast differences of bacterial carriage of antibiotic resistance. This was exemplified in a study of urinary and non-urinary isolates, in which trimethoprim resistance varied considerably between three hospitals; 59% of Hillbrow Hospital isolates, 50.6% of Baragwanath Hospital isolates and 37.6% of Johannesburg Hospital isolates were resistant (Wylie and Koornhof, 1989). While the high incidence of trimethoprim resistance amongst clinical isolates was reportedly related to the high dispension of this agent to both inpatients and outpatients (Wylie and Koornhof, 1989), both Hillbrow and Baragwanath hospitals have a high proportion of patients from poor community areas which may have influenced the carriage rate.

In the commensal faecal flora survey in South Africa, the carriage rate of antibiotic resistance was 88.6% for ampicillin and 74.2% for trimethoprim. Chloramphenicol resistance was found to be lower at 52.6%, while the carriage of gentamicin and nalidixic acid resistance were lower still at 7.5% and 10.2% respectively. This lower carriage of resistance may reflect the lower usage of these antimicrobials as opposed to ampicillin and trimethoprim, which are the

most widely used antibacterials in the study areas (H.J. Koornhof, Personal communication). The carriage rates of antibiotic resistance identified in South Africa are substantially higher than in Edinburgh. This is a true comparison as exactly the same methodology was employed in conducting each study.

These results strongly indicate that although South Africa is considered an industrialised developed country, the black communities in the study are representative of a developing country type under-culture. The findings of this research may be applicable to certain other closed population groups in developed countries. Such groups may include those on Indian reservations in the United States, where hygienic conditions are poor and suitable for spread of endemic disease (Blaser *et al.*, 1983). It may be speculated, therefore, that the carriage rates of antibiotic resistant bacteria amongst the faecal flora of similar groups such as the poor Americans in Ghetto areas, Aborigines in Australia and some population groups in Europe, may resemble those identified amongst the black communities in South Africa.

The highest incidence to date for the carriage of resistant bacteria within commensal populations was recently reported from South India (Amyes *et al.*, 1992); trimethoprim or ampicillin resistant bacteria were present in 98% of the specimens screened. The factors contributing to the maintenance of this high carriage of bacterial resistance included the contamination of the water supply in combination with the inappropriate dispension and widespread use of

antimicrobial agents (Young and Jesudason, 1990; Amyes *et al.*, 1992). The causal relationship between increased antibiotic resistance and increased antibiotic usage has been explained before.

High carriage rates of antibiotic resistant determinants amongst the normal flora of healthy children in the developing world have been demonstrated. In Caracus and Qin Pu, 60.9% and 64.1% respectively of the children examined contained trimethoprim resistant faecal flora. It has also been reported that the carriage of antibiotic resistant strains is much higher amongst children than adults (Linton *et al.*, 1972; Hawkey, 1986). Possible reasons for this include high antimicrobial prescribing amongst this group and lower standards of hygiene, which facilitates the transmission of resistant organisms (Singh *et al.*, 1990). Examination of the resistance levels within our population sub-groups, however, revealed a lack of correlation between age and antibiotic resistant faecal flora. This compares with the commensal study of Dutch urban communities which reported the same finding (Degener *et al.*, 1983). However, this contrasts with other findings; examination of child day care centres (DCC) (Reves *et al.*, 1990), revealed substantially higher levels of trimethoprim resistant faecal flora (30%) amongst the children attending the centre than is prevalent in the general population (6%). Similarly, ampicillin resistant faecal flora was reported as 70% in this particular population, which again is substantially higher than corresponding levels identified in the healthy community (34.9%) (Levy *et al.*,

1988). In the current study, approximately half the urban infants attended a childminder in SOWETO, the equivalent of a child day care centre, and yet the levels of resistance observed in this particular group are not noticeably higher than any other sub-group. This suggests the spread of antibiotic resistance factors may occur at the same frequency in all strata of society if the general levels of personal hygiene are low.

One of the distinctions between the developed and developing world is the lack of a uncontaminated potable water supply in the latter. This has obvious implications in facilitating the spread of infectious disease and had been speculated to have a role in the transmission of antibiotic resistant organisms (Amyes *et al.*, 1992; Young, 1993). This study reports contamination of the water and unsanitary conditions especially in rural areas which creates the opportunity for cross infection between bacteria. Similar conclusions have been drawn by other workers (Murray *et al.*, 1985; Lamikanra and Ndep, 1989; Amyes *et al.*, 1992). However, in contrast to the Indian study, which indicated contamination of the water supply coupled with high antimicrobial usage as being responsible for the high resistance levels in that area, these factors may only be contributors to the resistance levels witnessed in South Africa. This is because in spite of very basic conditions in the urban areas of SOWETO, all our participants came from houses with a tap supplying an uncontaminated supply of water. Surprisingly, although the water was contaminated in rural areas, it

did not seem to affect the carriage of antibiotic resistant *E. coli*; *E. coli* were not isolated in the water samples. However, ablution facilities in the rural villages were extremely limited increasing the opportunity for spread of resistant determinants through the faecal/oral route.

In addition, unprescribed use of antimicrobial agents may be a contributing factor to the high levels of resistance found in the developing world. Although the ability to buy antibiotics over the counter may contribute to high level resistance (Murray *et al.*, 1985; Lamikanra and Ndep, 1989; Amyes *et al.*, 1992), such purchasing is not usually possible in South Africa (Wylie and Koornhof, 1989). However, there might be a higher usage of antibiotics associated with this population group in order to treat endemic diseases. It should, however, be borne in mind that the justifiable prescription of an antibiotic does not guarantee that it will be used correctly. This is true in the developed world as well but in developing countries the temptation to save some of the course, divide the course amongst the family or even sell the antibiotic will undoubtedly be greater.

It is probable that the main influence determining and maintaining the high level of resistance in the black South African population is the severe overcrowding in the homes (>6.0 people/house). Close contact facilitates the transmission of resistant determinants (Singh *et al.*, 1990). Intrafamilial contact has been shown to be an important factor in the spread of resistant determinants (Rydberg and Cederberg, 1986).

Poor living conditions and poor sanitation, may be the major contributors in maintaining the high levels of resistant bacteria in this reservoir. Indeed, these factors may become more important than antibiotic usage itself.

As discussed previously, until now there has been little investigation into the genetic and biochemical basis for the development and spread of resistance amongst the non pathogenic commensal flora of healthy populations. In this study, the β-lactamases responsible for transferable ampicillin resistance and the genetic carriers for such resistance have been determined. Amongst the 158 transconjugants, restriction endonuclease fingerprinting identified 92 different plasmid types. Interestingly, the rural infant population group harboured three distinct plasmids which were all isolated on multiple (>6) occasions. However, the occurrence of such epidemic plasmids was restricted to this population group. The lack of a common plasmid in the urban infants was, perhaps, particularly surprising given the fact that 20 of the specimens were supplied from infants attending one childminder. Previous surveys have indicated an association between the presence of a common plasmid and children attending day care centres (Reves *et al.*, 1987). Furthermore, the widespread variation between plasmids was surprising given the similarity between many of the antibiogram profiles.

In contrast to the findings from the Edinburgh surveys, where an epidemic plasmid appeared to be dispersed across various population groups in the area, in this study the different plasmid types appeared to be restricted to specific

population groups. Only a single plasmid (Group 18) first recognised amongst the rural infant population was subsequently identified in other population groups; that is amongst rural infants and then again amongst rural children.

The identification of the same plasmid from the different strains isolated from the same donor was of interest and presumably resulted from plasmid transfer within the commensal flora rather than the acquisition of two strains carrying the same plasmid from an external source.

This study demonstrated that as in the Edinburgh surveys, the TEM-1 β-lactamase is the predominant mechanism of resistance involved in mediating resistance to ampicillin amongst the commensal faecal flora of the healthy population. This finding is indicative of this successful β-lactamase. However, unlike the results from the Edinburgh survey, where TEM-1 β-lactamase was the only β-lactamase recognised amongst the transconjugants, amongst the South African transconjugants, SHV-1 and TEM-2 β-lactamases were both isolated, although their occurrence was rare. It is not clear if the prevalence of these β-lactamases will persist or increase amongst the community or alternatively whether TEM-1 β-lactamase will replace them. The potential for TEM-2 β-lactamase, in particular, to spread throughout the community certainly exists. At present, TEM-2 β-lactamase is not frequently isolated. It may, therefore, be considered surprising that many of the identified extended-spectrum β-lactamases have been derived from

this particular β-lactamase (Amyes, 1989). It had been postulated that the high level of gene expression associated with TEM-2 β-lactamase may be conducive to the selection of variant β-lactamases (P. Courvalin, Personal communication).

The predominance of the TEM-1 β-lactamase as the determinant of β-lactam resistance amongst the commensal faecal flora of the healthy population has been reported before (Simpson *et al.*, 1986; Thomson *et al.*, 1993). Amongst 24 isolates obtained from the commensal faecal flora survey conducted in India, with the exception of one strain which produced OXA-1 β-lactamase, all the isolates produced the TEM-1 β-lactamase (Thomson *et al.*, 1993). The persistence of the TEM-1 β-lactamase may be facilitated in the healthy community where simple penicillins are probably the main selective pressure. The discovery of such a wide variety of plasmids encoding the TEM-1 β-lactamase was, perhaps, surprising as limited hygiene and sanitation are conditions in developing countries which are conducive to the spread of epidemic strains (Thomson *et al.*, 1993). This finding suggests that ampicillin resistance is not simply the result of spread of one particular strain or plasmid but rather from the spread and subsequent independent acquisition of the β-lactamase gene.

The TEM-1 β-lactamase is purportedly so ubiquitous as a result of the responsible genes residing on a series of transposons which facilitates the transmission of the resistance determinants (Heffron *et al.*, 1977; Chen and

Clowes, 1987). The success of the TEM-1 β-lactamase is also by virtue of its biochemistry; TEM-1 β-lactamase hydrolysis reaction of ampicillin and amoxycillin are reportedly completed with maximum efficiency (Christensen *et al.*, 1990).

The commensal faecal flora of the healthy individual acting as a reservoir of antibiotic resistant determinants (Levy *et al.*, 1988; Lester *et al.*, 1990), is a hypothesis strengthened by this research. However, whilst a picture of the mechanisms of resistance to β-lactam agents has been established, the identity of resistance plasmids in commensal flora begs the question as to their source. A number of potential avenues exist through which the acquisition of resistant *E. coli* may occur. It had been suggested that travel to developing countries, with or without the use of prophylactic treatment, is indeed associated with the acquisition of resistant *E. coli* (Murray *et al.*, 1990). In addition, it had long been speculated that the ingestion of contaminated meat products is a source of resistant bacteria (Shooter *et al.*, 1970; Corpet, 1987). Furthermore, animal feedlots are recognised as a reservoir of resistance determinants (Smith, 1968) and as both animal to man and person to person transmission of resistance determinants has been shown (Levy *et al.*, 1976; Linton, 1977; Linton, 1986), this is another potential avenue to the healthy population. Finally, the most recently elucidated avenue of resistant determinants is that of day care centres. A high carriage rate of resistant organisms has been demonstrated in this group (Reves *et al.*, 1987; 1990; Singh *et al.*, 1990).

This is to be expected because of the poor standard of hygiene between children which allows direct spread of resistant organisms (Goodman *et al.*, 1984; Singh *et al.*, 1990) and in addition this may be exacerbated by the higher antibiotic prescribing associated with this age group (Hawkey, 1986). Although children are not representative of the community at large, they may represent an avenue through which resistant determinants may spread into the community. Intrafamilial spread of resistant determinants had been demonstrated and may occur once the child returns home (Rydberg and Cederberg, 1986).

4.1. Conclusions

The non-pathogenic commensal gut flora of the healthy population clearly represents a reservoir of resistance determinants. Furthermore, it has been speculated that these resistant determinants may be the progenitors of those encountered in clinical isolates.

The recognition of the TEM-1 β-lactamase prevailing throughout the healthy community has confirmed the successful nature of this β-lactamase. Speculation has been made on the detrimental impact that TEM-1 β-lactamase may have on the healthy community. Not only will current therapy be complicated but, in addition, TEM-1 β-lactamase may act as a potential source of novel adapted β-lactamases as newer oral agents are introduced in to general practice.

The potential threat of this reservoir has been addressed but several questions do, however, remain unanswered. Little is known about the maintenance and transmission of these plasmids in the environment and, in particular, although an association of antibiotic resistance genes with plasmid-mediated virulence genes is purported to ensure greater survival of the strain, other factors affecting their stability in the absence of antibiotic selective pressure are unknown.

Chapter 5

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The Prevalence of Antimicrobial Resistance in Human Faecal Flora in South Africa.

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SUMMARY

Between January and March 1992, 361 faecal specimens were collected from the healthy black population in the Transvaal Province of South Africa. Each specimen was examined for the prevalence of antimicrobial resistance in commensal bacteria. Volunteers, from both rural and urban dwellings, were divided into four age groups. The overall carriage rate of resistance varied from 88.6% for ampicillin, 74.2% for trimethoprim, 52.6% for chloramphenicol, 10.2% for nalidixic acid to 7.5% for gentamicin. The carriage of resistance found to each individual antimicrobial agent was slightly higher in the rural population rather than the urban population but there was no correlation between the prevalence of antimicrobial resistance and the age group.

INTRODUCTION

The increasing prevalence of bacterial resistance to most antibiotics is of concern as the choice of agents available for treatment becomes limited and inevitably more complicated. Furthermore, the potential exists where no available therapeutic agent would be effective against many pathogens.[1]

The incidence of antibiotic resistance amongst clinical isolates is well documented. Antibacterial drug resistance has been shown to be higher in the developing world rather than the developed world.[2] Trimethoprim resistance amongst pathogenic Gram negative bacteria was found to be 64% in South India,[3] 49.1% in South Africa[4] and 63.3% in Nigeria.[5] In contrast, the levels of such resistance were found to be only 14-19% in Finland[6] and 23% in Scotland.[7] Reasons for the high levels of resistance in the developing countries may include the availability of antibiotics without prescription and the contamination of the water supply.[8]

In contrast, the incidence of such resistance amongst the normal non pathogenic flora such as E. coli of healthy individuals, in the absence of concurrent or recent antibiotic consumption, has been less extensively recorded.[8,9,10,11] The carriage of resistance determinants in these organisms may complicate treatment as the potential exists firstly, for their transfer to any invading pathogens and secondly, the resistant bacteria may cause an endogenous infection.[12]

It is recognised that transfer of resistance factors is encouraged when antibiotics are administered.[13] The acquisition of resistant bacteria may be achieved through ingestion of contaminated food, person to person or animal to man transmission.[14,15] A number of sources have been associated with the emergence of resistant bacteria including developing countries,[2] clinical practice, animal feedlots[16] and, more recently, day care centres (DCC).[17]

The normal commensal flora is increasingly recognised as a reservoir of antibiotic resistance. As in clinical isolates, levels of resistance in this group appear to be higher in the developing rather than developed countries. This study examines resistance in the commensal faecal flora in the black population of South Africa. This study area represents a unique situation in having third world living conditions with two important exceptions; there is generally no unprescribed use of antibiotics and the water supply is often bacteria-free.

METHODS

Population Description

The survey participants consisted of healthy volunteers resident in the Transvaal, South Africa. Candidates were excluded from the study if they had received any medical treatment in the three weeks prior to sampling. This time period allowed any changes in faecal flora, resulting from antimicrobial usage, to be reversed.[9,18] A completed questionnaire accompanied each specimen; it supplied information regarding the donor's

age, sex, address, medical history and data on social conditions. The study examined eight separate population groups from both urban and rural areas. In the urban area the four groups comprised infants attending either a childminder or a creche in SOWETO (0-5 years), urban children (6-11 years) and urban teenagers (12-19 years) attending a school in Kagiso, a town on the West Rand and urban adults (>19 years) who resided in SOWETO. The rural population was composed of infants attending a "well baby" clinic at Middleplaas in the KaNgwane district, rural children and rural teenagers attending a school at Hekpoort in the Magaliesburg district and lastly adults resident in KaNgwane district. Notably, the rural adults were all associated with the hospital at Shongwe Mission and consequently were in contact with patients. This specific population consisted of healthy mothers feeding sick children, mothers attending a nutrition centre for children and finally escorts to patients visiting the out patients department. Parental consent was obtained for all persons involved of <18 years.

Sample Collection and Processing

The survey collection was performed between January - March 1992; a total of 361 specimens were obtained. Rectal swabs or freshly passed faecal specimens were deposited in small plastic containers for immediate transport to the laboratory. Following the protocol established for such studies,[8] each faecal specimen was plated onto Oxoid MacConkey Agar plates containing either ampicillin (10mg/L), nalidixic acid (10mg/L), chloramphenicol (10mg/L), gentamicin (4mg/L) or no antibiotic. Each specimen was also plated onto Modified Difco Mueller Hinton Agar plates

containing trimethoprim (10mg/L).[19] The plates were incubated overnight at 37°C. Each plate was scored for the presence or absence of bacterial colonies which were subsequently classified as either lactose fermenters or lactose non- fermenters.

Water Sample Collection and Processing

From each study centre, except for Kagiso, a 100ml sample of the normal drinking water was collected in a sterile bottle. After immediate transportation to the laboratory, the water was filtered and the filter pad placed on an Oxoid MacConkey agar plate containing no antibiotic. The plate was incubated overnight at 37°C. Each plate was examined for the presence of bacterial colonies which were subsequently identified.

Bacterial Identification

Bacterial colonies were identified by standard biochemical analysis. Each strain was allocated to one of four groups; E. coli, Klebsiella species, Enterobacter species, or other enterobacteria.

Conjugation Studies

All the isolates resistant to ampicillin and trimethoprim were tested for the ability to transfer their resistance determinants by the method of Amyes and Gould (1984).[19] The conjugation experiments employed the rifampicin resistant E. coli K-12 strain J62-2 [20] as the recipient.

RESULTS.

Study Population

A total of 361 participants were included from both the rural (183) and urban (178) studies. The male/female distribution was 1:1.4 in both the groups. The adult population was predominantly female (Table 1).

Frequency of Antibiotic Resistance

The results of the survey are shown in Table 2. A high proportion of volunteers carried bacteria resistant to antibiotics; in particular to ampicillin (88.6%) and trimethoprim (74.2%). Five antibiotics were screened in the study and, for each, the corresponding level of resistance identified was slightly higher amongst the rural populations. There was no difference in the level of resistance to each antimicrobial agent and age group except that gentamicin resistance in the rural adults was much higher than it had been amongst their urban counterparts (38.6% versus 4%).

Identification of Ampicillin and Trimethoprim Resistant Strains

All the ampicillin and trimethoprim resistant strains were identified (Table 3). E.coli represented the largest proportion of resistant bacteria when either susceptibility to ampicillin or trimethoprim was measured.

Frequency of Transfer

A total of 608 ampicillin resistant strains and 357 trimethoprim resistant strains were collected from the survey. The results of the transfer experiments show that only 29% of the ampicillin resistant isolates contained self-transmissable plasmids. In contrast, 51.2% of trimethoprim resistant isolates contained self-transmissable plasmids while a further 3.4% contained trimethoprim resistant plasmids which could be mobilised with X^+ factor.

Environmental Conditions

In an attempt to establish if environmental conditions exerted any influence over the carriage of antibiotic resistance in bacteria, various factors were examined.

(i) Water supply. A representative water sample was taken in each study area except in Kagiso (table 4). Screening in the urban locations revealed the water, which is municipally piped, to be free of any bacteria. In contrast, the water tested from Middleplaas clinic, stored rain water, contained Pseudomonas aeruginosa, Serratia marcescens and a gram-positive coccus. The school in Hekpoort is provided with water from a bore hole. This was contaminated with four different bacteria, Bacillus cereus, Acinetobacter anitratus, Enterobacter cloacae and Pseudomonas aeruginosa. The rural adult population was not exposed to a contaminated water supply as the water utilized at Shongwe hospital was filtered and chlorinated river water. This water was completely bacteria-free.

(ii) Living conditions. The social conditions of the participants are reflected in Table 4. In both the rural and urban study, approximately 50% of the participants were associated with animals. Despite the possibility of animal to man transmission of bacteria, this was probably not a major contributory factor in the spread of resistance as resistant bacteria were as commonly observed in participants living in the absence of animals.

(iii) Population density. The most influential environmental factors observed were the number of people per family and per house. Many of the rural homes were wattle and daub huts with only one room for sleeping. Similarly, it was not uncommon for many urban homes to have just one bedroom. As similar numbers of residents per house (mean= 6.0 people/house) were recorded in both the rural and urban areas, it would seem that this widespread over-crowding is a large contributor to the high levels of resistance.

DISCUSSION

Commensal flora represent an increasingly well recognised reservoir of resistance genes. In this study the carriage rate of antimicrobial resistance amongst the commensal flora of selected black South African populations was examined. The incidence of antibiotic resistance carriage was 88.6% for ampicillin and 74.2% for trimethoprim. Chloramphenicol resistance was found to be lower at 52.6% while the carriage of gentamicin and nalidixic acid resistance were lower still at 7.5% and 10.2%

respectively. This lower carriage of resistance may reflect the lower usage of these antimicrobials as opposed to ampicillin and trimethoprim which are the most widely used antibacterials in the study areas.

The highest incidence to date for the carriage of resistant bacteria within commensal populations was recently reported from South India;[8] trimethoprim or ampicillin resistant bacteria were present in 98% of the specimens screened. The factors contributing to the maintainance of this high carriage of bacterial resistance included the contamination of the water supply and the inappropriate dispension and widespread use of antimicrobial agents.[8] The causal relationship between increased antibiotic resistance and increased antibiotic usage has been explained before.

It has been reported that the carriage of a antibiotic resistant strains is much higher amongst children than adults.[13] Possible reasons for this include high antimicrobial prescribing amongst this group and lower standards of hygiene which facilitate the transmission of resistant organisms. Examination of the resistance levels within our population sub-groups, however, revealed a lack of correlation between age and antibiotic resistant faecal flora. This compares with the commensal study of Dutch urban communities which reported the same finding.[21] However, this contrasts with other findings: examination of child day care centres (DCC)[17], revealed substantially higher levels of trimethoprim resistant faecal flora (30%) amongst the children attending the centre than is prevalent in the general population (6%). Similarly, ampicillin resistant faecal flora was reported as 70% in this particular population which again

is substantially higher than corresponding levels identified in the healthy community (34.9%).[9] In the current study, approximately half the urban infants attended a child-minder in SOWETO, the equivalent of a child day care centre, and yet the levels of resistance observed in this particular group are not noticeably higher than any other sub-group. This suggests the spread of antibiotic resistance factors may occur at the same frequency in all strata of society.

One of the distinctions between the developed and developing world is the lack of a uncontaminated potable water supply in the latter. This has obvious implications in facilitating the spread of infectious disease and had been speculated to have a role in the transmission of antibiotic resistant organisms.[8] This study reports contamination of the water and unsanitary conditions especially in rural areas which creates the opportunity for cross infection between bacteria.[5,8,22] However, in contrast to the Indian study which indicated contamination of the water supply coupled with high antimicrobial usage as being responsible for the high resistance levels in that area, these factors may only be contributors to the resistance levels witnessed in South Africa. This is because in spite of very basic conditions in the urban areas of SOWETO, all our participants came from houses with a tap supplying an uncontaminated supply of water. Surprisingly, although the water was contaminated in rural areas, it did not seem to affect the carriage of antibiotic resistant *E. coli*; *E. coli* were not isolated in the water samples. However, ablusion facilities in the rural villages were extremely limited increasing the opportunity for spread of resistant determinants through the faecal/oral route.

In addition, unprescribed use of antimicrobial agents may be a contributing factor to the high levels of resistance found in the developing world. Although the ability to buy antibiotics over the counter may contribute to high level resistance,[5,8,22] such purchasing is not usually possible in South Africa.[4] However, there might be a higher usage of antibiotics associated with this population group in order to treat endemic diseases.

It is probable that the main influence determining and maintaining the high level of resistance in the black South African population is the severe overcrowding in the homes (>6.0 people/house). Close contact facilitates the transmission of resistant determinants.[23] Intrafamilial contact has been shown to be an important factor in the spread of resistant determinants.[24]

Once established, poor living conditions and poor sanitation, may be the major contributors in maintaining the high levels of resistant bacteria in this reservoir. Indeed, these factors may become more important than antibiotic usage itself.

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TABLE 1. Volunteers in the Study.

Location/ Study Group	Total No. of Individuals	No. Females	AGE Range (yrs)	Mean (yrs)	SD	Mode Meat/Week
Rural Study						
Infants	50	29	0-5	0.75	0.82	3
Children	47	26	6-11	9.1	1.42	3
Teenagers	36	19	13-18	14.0	1.24	3
Adults	50	35	>19	28.4	10.12	3
Urban Study						
Infants	45	23	0-5	2.4	1.28	4
Children	42	18	6-11	9.1	1.69	4
Teenagers	47	22	13-18	13.1	0.93	7
Adults	44	42	>19	33.0	6.06	3

TABLE 2 Percentage of specimens with organisms resistant to five antibiotics in South Africa.

	URBAN					RURAL				
ANTIBIOTIC	Ap	Tp	Cm	NA	Gm	Ap	Tp	Cm	NA	GM
AGE (years)										
0 - 5	94	80	66	4	10	100	88.9	66.7	44	2.2
6 - 11	89.4	72.3	38.3	4.2	0	76.2	54.8	40.5	14.2	0
12 - 19	75	61.1	30.5	13.9	0	87.2	72.3	44.7	14.9	4.2
>19	84	68	44	10	4	100	93.2	86.4	18.2	38.6
TOTAL	86.3	71	45.9	7.7	3.8	91	77.5	59.9	12.9	11.2

Ap, ampicillin; Tp, trimethoprim; Cm, chloramphenicol; NA, nalidixic acid; Gm, gentamicin.

TABLE 3. The Incidence of Organisms Isolated from the Study.

	Number Isolated (%)			
Organism	Ap resistant strains		Tp resistant strains	
E. coli	445	(73)	297	(83)
Klebsiella species	104	(17)	46	(13)
Citrobacter/*Enterobacter*	35	(6)	8	(2)
Other	24	(4)	6	(2)

TABLE 4. Social Conditions of Volunteers in the Survey.

Location	Mean No./Family	Mean No./House	% with Animals	% Water Source= TAP	Water Contaminated
Rural Study					
Hekpoort	7.0	6.2	61	79	+
Shongwe	7.4	7.7	41	88	+
Urban Study					
SOWETO	8.6	8.7	68	99	-
Kagiso	6.0	6.3	50	100	NT

NT, Not tested.

β-lactam Resistance in Aerobic Commensal Faecal Flora

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Faecal specimens from 100 healthy volunteers living in Edinburgh were examined for the presence of antibiotic resistant bacteria. A high incidence of ampicillin resistance was found as 42% of specimens containing normally sensitive bacteria were resistant to the drug; however only 12% of the specimens contained trimethoprim resistant bacteria. There was no detectable resistance to the third generation cephalosporin, ceftazidime or the 4-quinolone, ciprofloxacin. Identification of the β–lactamases produced by the ampicillin resistant isolates demonstrated that the TEM-1 β–lactamase predominated particularly in *E. coli* where it was identified in 86% of isolates. Thirty three percent of the ampicillin resistant isolates were able to transfer their resistance to *E. coli* K12 strain J62-2 and analysis of these transconjugants by iso-electric focusing revealed that the TEM-1 β–lactamase was present in 100% of the transconjugants. Restriction endonuclease fingerprinting of the TEM-1 containing plasmids revealed an epidemic plasmid in the community.

Introduction

There is increasing evidence to suggest that the healthy members of a community may contain the largest reservoir of bacterial antibiotic resistance genes [1,2]. Such resistance determinants may complicate treatment as the potential exists, firstly, for their transfer to any invading pathogens and, secondly, the resistant bacteria may cause an endogenous infection. Consequently, it has been suggested that knowledge of these resistance levels may improve the success of initial antibiotic therapy [2]. A number of surveys examining antimicrobial resistance in selected healthy volunteers have recently been reported [1-6]. In clinical isolates, the levels of antibiotic resistance are significantly higher in the developing world than in the developed world [7]. This may also be true of the carriage of antibiotic resistance in commensal flora [5,6]. In particular, the incidence of ampicillin resistance in both areas is high. The genetic and biochemical mechanisms of resistance to β–lactam agents have been extensively investigated in clinical isolates and it has been shown that resistance to β–lactam agents in Gram-negative bacteria is most commonly mediated by the TEM-1 β–lactamase [8-12]. This is especially true for transferable resistance where the TEM-1 gene has been shown to account for up to 80% of all plasmid-mediated resistance [10].

Although the significance of the commensal flora as a reservoir for resistance determinants is increasingly realised, there have been few studies as yet to address the resistance mechanisms involved in such isolates. This study establishes the levels of resistance to various antimicrobial agents in the healthy population in Edinburgh and then examines the genetic and biochemical basis for the ampicillin resistance identified.

Materials and Methods

Sample population

A total of 100 faecal specimens were collected from three separate healthy populations in Edinburgh between January - June 1991. No volunteer was allowed to participate in the study if they had received any antimicrobial treatment in the three weeks prior to sampling. The study group consisted of office workers and members of the university chaplaincy centre (42), patients of a general practice in Loanhead (34) and medical students at the University of Edinburgh (24).

Sample collection and processing

The carriage of antibiotic resistance was assessed following the protocol established for such studies and described previously [5]. The fresh faecal samples were collected in sterile plastic bottles and transported to the laboratory the same day. Each specimen was plated onto Oxoid MacConkey agar (Oxoid, Basingstoke, U.K.) plates containing either ampicillin (10mg/L), ceftazidime (2mg/L), nalidixic acid (10mg/L), ciprofloxacin (1mg/L), gentamicin (4mg/L), tetracycline (10mg/L) or no antibiotic. In addition, the specimens were also plated onto a modified Mueller Hinton Agar (Difco, Michigan, U.S.A.) containing trimethoprim (10mg/L) [13]. The plates were incubated at 37°C for 24h. Non-mucoid, lactose-fermenting colonies were recorded and purified onto media containing the same antibiotics as in the initial isolation.

Identification of ampicillin resistant isolates

All ampicillin resistant isolates were identified by the API 20E system.

Transfer of ampicillin resistance determinants

All the ampicillin resistant isolates were investigated for their ability to transfer their resistant determinants to an isogenic background. Transfers were carried out in nutrient broth at 37°C for 18h employing *E.coli* K-12 strain J62-2 as the recipient

strain [14]. The mating mixture was washed and resuspended in Davis and Mingioli (DM) minimal medium [15], before they were plated onto a selective medium containing ampicillin (10mg/L) and rifampicin (25mg/L) [13]. The auxotrophic requirements of the transconjugants were checked on DM minimal media plates containing L-proline, L-histidine and L-tryptophan (each at 50mg/L) and also on similar plates lacking L-histidine in order to confirm their status.

Plasmid and restriction endonuclease analysis

Plasmid DNA was isolated by the method of Takahashi and Nagano [16]. Restriction analysis was carried out with *Eco* R1 (Boeringer Mannheim, Sussex, U.K.) according to the manufacturer's instructions.

Minimum inhibitory concentration and antibiogram analysis

All the ampicillin resistant organisms isolated were investigated further. The minimum inhibitory concentration (MIC) of each ampicillin resistant bacterium was determined by diluting an overnight nutrient broth culture in DM minimal medium. Approximately 10^4 cfu was inoculated onto the surface of Diagnostic Sensitivity Test Agar (Oxoid) (DSTA) plates containing doubling concentrations of amoxycillin, amoxycillin plus clavulanic acid in a 2:1 ratio, cefuroxime, cefotaxime and ceftazidime. The plates were incubated aerobically at 37°C for 24h. The MIC was taken as the lowest concentration which gave no visible growth. The antibiogram of each transconjugant was obtained by following the same method as for the MIC except one fixed concentration of antibiotic was incorporated into the test plates.

β–lactamase identification

Sonicated β–lactamase extracts were prepared as described previously [17]. The enzymes were identified by analytic isoelectric focusing by comparison with known β–lactamases isolated from control strains [17,18] and visualised by staining with nitrocephin (10^{-3}M). β–lactamase activity was measured by spectrophotometric assay

(λ 2 UV/VIS Spectrophotometer) of the hydrolysis of nitrocephin (10^{-4}) at 384nm (sodium phosphate buffer pH7.0, 37°C) and the specific activity was obtained with reference to the protein concentration measured by the method of Waddell [19]

Results

Incidence of antibiotic resistance in the commensal faecal flora

A total of 100 faecal specimens were obtained from healthy populations in Edinburgh. The sex distribution of the volunteers consisted of 46 males, 45 females and 9 unknown. The carriage rates of resistance identified in the faecal strains is shown in Table 1. The highest incidence of resistance was against ampicillin as 42% of the specimens contained aerobic bacteria not susceptible to the drug. However, no resistance was found to the third generation cephalosporin, ceftazidime. Little resistance was detected to nalidixic acid (2%) and none to ciprofloxacin. Resistance to gentamicin was low (1%) while 27% of all volunteer's specimens contained bacteria resistant to tetracycline. Trimethoprim resistance was 12%. There was no significant difference in the levels of resistance found from the different populations.

Identification of ampicillin resistant isolates

Identification of the ampicillin resistant isolates demonstrated that *E.coli* was the most common isolate (Table 2). Other strains identified were *Klebsiella*, *Citrobacter* and *Hafnia* spp.

Sensitivity of ampicillin resistant strains to β–lactam antibiotics

The 42 ampicillin resistant isolates were examined for their resistance to a range of β–lactam agents. The minimum inhibitory concentration (MIC) of the strains was determined for amoxycillin, amoxycillin in combination with clavulanic acid (2:1 ratio), cefotaxime, ceftazidime and cefuroxime (Table 3). Isolates with a high MIC of amoxycillin (MIC > 512mg/L) were predominantly *E.coli*. Amongst the *E.coli* there

was almost universal sensitivity to cefotaxime and ceftazidime (MIC < 2mg/L) and the addition of clavulanic acid reduced the MIC to amoxycillin to below 32mg/L in all but two cases. Cefuroxime resistance amongst the *E.coli* (MIC > 4mg/L) was 15%.

Those isolates identified as *Klebsiella* spp had lower MICs of amoxycillin and amoxycillin plus clavulanic acid than the *E.coli* and universal sensitivity to cefuroxime, cefotaxime and ceftazidime. The small number of *Citrobacter* and *Hafnia* spp identified showed variable resistance to amoxycillin, amoxycillin/clavulanic acid and cefuroxime but no resistance to ceftazidime and cefotaxime.

Examination of transferable ampicillin resistance

Amongst the ampicillin resistant strains, the ability to transfer the resistant determinants by conjugation to the standard bacterial strain *E. coli* J62-2 was exhibited in 14 of the 42 isolates (33%). Those strains which were able to transfer their ampicillin resistance determinants were all identified by API 20E system as *E. coli*. Each transconjugant was examined for its plasmid content and the plasmids were characterised by their size and resistance profile (Table 4). Although three transconjugants contained more than one plasmid, the remainder of the transconjugants contained only a single plasmid. The plasmids were investigated further by restriction analysis with *Eco* R1 (Figure 1). Plasmids with closely related restriction digest patterns were assigned to the same category; that is they did not differ by changes in no more than two bands. However, if the profiles differed by three bands or more, the plasmids were assigned to a separate group. In addition, the antibiogram of the plasmids was established by testing the sensitivity to various antibiotics at given breakpoints (Table 4). The results from the restriction analysis revealed a number of different plasmid types (9) were present. Amongst the transconjugants, a common plasmid type was isolated on five occasions (Tracks 1,2,9,13 and 14) conferring resistance to amoxycillin, cephaloridine, cefuroxime, streptomycin and spectinomycin. Another plasmid was present on two occasions (Tracks 5 and 12) and appeared to be

related to the common plasmid because, although the restriction digest profile differed by three bands, there was a number of common bands, and the antibiograms were identical. The remaining seven plasmids each occurred on a single occasion and although the restriction digest was quite different for each, the antibiogram showed that, in common with the other plasmids isolated, resistance was mediated to similar agents.

Distribution of β–lactamases

The prevalence of different β–lactamase was determined by iso-electric focusing (Table 3). Amongst *E.coli* the TEM-1 β–lactamase predominated as only two β-lactamase producers did not mediate this enzyme; in one isolate no β–lactamase was detected whilst in the second only chromosomal β–lactamase was observed. All the *E.coli* that could transfer ampicillin resistance were TEM-1 producers. Greater heterogeneity in β–lactamase production was observed with the Klebsiella isolates with TEM-1, SHV-1 and K-1 all present. In a single isolate no β–lactamase was detected. The Citrobacter and Hafnia isolates produced only chromosomally encoded and TEM-1 β–lactamases. The specific activity of the TEM-1 β–lactamase was determined for the ampicillin resistant transconjugants. No significant difference could be identified between the plasmid type and the specific activity of the enzymes.

Discussion

The incidence of antibiotic resistance amongst clinical isolates is of worldwide concern and has been extensively studied [20,21]. Much less attention has been paid to the carriage of antibiotic resistance genes in the normal non pathogenic aerobic gut flora of healthy individuals. However recent studies indicate that this group may represent an important pool of resistant organisms demonstrating significant carriage rates of *E.coli* resistant to commonly used antimicrobials [1,2,4]. Amongst clinical isolates the levels

of antibiotic resistance in the Third World are considerably higher than those found in the West [7]. This would also appear to be the case with resistance in commensal faecal flora. In South India there is almost universal carriage of ampicillin (96.8%) and trimethoprim (96.8%) resistant strains in the normal healthy population [5]. In South African townships there is also a high carriage rate of resistant organisms to ampicillin (88.6%) and trimethoprim (74.2%) [6]. In this study in Edinburgh the carriage rate of antibiotic resistance amongst 100 healthy volunteers was 42.0%, 27.0% and 12.0% for ampicillin, tetracycline and trimethoprim respectively. No resistance was detected to the third generation cephalosporin ceftazidime and only a low level of resistance to gentamicin perhaps reflecting the restricted use of these compounds to hospitals only. There was only a low level of resistance to the quinolone antibacterials, this has been noted before in surveys of resistance in commensal faecal flora [5] and may result from the lack of plasmid mediated resistance to these agents. In common with other surveys of the carriage of resistance in commensal faecal flora the highest carriage rate of resistance in the Edinburgh population was to ampicillin. Ampicillin has been the most widely used antibiotic worldwide and this may partly explain the high levels of resistance encountered. In addition ampicillin is poorly absorbed from the gut facilitating the selection of resistant gut flora [22]. Further examination of the ampicillin resistant isolates for their resistance to other β–lactam agents revealed that amongst the *E.coli* there was complete sensitivity to the later generation cephalosporins cefotaxime and ceftazidime and in addition clavulanic acid was effective in restoring the sensitivity to amoxycillin (MIC < 32mg/L) in 96% of cases. This resistance pattern results from the fact that the TEM-1 β–lactamase predominates as the mediator of ampicillin resistance in these strains.

The genetic and biochemical basis for the development and spread of resistance is well documented in clinical settings [20,21]. Unfortunately few of the studies on normal *E.coli* have proceeded to examine the mechanisms of resistance in the strains isolated. The value of such studies is obvious to establish whether the resistance genes and

plasmids in the normal flora are indeed the progenitors of those encountered in clinical isolates. In this present study the β–lactamases responsible for transferable ampicillin resistance and the genetic carriers for such resistance have been determined. Restriction endonuclease fingerprinting of the transconjugant plasmids showed that there were nine different plasmid types. The fact that one plasmid type not only occurred on five occasions but was also isolated from each of the different sample groups and another, very similar, plasmid type occurred on two occasions may indicate the presence of an epidemic plasmid in the community. Although spread of epidemic plasmids in hospitals is well reported [23-25] this study represents the first indication that this may also happen amongst the healthy community. Examination of the β–lactamases produced by the transconjugants demonstrated that ampicillin resistance resulted from the presence of the TEM-1 β–lactamase in all cases, this is similar to a recent study in India which demonstrated that production of the TEM-1 β–lactamase predominated as the mechanism of ampicillin resistance amongst normal *E.coli* [26]. This would suggest that the situation in normal *E.coli* is the same as in the clinical setting where TEM-1 is by far the most commonly produced β–lactamase in clinical isolates [10,21].

This study has demonstrated significant carriage of antibiotic resistant strains in the healthy population in Edinburgh. In particular, there is a high carriage rate of resistance to ampicillin. The widespread presence of the TEM-1 β–lactamase on specific plasmids throughout the community is of concern. The TEM-1 β–lactamase is the progenitor of many extended-spectrum β–lactamases [20]. The presence of the TEM-1 β–lactamases in the healthy community may therefore not only complicate current therapy but may also act as potential source of novel adapted β–lactamases as newer agents are introduced into general practice.

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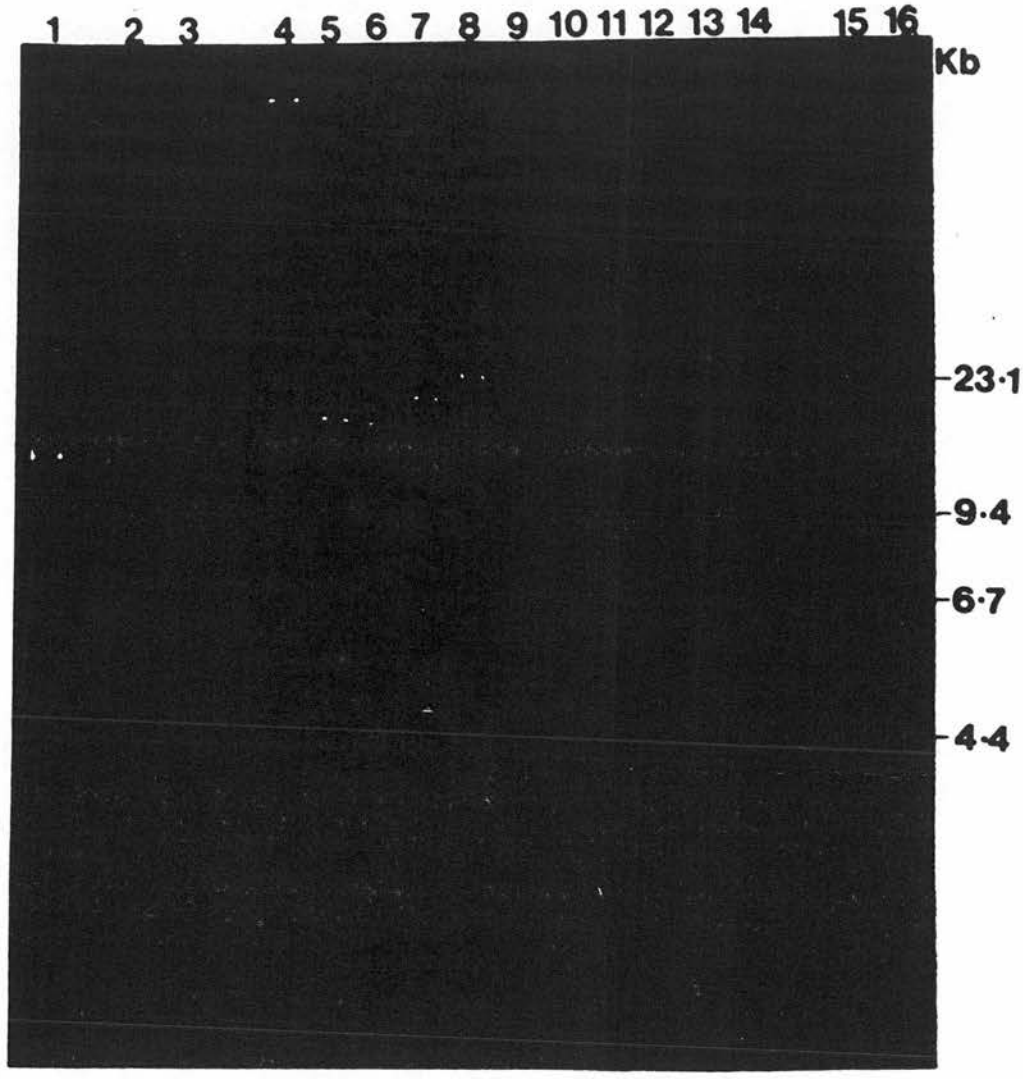
FIGURE 1 Restriction digest of the ampicillin resistant transconjugants



***Eco* R1 restriction endonuclease digests of plasmids isolated from the ampicillin resistant transconjugants.**

1) ES20, 2) ES59, 3) ES60, 4) ES62, 5) ES63, 6) ES64, 7) ES65, 8) ES67, 9) ES68, 10) ES69, 11) ES72, 12) ES73, 13) ES75, 14) ES76, 15)λ phage restricted with *Eco* R1, 16) λ phage restricted with *Hin* dIII

TABLE 1 Proportion of volunteers with antibiotic resistant bacteria in their commensal faecal flora

Antimicrobial agent	Concentration mg/L	Percentage volunteers
Ampicillin	10	42
Ceftazidime	2	0
Nalidixic acid	10	2
Ciprofloxacin	1	0
Gentamicin	4	1
Tetracycline	10	27
Trimethoprim	10	12

TABLE 2 Identification of ampicillin resistant isolates

Organism	No. of isolates	Percentage
Escherichia coli	28	67
Klebsiella spp	9	21
Citrobacter spp	4	10
Hafnia spp	1	2

TABLE 3 Minimum inhibitory concentrations of β-lactam antibiotics and the production of β-lactamase in the original isolates

PS No.	Isolate No	Minimum Inhibitory Concentration (mg/L)					Original Strain	β-lactamase
		Amox	Amox/ clav	CTX	CAZ	CXM		
*275	1	1024	16	2	2	4	*E.coli*	TEM-1
*277	2	1024	16	2	2	2	*E.coli*	TEM-1
*279	3	>1024	16	2	2	4	*E.coli*	TEM-1
*283	4	1024	16	2	2	4	*E.coli*	TEM-1
295	5	1024	8	2	2	8	*E.coli*	TEM-1
12	6	1024	8	2	2	2	*E.coli*	TEM-1
*17	7	1024	16	2	2	4	*E.coli*	TEM-1
19	8	>1024	16	2	2	2	*E.coli*	TEM-1
27	9	1024	32	2	8	32	*E.coli*	CHRM
*31	10	1024	16	2	2	2	*E.coli*	TEM-1
43	11	>1024	16	2	2	4	*E.coli*	TEM-1
*47	12	>1024	16	2	2	2	*E.coli*	TEM-1
*503	13	512	16	2	2	2	*E.coli*	TEM-1
*512	14	1024	16	2	2	4	*E.coli*	TEM-1
*516	15	>1024	16	2	2	2	*E.coli*	TEM-1
*520	16	1024	16	2	2	2	*E.coli*	TEM-1
526	17	512	2	2	2	2	*E.coli*	TEM-1
*529	18	>1024	16	2	2	4	*E.coli*	TEM-1
530	19	32	2	2	2	2	*E.coli*	ND
302	20	>1024	16	2	2	4	*E.coli*	TEM-1
305	21	1024	8	2	2	4	*E.coli*	TEM-1
*307	22	>1024	16	2	2	2	*E.coli*	TEM-1
319	23	1024	8	2	2	4	*E.coli*	TEM-1
*320	24	1024	16	2	2	4	*E.coli*	TEM-1
322	25	>1024	16	2	2	4	*E.coli*	TEM-1
327	26	1024	8	2	2	8	*E.coli*	TEM-1
332	27	256	32	2	2	16	*E.coli*	TEM-1
336	28	16	2	2	2	4	*E.coli*	CHRM
293	29	64	2	2	2	2	*K.pneumoniae*	SHV-1
35	30	64	2	2	2	2	*K.oxytoca*	SHV-1
40	31	64	2	2	2	2	*K.oxytoca*	ND
42	32	32	2	2	2	2	*K.oxytoca*	SHV-1
508	33	64	4	2	2	2	*K.oxytoca*	SHV-1
522	34	32	2	2	2	2	*K.oxytoca*	TEM-1
308	35	32	2	2	2	2	*K.oxytoca*	SHV-1
312	36	32	2	2	2	2	*K.pneumoniae*	SHV-1
318	37	64	2	2	2	2	*K.oxytoca*	K1
285	38	1024	8	2	2	2	*C.freundii*	TEM-1
290	39	256	64	2	2	2	*C.freundii*	CHRM
21	40	256	64	2	2	4	*C.freundii*	CHRM
50	41	16	16	2	2	16	*C.diversus*	TEM-1
3	42	32	16	2	2	2	*H.alvei*	TEM-1

Amox	Amoxycillin
Amox/Clav	Amoxycillin plus clavulanic acid
CTX	Cefotaxime
CAZ	Ceftazidime
CXM	Cefuroxime
CHRM	Chromosomal β-lactamase
ND	No detectable β-lactamase
*	Strain carries a self-transmissible plasmid

TABLE 4 Plasmid analysis of the TEM-1 β-lactamase-producing transconjugants

Transconjugant No.	No. of plasmids	Size Kb	Restriction pattern group	Specific activity*	Antibiogram
ES20	1	80.9	A	2.2	Ap Cf Sm Sp
ES59	3	80.9	A	19.3	Ap Cf Sm Sp
ES68	1	80.9	A	5.2	Ap Cf Sm Sp
ES75	1	84.1	A	3.4	Ap Cf Sm Sp
ES76	1	82.9	A	2.8	Ap Cf Sm Sp
ES60	1	87.9	B	6.8	Ap Cf Sm Sp Cm Tc
ES62	1	87.0	C	12.7	Ap Cf Sm Sp Tc
ES63	3	81.9	D	28.9	Ap Cf Sm Sp
ES73	1	81.9	D	5.7	Ap Cf Sm Sp
ES64	1	85.7	E	17.7	Ap Cf Sm Sp
ES65	3	121.5	F	18.1	Ap Cf Sp
ES67	1	46.5	G	35.5	Ap Cf Sm Sp Tp
ES69	1	54.8	H	4.2	Ap Cf Sm Sp
ES72	1	46.1	I	3.1	Ap Cf Sm Sp

* nmol nitrocephin hydrolysed/min/mg protein
Ap Ampicillin (10mg/L)
Cf Cefuroxime (4mg/L)
Sm Streptomycin (10mg/L)
Sp Spectinomycin (10mg/L)
Cm Chloramphenicol (10mg/L)
Tc Tetracycline (10mg/L)
Tp Trimethoprim (10mg/L)

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DETECTION OF BETA-LACTAMASE PRODUCTION BY ENTEROBACTERIACEAE; SUS.PTATE PROFILES OF HOSPITAL BETA-LACTAMASES.

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Our study group consists of 449 Enterobacteriaceae strains isolated in Moldavia in 1988-1990 period. Among these strains, 16 strains (3 Proteus strains, 6 Salmonella non-typhoidic strains, 7 Escherichia coli) remained resistant both at Ampicillin-Sulbactam and Ampicillin-Clavulanic acid association. In our study, we have characterized the activity profiles of the beta-lactamases produced by these resistant strains. We have monitorized the effects of the enzyme filtrates on: Ampicillin, Cloxacillin, Cefuroxil, Cefuroxime, Cefotaxime, Ceftriaxone. The test strain used was Sarcina lutea ATCC 9341. The enzyme types were established after the inhibitor activity of the beta-lactamins tested.
Conclusion: According to Bau classification, all the beta-lactamases produced by these strains were class I.

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REDUCED SYNTHESIS OF β-LACTAMASE BY *Enterobacteriaceae* UNDER ANAEROBIC CONDITIONS.

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It is known that under anaerobic conditions the expression of several genes is reduced while that of others is increased. Since different Authors have reported that the bactericidal activity of various new oral third generation cephalosporins against β-lactamase producing *Enterobacteriaceae* is affected by the analytical techniques adopted, in this study we have evaluated the extent of β-lactamase synthesis and the susceptibility of some gram-negative enteric bacteria to the aforementioned drugs under aerobic and anaerobic conditions. MIC values obtained with *E.coli* and *K.pneumoniae* differed widely by microtiter, macromethod, and under anaerobic environment in comparison to those noted in cultures maintained under vigorous aeration. In particular, tests conducted in aerobiosis showed MIC values 8-fold higher than those observed in the anaerobic assays. The same findings were noted with *P.mirabilis, S.marcescens,* and *E. cloacae* in experiments that included cefdinir and cefuroxime. On the other hand, a reference non β-lactamase producing *E.coli* strain exhibited the same MIC values in all the experimental conditions examined. Under anaerobic conditions β-lactamase production by *E.coli* and *K.pneumoniae*, as assessed by a colorimetric method, was reduced (63 and 77% respectively) in comparison with the amounts found in aerobiosis. These findings suggest that *in vivo*, where low oxygen tensions exist in several infected sites, the sluggish rate of β-lactamase synthesis by some gram-negative pathogens might render the threat of inactivation of these antibiotics negligible, thus improving their therapeutic margin.

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EXTENDED-SPECTRUM ß-LACTAMASES CONFERRING RESISTANCE TO THIRD-GENERATION CEPHALOSPORINS AND CEPHAMYCINS IN KLEBSIELLA PNEUMONIAE CLINICAL ISOLATES.

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The extended spectrum ß-lactamases (ESßs) have been reported with increasing frequency mainly in K.pneumoniae and have been recognized as a serious threat to current ß-lactam therapy. ESß producing strains are involved in epidemic spreads throughout institutions particularly in Europe. We have studied twenty isolates of K.pneumoniae from patients in intensive care units, resistant to cefotaxime, ceftazidime, aztreonam and cefoxitine. The ß-lactamases extracted from uninduced and induced strains have been studied by isoelectro focusing. Plasmid DNA obtained by alkali-lysis has been electrophoresed in agarose gels. Transfer of resistance markers to E.coli K12 has been also carried out. All strains produced a ß-lactamase with pI 7.6, four strains produced a ß-lactamase with pI 5.5 and three strains produced a ß-lactamase with pI 8.4. All strains showed a common plasmid of about 31.7 M dal and three strains another one slightly smaller.
CONCLUSION: The clinical isolates showed a similar extended-spectrum ß-lactamases and plasmid pattern supporting the impression that the outbreak was caused by dissemination of a single organism throughout the hospital.

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A CARBAPENEM-HYDROLYSING β-LACTAMASE FROM ENTEROBACTER CLOACAE: SEQUENCE ANALYSIS AND REGULATION.

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As previously reported (ICAAC, 1990), a clinical isolate of Enterobacter cloacae produces a carbapenemase along with an AmpC cephalosporinase. From this strain, a 5.2 kb Sau3A genomic DNA fragment containing the carbapenemase gene was cloned into pACYC184 plasmid in E. coli. Analysis of the deduced protein from its determined DNA sequence revealed that the protein (NOR-BLA) is a 30 kDa Ambler class A β-lactamase. It shares 50% homology with chromosomal β-lactamase from Klebsiella oxytoca. Deletion experiments of the 5' upstream sequences of the carbapenemase gene lead to a decrease in carbapenem resistance level, therefore suspecting the existence of regulatory elements. In the opposite orientation from the NOR-bla promotor, an overlapping and divergent promotor was detected along with an open reading frame which encoded for a 32 kDa protein (NOR-R). NOR-R sequence displayed homology with the LysR regulatory family proteins including, in particularly, the conserved residues nearby the NH_2 end which are thought to be involved in their DNA binding functions. NOR-R homology was 43 % with AmpR of the inducible cephalosporinase system of E. cloacae. Protein fusion of NOR-bla with the β-galactosidase gene revealed that NOR-BLA expression was (i) strongly decreased in the absence of NOR-R, (ii) and induced in presence of NOR-R with 6-aminopenicilloic acid (500 µg/ml), imipenem (0.1 µg/ml) and ampicillin (100 µg/ml).
CONCLUSION: This is the first report of a carbapenemase sequence in Enterobacteriaceae. As opposed to the carbapenemases previously sequenced (B. fragilis, B. cereus) NOR-BLA is a serine β-lactamase and not a metallo-enzyme. Moreover, its biosynthesis is regulated by a LysR-type protein which acts as a positive regulator and which is required for NOR-BLA induction.

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INCIDENCE OF THE TEM-1 βETA-LACTAMASE IN COMMENSAL FAECAL FLORA.

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There is increasing evidence to suggest that commensal flora may act as a reservoir for antibiotic resistance genes. In 1991, a survey was carried out in Edinburgh in which the levels of resistance to β-lactam agents in the commensal faecal flora of healthy volunteers and the genetic basis for this resistance were determined. Amongst the 100 participants, 42% and 38% carried ampicillin and cefuroxime resistant bacteria respectively but there was no resistance to either ceftazidime or cefotaxime. Of the ampicillin resistant strains, 38% could transfer their resistance to E. coli J62-2 at 37°C. Biochemical and molecular analysis of the transconjugants revealed that ampicillin resistance resulted from the presence of the TEM-1 β-lactamase which was located on a wide variety of plasmids. In the presence of clavulanic acid sensitivity was restored to ampicillin in 98% of the ampicillin resistant isolates. The discovery of commensal ampicillin resistance levels, similar to those found in clinical isolates, is worrying as is the prevalence of the TEM-1 β-lactamase; however, there is almost universal sensitivity to the widely used combination of amoxycillin/clavulanate.

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EFFECT OF AMOXYCILLIN CHALLENGE ON THE β-LACTAMASE TRC-1.

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TRC-1 is a TEM derived β-lactamase with increased resistance to β-lactamase inhibitors but acquisition of this resistance appears to have been at the expense of activity against amoxycillin. To investigate if the rarity of these β-lactamases results from their reduced efficiency against amoxycillin the effect of amoxycillin challenge on TRC-1 was examined. The TRC-1 producing strain E. coli J62, (pUK901) was challenged in Isosensitest broth containing amoxycillin concentrations (0, 128, 256 and 512mg/L). Every 24 h, the culture was inoculated into identical fresh media before harvesting the bacteria to examine the β-lactamase present by iso-electric focusing. This procedure was repeated 5 times. At amoxycillin concentrations 0, 128 and 256 mg/L, all isolates produced TRC-1 but at 512 mg/L a TEM-1 producing mutant appeared at day 4. The revertant TEM-1 β-lactamase had restored sensitivity to clavulanic acid (ID_{50} reduced from 4.2 uM to 0.15 uM). In high amoxycillin concentrations the TEM-1 revertant outgrew its TRC-1 isogenic parent. It is not clear therefore if such inhibitor resistant TEM β-lactamases will pose a major clinical problem.

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COMPARISON OF RESISTANCE IN COMMENSAL BACTERIA IN BRITAIN AND INDIA.

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This study compares the proportion of healthy volunteers in the community that possess aerobic Gram-negative rods that are resistant to common antibacterial drugs to assess the impact that drug usage has on the community. The proportion of trimethoprim resistance in India was 99%, even in rural areas where drug availability should have been low. In Scotland, the proportion of trimethoprim resistance was only 12.6%. The proportion of people carrying ampicillin resistant bacteria in India was 98% whereas the comparable proportion in Scotland was 43%. The reason that the ampicillin figure in Scotland was so high is believed to result from the poor absorption of this drug and amoxycillin from the gut. In India the proportion of nalidixic acid resistance was 20% compared with 3% in Scotland, but ciprofloxacin resistance was negligible in both areas. These results show that control of the distribution of antibiotics is essential.

Comparison of resistance in commensal bacteria in Britain and India