BETA-LACTAM RESISTANCE IN GRAM-NEGATIVE BACTERIA ISOLATED IN INDIA

by

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ABSTRACT

The most important resistance mechanism to beta-lactam antibiotics is the plasmid-mediated beta-lactamase and the common criterion for the epidemiology of these enzymes is the determination of their biochemical characteristics. Surveys of plasmid-encoded beta-lactamases of Gram-negative bacteria used to investigate their relative clinical importance have been poorly performed and rarely conducted outside the developed world.

A survey of uropathogenic strains and of *Salmonellae* and *Shigellae,* isolated in South India in 1984 revealed a higher incidence of ampicillin resistance (Minimum Inhibitory Concentration [MIC] >10mg/l) than had ever been reported before (Enterobacteriaceae 80.9%, *Salmonellae* 90%, *Shigellae* 66%). Only the enterobacterial strains showed any significant resistance to the first generation cephalosporin, cephaloridine (MIC >10mg/l). However, 65.7% of the *Salmonella* strains were cefuroxime resistant. Cefuroxime resistance in these *Salmonella* strains was accompanied by the widespread distribution of the novel OXA-E1 like enzyme with more hydrolytic activity against the drug, even though other resistance mechanisms may play subsidiary role. A small proportion of all species conferred resistance to third generation cephalosporins. In the individual species, there was a very high incidence of ampicillin resistance *(E.coli* 77%; *Klebsiella* spp 69%) and cephaloridine resistance (*E.coli* 57%; *Klebsiella* 96%). Many of the ampicillin resistant strains harboured either auto-transferable or mobilisable plasmids (41.8%). Characterisation of the plasmid DNA from the *E.coli* transconjugants revealed the existence of 37 different plasmids types. The transconjugants from *Klebsiella, Salmonella* and *Shigella* possessed fewer plasmids types than those from *E.coli.* Most plasmids possessed resistance genes to aminoglycosides and to six or more

drugs. Beta-lactamase studies revealed that TEM-1 was the most predominant enzyme in all transconjugant strains followed by OXA-1, SHV-1, TEM-2, OXA-2 and the novel enzyme SAR-2. The SAR-2 enzyme was fully characterised and had a higher pI (8.3) than any previously characterised plasmid-mediated beta-lactamase. It had a broad-spectrum activity with the molecular weight of 36000. In addition, the unusual observations of *E.coli* strains producing both the PSE-1 and PSE-2 beta-lactamases and strains hyperproducing the TEM-1 were made and these strains were studied further.

The development and mechanisms of resistance to beta-lactam/beta-lactamase inhibitor combinations (ampicillin and clavulanic acid) have been performed with laboratory strains possessing the ampicillin resistance plasmids R1, R1010 and extended-broad spectrum beta-lactam resistance plasmids. The results show that challenge with clavulanic acid alone did not affect the expression or integrity of the beta-lactamase whereas challenge with the combination of ampicillin and clavulanic acid caused radical changes in the expression of the beta-lactamase. In some cases there were multiple copies of genes which resulted in hyperproduction of TEM-1 enzyme and this was sufficient to resist the combinations. Similarly, elevated SHV-1 enzyme production resulted in a resistance mechanism with no concurrent reassortment of the plasmid R1010. Unfortunately, these variants also conferred resistance to second and third generation cephalosporins. The enzymes TEM-3, TEM-5 and TEM-7 were not hyperproduced but the corresponding strains were stable against the combination at higher inoculum density. Importantly, the SHV-1 beta-lactamase had become active against cefotaxime as it was able to hydrolyse the drug. Evidence of this type of resistance (hyperproduced TEM-1 and SHV-1 enzymes) to clavulanic acid is now emerging in clinical practice.

If you ever
feel afraid of anything
always turn around and face it
Never think of running away.

SWAMI VIVEKANANDA
(1862-1902)

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ABBREVIATIONS

Amp, Ap	ampicillin
Aug	augmentin
Az	aztreonam
bp	base pairs
Carb	carbenicillin
Caz	ceftazidime
Ced	cephradine
Cep	cephalothin
Cer	cephaloridine
cfu	colony forming unit
Cfx	cefoxitin
Cfz	cefozolin
Clav	clavulanic acid
Clox	cloxacillin
Cm	chloramphenicol
Cmd	cephamandole
Ctn	cefotetan
Ctr	ceftriaxone
Ctx	cefotaxime
Cxm	cefuroxime
DM	Davis and Mingioli
DSTA	Diagnostic Sensitivity Test Agar
EDTA	ethylenediaminetetra acetic acid
FIC	fractional inhibitory concentration
Gm	gentamicin
his	histidine
IEF	isoelectric focusing
Imp	imipenem
K, Kd	kilodaltons
kb	kilobases
Km	kanamycin
L	litres
Log	logarithim
M	Molar
Md	megadaltons
met	methionine
MIC	minimum inhibitory concentration
mM	millimolar
MW	molecular weight
Mox	moxalactam
NaCl	sodium chloride
ND	not done
ng	nanogram
NH	no hydrolysis
NM	not measurable
nM	nanoMolar
NMH	North Middlesex Hospital
OD	optical density
OMP	outer membrane protein
PAGE	polyacrylamide gel electrophoresis
PBP	penicillin binding protein
pCMB	para-chloromercuribenzoic-acid
pI	isoelectric focusing
pro	proline
R,r	resistance
Rif	rifampicin
RMH	Royal Marsden Hospital, London
S	substrate
SDS	sodium dodecyl sulphate
Sm	streptomycin
Sp	spectinomycin
Sulb	sulbactam
Su	sulphonamide
Sx	sulphamethoxazole
try	tryptophan
Tc	tetracycline
TEMED	N,N,N',N'-tetramethylethylenediamine
Tn	transposon
Tp	trimethoprim
ul	microlitres
um	microMolar
UM	unmeasurable
V	velocity
3GC	"third generation cephalosporins"

CHAPTER 1

INTRODUCTION

1.1 Development of Beta-lactam Family

The beta-lactam antibiotics are widely used clinically because they have a broad spectrum, are well tolerated and have a high degree of safety. A combination which is found in few other therapeutic agents. The antibacterial activity of all the beta-lactams is attributed to the active beta-lactam ring. Beta-lactams act by binding and inhibiting the action of specific proteins (Penicillin Binding Proteins or PBPs) located in the cytoplasmic membrane and are necessary in the last stage of peptidoglycan synthesis. Beta-lactam antibiotics penetrate the outer membrane of the bacterial cell, enter the periplasm and finally reach the cytoplasmic membrane where the bacterial cell's PBPs are located (Tomasz, 1983).

It is remarkable that research into beta-lactam antibiotics has continued so long after the first discovery of penicillin 60 years ago. This demonstrates how truly revolutionary and versatile the drugs were; nevertheless, the development of beta-lactam drugs over the years has been hampered by the evolution of beta-lactam resistance.

The history of beta-lactam resistance began in 1929 with the observation of the penicillin resistant typhoid bacteria (Fleming,1929). In 1940 the mechanism of resistance was found by extraction of a penicillinase (Abraham and Chain, 1940) from *Escherichia coli*; however,

Staphylococcus aureus was found to be penicillin sensitive at that time. Subsequently, the discovery of penicillinase production in penicillin-sensitive strain of *Micrococcus lysodeikticus* and other species of bacteria led to the conclusion that the penicillinase may have a metabolic function in some bacterial cells (Richmond and Sykes, 1973; Saz and Lowery, 1979; Medeiros, 1984).

1.1.1 Semisynthetic Penicillins

In 1944, penicillinase producing clinical strains of *Staphylococci* were first isolated (Kirby, 1944). From then until 1960, the increasing use of penicillin led to the rapidly increasing spread of penicillin-resistant *Staphylococci*. This soon reached epidemic proportions and rendered the original penicillin less useful (Richmond, 1965). The problem of the penicillinase producing *Staphylococci* was solved initially by the development of methicillin (1960) (Stewart, 1960). Penicillin G was prepared by conventional fermentation and the molecule was chemically modified to produce 6-amino-penicillinic acid. Addition of a 2,6 dimethyl phenyl group to the penicillin nucleus instead of the benzyl methyl group

gave methicillin (Hoover, 1983). Methicillin was insusceptible to penicillinase attack but was a less effective antibiotic than penicillin G, therefore, a compromise has had to be made, beta-lactamase stability in favour of antibacterial efficacy. Methicillin was the first of the semisynthetic penicillins and it spawned a series which included oxacillin (Kirby, Rosenfeld and Brodie, 1962), cloxacillin and finally flucloxacillin (Sutherland, Croydon and Rolinson, 1970) (isoxazolyl penicillins). Unfortunately, neither penicillin G nor any of the semi-synthetic penicillins had any effect on large Gram-negative rods. This problem was initially tackled by the discovery of ampicillin, another semisynthetic derivative, which produced significant activity against Gram-negative species (Rolinson and Stevens, 1961).

1.1.2 Introduction of First Generation Cephalosporins and Antipseudomonas Agents

An important advance in the design of beta-lactam antibiotics was made in the early 1950s. Cephalosporin C, the first of the cephalosporins as they are known today, was first discovered by Professor Guiseppe Brotzu from a strain of *Cephalosporium acremonium* isolated from the sea near a sewage outfall (Abraham, 1983). The first crude material extracted from this strain by Brotzu, was found to be active against both Gram-positive and Gram-negative bacteria. Later in 1953 it was purified and studied in detail. In 1959 it was shown to be penicillinase-stable with low toxicity (Abraham, 1983). Cephalosporins have an inherently greater resistance to beta-lactamases than the penicillins because the functional group at the C-7 position of the beta-lactam prevents the molecule entering the active site of many common beta-lactamases (O'Callaghan, 1975); however, the limitation was that earlier cephalosporins were more active against Gram-positive than the Gram-negative organisms. Much the same is true for the case of the first group of cephalosporins (cephalothin, cephapirin and cephacetrile) even though they are regarded as effective broad-spectrum antibiotics. In addition, they were found to be unstable in the body; esterases convert their ester groups to the corresponding desacetyl compound (O'Callaghan and Muggleton, 1963) which was formed in variable amounts and had low antibacterial activity. The failure of first group cephalosporins (cephalothin, cephapirin and cephacetrile) (O'Callaghan, 1975) opened the door for other semisynthetic cephalosporins cephaloridine in 1964 with a pyridinium as a replacement for the ester group (Muggleton, O'Callaghan and Stevens, 1964) and cefazolin (Nishida et al., 1969) followed by oral cephalosporins, cephalexin (Guttman, 1970) and cephradine (Neiss, 1973). At the same time new penicillins, such as amoxycillin (Sutherland, Croydon and

Rolinson, 1972), carbenicillin (Knudsen, Rolinson and Sutherland, 1967) and ticarcillin (1965) (Bergan, 1978; Neu, 1983) were brought into the chemotherapeutic field, the latter two drugs specifically designed for *Pseudomonas* spp.

The introduction of broad-spectrum penicillins and cephalosporins during the 1960s rapidly led to the increased prevalence of ampicillin resistance among Gram-negative bacteria, especially *E.coli*, *Salmonella* spp, *Klebsiella* spp, *Haemophilus* spp and methicillin-resistant *Staphylococcus* (review: Medeiros, 1984). This has been particularly well documented in the type 29 *Salmonella typhimurium* (Anderson, 1968) in which the complete development of resistance was followed from the introduction of the drugs to the emergence of multiple resistance. A major defect of all the first generation cephalosporins was their sensitivity to hydrolysis by the beta-lactamases of many Gram-negative bacteria. Organisms such as *Enterobacter* spp., many *Klebsiella* spp. and indole-positive *Proteus* owe much of their resistance to cephalosporins to the plasmid-mediated or chromosomal beta-lactamases. Resistance to beta-lactam antibiotics rapidly became widespread and this initially resulted from the transfer of the gene from one species to another. The resistance determinants were located on extra-chromosomal genetic elements, known as plasmids (Anderson and Datta, 1965; Datta and Kontomichalou, 1965; Anderson, 1968); however, beta-lactam resistance became more ubiquitous and the main genes were found on many plasmid types. It was demonstrated that the resistance genes were being spread from one plasmid to another by transposons (Hedges and Jacob, 1974).

1.1.3 Second and Third Generation Cephalosporins

In order to overcome beta-lactam resistant beta-lactamases, resistant second generation cephalosporins such as cephamandole (Wick and Preston, 1972) cefoxitin

(Onishi et al., 1974) and cefuroxime (O'Callaghan et al., 1976; O'Callaghan, 1979) were developed. These were found to be metabolically-stable and also beta-lactamase stable drugs with wide antibacterial activity against many Gram-negative bacteria. Their activity is associated with the presence of a 7-acyl group with a small amount of steric hindrance in cephamandole (Wick and Preston, 1972; Williams and Andrews, 1974). In cefoxitin the methoxy substituent is directly attached to the beta-lactam ring at position 7 (Onishi et al., 1974) and a carbamoyloxymethyl ester attached at position 3 in the case of cefuroxime (O'Callaghan, 1975). Even these drugs did not solve the problem as the gradual selection of mutants that overproduce chromosomally-mediated beta-lactamase occurred. In addition, some of the drugs promote the induction of chromosomal beta-lactamases in clinical isolates (Livermore, 1987a). During the 1970s the frequent occurrence of beta-lactam resistances by these two mechanisms urged the introduction of a new generation of highly beta-lactamase resistant antibiotics, namely third-generation cephalosporins. The discovery of cefotaxime in 1976 (Heymes, Lutz and Schrinner, 1977) and ceftazidime in 1978 (O'Callaghan et al., 1980) led to their clinical use in 1978 in Europe and in 1982 in the USA. The third-generation cephalosporins and the monobactam aztreonam (Sykes et al., 1982) have considerably expanded the therapeutic armamentarium available for treating Gram-negative infections and even allowed the treatment of infections by *Ps. aeruginosa.*

Before the discovery of the third-generation cephalosporins, pharmaceutical companies considered an alternative strategy to overcome the problem of plasmid-mediated beta-lactam resistance. This approach was to add a beta-lactamase inhibitor, clavulanic acid, to penicillin thus broadening its spectrum of activity against beta-lactam resistant bacteria (Fisher, Charnas and Knowles, 1978; Farmer and Reading, 1988). This inhibitor, first discovered in 1974, was followed by

another, sulbactam, in 1977 (English et al., 1978).

Both combination therapy and the third-generation cephalosporins were developed in order to overcome the increasing diversity of beta-lactam resistance problems in clinical bacteria.

1.2 Mode of Action of Antibiotics

1.2.1 General Antibiotic Action

The antibiotics can, in general, be divided into three main classes according to their sites of attack on the cellular metabolism of bacteria (Reiner, 1982a). These are: 1) antibiotics interfering with nucleic acid or protein synthesis (e.g. aminoglycosides, chloramphenicol, fusidic acid, gentamicin, quinolones, rifamycins, sulphonamide, trimethoprim, tetracycline); 2) antibiotics interfering with the cell-wall bio-synthesis (e.g. penicillins, cephalosporins, bacitracin, fosfomycin, mecillinam, thienamycin, vancomycin); 3) antibiotics affecting the function of the cytoplasmic membrane (e.g. amphotericin B, candicidin, gramicidins, hachimycin, natamycin, nystatin, polymixins, tyrocidins).

The antibiotics can also be sub-divided into four categories depending upon their bactericidal or bacteriostatic properties (Reiner, 1982b). These categories are: group 1 bactericidal, but able to exert an effect on resting micro-organisms (e.g. amikacin, gentamicin, kanamycin, polymixins, streptomycin); group 2 bactericidal, but only able to act on proliferating micro-organisms (e.g. penicillins, cephalosporins, thienamycins, bacitracin, vancomycin, rifamycins); group 3. bacteriostatic but in high concentrations may also be bactericidal (e.g. chloramphenicol, erythromycin,

fusicid acid, novobiocin, tetracyclines) and group 4, exclusively bacteriostatic, even in high drug concentrations (e.g. capreomycin, d-cycloserine, viomycin etc.).

1.2.2 Mode of Action of Beta-lactam Antibiotics

The beta-lactam family interfere with the biosynthesis of the bacterial cell-wall by acting as analogues of the acyl-D-alanyl-D-alanine moiety of the lipid-linked disaccharide-peptapeptide substrate of the enzymes (PBPs) that catalyse the synthesis of cross-linked peptidoglycan; therefore, beta-lactam agents irreversibly acylate the PBPs resulting in an inactive penicilloyl-enzyme. This acylation is analogous to acyl-enzyme formed during the processing of their normal peptide substrate (Spratt, 1989). The irreversibility of their binding ensures that beta-lactams are bactericidal and active against only proliferating bacterial cell. Some new beta-lactam agents (i.e. Mt-181), unlike the previous ones, are able to lyse non-growing cells of E. coli because they bind the poorly characterised low molecular-weight PBP 7 (Tsuruoka et al., 1985; Tuomanen and Schwartz, 1987).

1.3 Resistance Mechanisms

Depending upon the mode and route of action of antibiotics, different types of resistance mechanisms have been evolved. Both Gram-positive and Gram-negative bacteria fundamentally differ in their response to beta-lactam agents. This has been discussed in an overview by Greenwood (1986). These resistance mechanisms which regulate the antibacterial activity have been detailed in recent reviews (Gootz, 1985; Piddock and Wise, 1985; Nayler, 1987). The main resistance mechanisms to beta-lactam antibiotics are: (1) the inability of the drug to penetrate through the outer membrane (Nikaido, 1989); (2) the affinity of the target PBPs for beta-lactams (Spratt, 1989);

(3) the production of enzymes which can hydrolyse the beta-lactams, the so-called beta-lactamases (Medeiros, 1989; Sanders, 1989) and (4) the affinity of the drug for the beta-lactamases. Despite the number of different types of resistant mechanisms, beta-lactamases constitute the most widely distributed in bacteria, and are the major single cause of beta-lactam resistance.

1.3.1 Outer Membrane Permeability

All bacterial cells have "permeability" barriers; however, there is a very considerable variation between permeabilities of different strains within a species. The permeability barriers are phospholipids, lipopolysaccharides and various proteins. Different outer membrane proteins (OMPs) or "porins" have different permeability characteristics (Nikaido and Vaara, 1985). Porins are acidic in nature (Nikaido and Rosenberg, 1983) and are considered to provide a major route of entry for hydrophilic molecules of anionic or zwitterionic type, including all the important beta-lactams (Nayler, 1987). They are peptidoglycan-associated and have molecular weights within the same range (30,000-40,000) (Nikaido, 1989); however, porin channels differ in their diameter and in the number of open channels. This considerably affects the rate of penetration of the solutes (Nikaido and Rosenberg, 1983) through the outer membrane. Transport of low-molecular weight compounds into the periplasm of Gram-negative bacteria is dependent on both the size and the hydrophobicity of the molecule (Acar and Minozzi, 1986); therefore, many factors influence the ability of a beta-lactam antibiotic to enter a Gram-negative organism and it is often expressed as a permeability coefficient (Acar and Minozzi, 1986).

Haemophilus influenzae is very sensitive to beta-lactam antibiotics since there is

apparently no major barrier to antibiotic penetration. Indeed, this species is at least one order of magnitude more permeable than *E.coli* (Zimmermann and Rosselet, 1977; Coulton, Mason and Dorrance, 1983); however, in their turn, *E.coli* strains are about 14 times more permeable than *Enterobacter cloaceae* (Vu and Nikaido, 1985). Most strains of *Pseudomonas aeruginosa* are intrinsically highly resistant because of the relative impermeability of their membrane to beta-lactams (Angus, et al., 1982) and the permeability coefficients for several beta-lactams revealed that the *P. aeruginosa* is far less permeable than *E. cloacae* (Nicas and Hancock, 1983).

Reduced permeability is a widely accepted potential source of beta-lactam resistance. In the case of *E.coli*, three porin types, OMP C, OMP F and OMP E (Harder, Nikaido and Matsuhashi, 1981) have been identified. Of these, the first two types are the most important for uptake of beta-lactams. Mutational reduction in the expression of OMP F elevates the minimum inhibitory concentrations (MICs) of many beta-lactam drugs. This phenomenon is even more striking when the expression of both OMP C and OMP F are reduced (Harder, Nikaido and Matsuhashi, 1981; Komatsu, Murakami and Nishikawa, 1981; Jaffe, Chabbert and Semonin, 1982); and, a good example of this was an *E.coli* mutant resistant to cephalosporins (Yamaguchi et al., 1985). Another example is the OMP C and OMP F deficient mutants of *E. coli* (Nikaido, Rosenberg and Foulds, 1983) in which the penetration constant (C) value, characteristic to the particular organism and antibiotic, was decreased and led to the cephalosporin resistance. Similar results have been reported for other enterobacteria such as *Proteus*, *Enterobacter*, *Klebsiella*, *Serratia* and *Salmonella* spps (Sawai et al., 1982; Gutmann et al., 1985).

Nayler (1987) reviewed that in *Salmonella typhimurium* mutants, deficiency of only two porins of the several types normally present led to 10-fold decrease in permeability

cephaloridine; however, to put this in perspective, the resistance from the OMPs modifications is usually very moderate in comparison with the derepressed cephalosporinases produced by *Enterobacter cloacae* (Vu and Nikaido., 1985; Werner et al., 1985).

In *Pseudomonas aeruginosa*, it has been assumed that a single porin (protein F) provides a channel of two different types. These channels exist in very unequal proportions and there is a possibility that mere conformational change may be all that is required to produce significant changes in permeability to produce multi-resistant mutants (Woodruff et al., 1986). A diminished expression of a 52,000 dalton OMP of *Pseudomonas aeruginosa* has led to resistance to imipenem both *in vivo* and *in vitro* (Quinn et al., 1986; Buscher et al., 1987). There may also be other significant mechanisms of beta-lactam entry in some species besides the porins (Nayler, 1987; Livermore, 1988). This has been confirmed when both an *E. coli* and a mutant deficient in porins in the phospholipid bilayer were shown to be more permeable to penicillins than to cephalosporins (Yamaguchi, Hiruma and Sawai, 1982; Yamaguchi et al., 1985). Another example was the role of lipopolysaccharide layer in contribution to the development of resistance in *P. aeruginosa* (Godfrey, Hatlelid and Bryan, 1984). In this study, the subtle changes within the LPS were correlated with increased MICs of beta-lactams. The authors noted that while the interaction of all the outer membrane components contributed to an overall level of permeability, this was a major role for the LPS layer in the development of the resistance.

The presence of an exopolysaccharide layer outside the outer membrane, comprised of slime and capsular material (Livermore, 1988), can contribute towards antibiotic resistance. The slime may protect bacteria by binding antibiotic molecules; however, there is no significant evidence to suggest this is a resistance

mechanism for beta-lactams, even in the species, such as "mucoid" *P.aeruginosa* isolated from cystic fibrosis patients (Slack and Nichols, 1982; Lambert, 1983)

In some cases it is hard to say whether high-level resistance to beta-lactam antibiotics can be ascribed entirely to permeability, because both very low levels of periplasmic beta-lactamases and extremely low rates of enzymatic hydrolysis are difficult to detect. It is quite evident that minimum inhibitory concentrations of antibiotics change in accordance with the physiocochemical characteristics of the bacterium. These changes may be related to alterations in diffusibility, the part played by beta-lactamases, the level of these enzymes in the periplasm and finally permeability of bacterial cell-wall (Nayler, 1987).

1.3.2 Penicillin Binding Proteins

The only target molecules for beta-lactam antibiotics in all bacteria are the penicillin binding proteins (PBPs). These proteins are bound by beta-lactam antibiotics by covalent bonding after the drugs have penetrated the outer membrane. The bacterial cytoplasmic membrane contains at least one, or more often more than one type of PBP (Tomasz, 1986); however, recent work with *E.coli* has shown that some PBPs are actually associated with the outer membrane although the inner membrane adhesion sites are considered as the major location (Barbas et al., 1986). Detailed studies on the biochemistry, physiology (Frere and Joris, 1985), genetics and structural analysis of PBPs (Waxman and Strominger, 1983) have shown that they are a diverse group of proteins with various peptidase functions. They have been most closely studied in Gram-negative bacteria (Spratt and Cromie, 1988; Spratt, 1989). It is thought that there is distant evolutionary relationship between PBPs and beta-lactamases (Pollock, 1967) and this is strongly supported by recent findings,

such as close similarities in the amino-acid sequence of several low and high molecular weight PBPs and active-site serine beta-lactamases (Waxman, Amanuma and Strominger, 1982; Samraoui et al., 1986) by the similarities in tertiary structures of PBPs and beta-lactamases (Kelly et al., 1986; Samraoui et al.,1986).

PBPs do not all have the same function nor do they react in the same way to beta-lactams. In *E.coli* for example, there are seven PBPs, each corresponding to a distinct penicillin-sensitive enzyme. The main PBPs which are involved in the final stages of peptidoglycan biosynthesis are the high molecular weight molecules (i.e. PBP 1, PBP 2 and PBP 3) which were considered as the essential or physiologically important PBPs. On the other hand, the low molecular weight PBPs 4, 5, 6 have been suggested to be non-essential for cell survival and were not considered as killing targets for beta-lactam antibiotics (Spratt, 1983; Waxman and Strominger, 1983).

Depending on their affinity for a particular type of PBP, beta-lactams may affect the host organism in one of the three ways (Spratt and Pardee, 1975; Waxman and Strominger, 1983): (1) rapid lysis of the bacterium (inhibition of PBP 1); (2) change of the organism into a spherical shape and eventual cell lysis (inhibition of PBP 2); (3) inhibition of septum formation and thus cell division leading to filamentous growth and eventual cell death (inhibition of PBP 3).

Beta-lactam resistance has recently emerged which has been attributed to mutational alterations in the PBPs. These may arise in three different ways (Malouin and Bryan, 1986): (1) reduction in affinity of the normal PBPs for beta-lactams; (2) loss in the level of one or more types of PBPs; (3) synthesis of additional new types with lower affinity for beta-lactams. Resistance in Gram-positive bacteria can also result from these changes. In Gram-negative organisms, resistance is mainly by reduction

in their affinity. Even minor changes in affinity may be of considerable importance with the newer, poorly hydrolysed beta-lactams such as the third generation cephalosporins (Spratt, 1989).

The examples where resistance resulting from alterations of PBPs have been convincingly demonstrated are in three types of bacteria. The first are those organisms without beta-lactamases such as *Streptococcus pneumoniae* (Hakenbeck, Tarpay and Tomasz, 1980; Bryan, 1988). In the second group those strains where beta-lactamases have recently appeared, such as Neisseria gonorrhoeae, (Dougherty, Koller and Tomasz, 1980; Faruki et al., 1986; Dougherty, 1986; Bryan, 1988) and Haemophilus influenzae (Serfass et al, 1986; Reid et al., 1987a) where resistance derives from an alteration of PBP 2. The third type is that in which beta-lactamases are of relatively little clinical significance, such as Staphylococcus aureus (Bryan, 1988), because beta-lactamase resistant antibiotics have been in use for many years.

Recent studies have shown that some PBPs, notably the low-affinity PBP (PBP 2) found in methicillin-resistant *Staphylococcus aureus*, are also inducible (Chambers, Hartman and Tomasz, 1985; Ubukata, Yamashita and Konno, 1985). Indeed, the regulation of the PBP that determines methicillin resistance in *Staphylococcus aureus* may be linked to regulation of staphylococcal beta-lactamases (Boyce and Medeiros, 1987).

The development of beta-lactam resistant PBPs in clinical strains involves the introduction of multiple amino-acid substitutions that restabilise the enzyme (Spratt and Cromie, 1988). For example, the development of cephalexin-resistant forms of *E.coli* in the laboratory (Hedge and Spratt 1985a) resulted from an altered form of PBP 3 with substantial levels of resistance to beta-lactam antibiotics.

This was due to the introduction of several amino-acid substitutions that remodel the active site of the enzyme to exclude beta-lactam agents without impairing its ability to process its normal peptide substrates (Hedge and Spratt, 1985b). The other examples were the isolates of *Neisseria gonorrhoeae* where multiple amino-acid substitutions had taken place within the transpeptidase domain of the enzymes (Faruki et al., 1985; Dougherty, 1986). With particular regard to cephalosporin resistance, there is clinical evidence that evolution of third-generation cephalosporin resistant *Pseudomonas aeruginosa* and *Serratia marcescens* can result from multiple substitutions of amino-acids leading to altered penicillin-binding proteins (Bryan, 1988)

The recently-acquired ability to study the protein crystallography of beta-lactamases and low molecular-weight PBPs, coupled with the likely feasibility of obtaining water-soluble forms of high molecular-weight PBPs, suggest that the crystallography of the high molecular-weight PBPs should soon be achievable. This capability should allow the design of potent new beta-lactam antibiotics and also provide an insight into the remodelling events that allow the PBPs of clinical isolates to reduce drastically their affinity for beta-lactam antibiotics (Spratt and Cromie, 1988).

1.3.3 Beta-lactamases

Beta-lactamases are ubiquitous proteins, present in almost all bacteria; they have structural and functional characteristics in common with the penicillin-binding proteins responsible for the assembly of the bacterial cell-wall and they probably share a common evolutionary origin (Medeiros, 1989). In all likelihood, the primary role of beta-lactamases is not to protect bacteria from beta-lactam antibiotics but rather to break transitory intermediates of beta-lactam structure during bacterial

peptidoglycan metabolism. Indeed, beta-lactamases were produced by bacterial strains that were isolated before beta-lactam antibiotics were used in chemotherapy (Pollock, 1967; Segalove, 1974).

Beta-lactamases are produced by various organisms, both Gram-positive and Gram-negative bacteria as well as *Mycobacteria* and *Nocardia* (Kasik, 1979). Many different beta-lactamases are known and, when produced in large quantities, they act as the principal resistance determinants to beta-lactam agents. The various beta-lactamases differ in their specificity for each type of beta-lactam antibiotic (Nayler, 1987; Medeiros, 1989). There are a number of different enzymes that could be termed beta-lactamases as they all attack beta-lactam antibiotics. Amidases and acylases of Gram-negative bacteria eliminate the acyl side chain of beta-lactam antibiotics. Cephalosporins that carry an acyl ester are sensitive to bacterial and mammalian esterase (Bauernfeind, 1986); however, it is the enzymes that hydrolyse the amide bond in the beta-lactam ring that are the most important enzymes in the bacterial defence system against beta-lactam drugs and these are the proteins that I shall term beta-lactamases. Typically they are soluble enzymes which in Gram-negative bacteria are confined to the periplasmic space between the inner and outer membrane; and in Gram-positive bacteria, they are excreted in the environment (Wiedemann, Kliebe and Kresken, 1989).

Beta-lactamases are responsible for therapeutic failures where previously active antibiotics are no longer effective. The resistant bacterium may either acquire a beta-lactamase-producing plasmid and its chromosomal beta-lactamase may be induced or become derepressed.

1.3.3A Identification Methods for Beta-lactamases

Most beta-lactamases can be easily detected by isoelectric focusing on polyacrylamide gels and subsequent identification with the chromogenic cephalosporin nitrocephin (Matthew et al., 1975). In addition, the other chromogenic cephalosporin, PADAC, can also be used in agar plates to detect these enzymes in growing bacteria. This may be achieved in the presence or absence of the inhibitor clavulanic acid and is considered a particularly useful tool, permitting easy, semiquantitative determination of beta-lactamase activity (Kobayashi et al., 1988). Another method is the combination of polyacrylamide-agarose gel electrophoresis and pH gradient electrophoresis (titration curve) which can provide a powerful approach to the study of the molecular structure of these enzymes. It is useful for rapid and easy identification of the major penicillinases in Gram-negative bacteria (Vedel, Paul and Picard, 1988).

1.3.3B Classification of Beta-lactamases

There is a great variety of beta-lactamases produced by bacteria. The diversity led to several classification schemes based on different criteria: substrate profile, inhibitor profile, molecular weight, isoelectric points, genetic determination (DNA homology) and amino-acid sequencing of enzymes (Richmond and Sykes, 1973; Matthew et al., 1975; Sykes and Matthew, 1976; Ambler, 1980; Bush, 1988a). None of these classification systems has achieved the ability to differentiate all beta-lactamases adequately.

The grouping of beta-lactamases was described by Sawai in 1968 (Medeiros, 1989) by the substrate profile of both plasmid and chromosomal enzymes in Gram-

negative bacteria. He listed three types of beta-lactamases; 1) inducible cephalosporinases; 2) cephalosporinases that had the properties of penicillinase, or specifically broad-spectrum beta-lactamases identified from *Proteus vulgaris* ; 3. penicillinases. Later, in 1970, this system was expanded by the addition of inhibitor profiles and reaction to antisera which provided an additional discriminator (Jack and Richmond, 1970). In 1973, Richmond and Sykes proposed one of the most widely used classification schemes, where five classes (1-5) of beta-lactamases from Gram-negative bacteria were outlined. Class 1 enzymes were predominantly cephalosporinases; class 2 were penicillinases; class 3 enzymes showed broad-spectrum activity and were sensitive to inhibition by cloxacillin but resistant to p-chloromercuribenzoate (pCMB); class 4 were also broad-spectrum enzymes but resistant to inhibition by cloxacillin and sensitive to pCMB and the final class 5 enzymes were penicillinases able to hydrolyse cloxacillin and resistant to pCMB inhibition.

A major advance in the classification of these enzymes took place when Matthew et al (1975) introduced a new system of flat-bed isoelectric focusing in polyacrylamide gels for identification of specific beta-lactamases. This identification method was further developed by an alternative method i.e. application of a matrix of highly purified agarose for isoelectric focusing (Vecoli et al., 1983).
Polyacrylamide gel is the most widely accepted method and this system can be further refined by overlaying the gel with an inhibitor prior to applying the nitrocephin overlay (Sanders, Sanders and Moland, 1986). The revolutionary system of classification by Sykes and Matthew (1976) enabled very closely related beta-lactamases to be distinguished. These enzymes could then be further typed by their molecular weights, substrate profiles and susceptibilities to inhibitors. Only Gram-negative bacterial beta-lactamases have been included in this scheme, for it does not

work well with Gram-positive beta-lactamase. The enzymes fall into two major groups, classes A and B. Class A enzymes are chromosomally mediated and be divided into the subclasses (a) penicillinases, (b) cephalosporinases and (c) broad-spectrum beta-lactamases. The class B enzymes are plasmid-mediated and subdivided into the sub-classes (a) isoxazolyl-non-hydrolysing, (b) isoxazyl-hydrolysing and (c) other beta-lactamases.

A new type of taxonomy was proposed by Ambler (1980) based on the amino acid sequence of the active site. It has been extended by Jaurin and Grundstrom (1981) and Bush (1988a) and now it includes beta-lactamases from both Gram-positive and Gram-negative bacteria. The enzymes could be divided into three classes; 1.) penicillinases with active serine site residue; 2.) metallo enzymes, such as the enzyme from *Bacillus cereus* and related bacilli which require bivalent metal cations such as Zn^{2+}; and 3) enzymes which also have an active site serine residue, but with a distinct amino-acid sequence around the active site. It has been found that all these class C enzymes have substrate profiles favouring cephalosporin hydrolysis, whereas those beta-lactamases in class A have substrate profiles indicating either penicillinase or broad-spectrum activity. However, Bush (1988a) modified this system further. She proposed that there are three classes 1, 2 and 3 : class 1, cephalosporinases ; class 2, subdivided into five groups, namely a-e (a = cephalosporinases, b = broad-spectrum enzymes, c = carbinicillinases, d = cloxacillinases and e = inducible cephalosporinases); class 3, metalloenzymes.

Many plasmid-mediated beta-lactamases which have been classified on the basis of their biochemical properties have now been subjected to further investigation for their relatedness by applying the methods of immunological cross-reactivity, DNA-hybridisation and nucleotide sequencing.

1.3.3B1 Immunological Cross-Reactivity

This technique was carried out with polyclonal antisera within, but not between, the classes of plasmid-mediated beta-lactamases. According to these results TEM-1, TEM-2 and TLE-1 are closely related. They are partially related to SHV-1 but show no cross-reactivity with HMS-1, OXA- or PSE- type beta-lactamases (Sykes and Matthew, 1979; Paul et al., 1981; Paul, Philippon and Nevot, 1985). There is also a close relationship between beta-lactamases from *Proteus mirabilis* strains N-3, N-29 and the PSE-1, PSE-4 and CARB enzymes (Takahashi et al., 1983). Amongst the OXA-group, there is a considerable homology between OXA-1 and OXA-4 beta-lactamases (Paul, Philippon and Nevot, 1985; Philippon, Paul and Jacoby, 1986); but, there were less clear relationships between OXA-2 and OXA-3 (Holland and Dale, 1985; Medeiros, 1989).

Studies with monoclonal antibodies have revealed a similar sort of relationship between TEM-1 and other enzymes (TEM-2 and TLE-1) and to a lesser extent, SHV-1. Different levels of cross reactivity were also observed with OXA-6, OXA-7 and AER-1 (Morin et al., 1987). It can be deduced from these results that biochemically distinct plasmid-mediated beta-lactamases share common epitopes; and, although there might not be any immunological relationship within the same class of enzymes, the enzymes can still posses structural homology in their amino-acid sequences. This can be demonstrated by comparing the relatedness between beta-lactamases of *B.licheniformis* and *S.aureus* (Pollock, 1964; Ambler, 1980). The extensive cross-reactivity of these monoclonal antibodies is a limiting factor for using the anti-TEM-1 monoclonal antibodies as a tool for the identification of a given plasmid-mediated beta-lactamases (Morin et al., 1987).

1.3.3B2 DNA-Hybridisation Studies

A 1000 base pair TEM-1 probe showed cross-hybridisation with both TEM-2 and OXA-2 enzymes (Cooksey, Clark and Thornsberry, 1985); however, oligonucleotide probes of TEM-1 and TEM-2 hybridised only with their respective beta-lactamases (Ouellette et al., 1987). Smaller plasmids of intragenic fragments of the TEM-1 gene showed cross-reactivity only with TEM-2 or TLE-1 beta-lactamases (Cooksey, Clark and Thornsberry, 1985).

An intragenic probe of the OXA-1 enzyme had shown a relationship with OXA-4 (Levesque, Medeiros and Jacoby, 1987) and slight reactivity with OXA-2 (Ouellette and Roy, 1986). Four oligonucleotide probes of the OXA-1 were constructed by Boissinot, Mercier and Levesque (1987); three reacted only with their respective genes, but one did cross hybridise with the OXA-3 gene. Amongst PSE enzymes, a PSE-1 probe showed a relationship with PSE-4 and CARB-3 and PSE-2 positively reacted with OXA-6 (Huovinen, Huovinen and Jacoby, 1988a, 1988b).

These results supported the similarities revealed with the immunological assays. Recently it has been suggested that there may be "silent" TEM-genes that are not phenotypically expressed from a probing experiment conducted by Jouvenot et al (1987). When isoelectric focusing was compared with colony hybridisation for 122 strains, the use of DNA probes for the detection of beta-lactamases produced by clinical isolates, resulted in only six strains giving false positive reactions.

It was assumed that colony hybridisation may be useful as a screening method for detection of plasmid-mediated beta-lactamases (Huovinen, Huovinen and Jacoby, 1988b).

1.3.3B3 Nucleotide and Amino Acid Sequencing

Hybridisation studies can be performed with sequences as short as 12 nucleotides coding for four amino-acids (Ouellette, Bissonnette and Roy, 1987). So far, only five plasmid-mediated beta-lactamases genes have been sequenced i.e TEM-1 (Sutcliffe, 1978), TEM-2 (Chen and Clowes, 1987), OXA-1 (Ouellette, Bissonnette and Roy, 1987) OXA-2 (Dale et al., 1985) and PSE-2 (Huovinen, Huovinen and Jacoby, 1988a). The two OXA enzymes share greater homology but have no significant homology in their amino-acid sequences with TEM enzymes. They do not show homology with class A or class C enzymes, except at the region adjacent to the active site; this is also true for same with the class B beta-lactamases. It was proposed by Ouellette, Bissonnette and Roy (1987) that OXA-1 and OXA-2 be designated as class D beta-lactamases.

Nucleotide sequencing studies revealed that both *S. aureus* and *B.licheniformis* (Chan, 1986; Wang and Novick, 1987) have very low nucleotide homology despite their high degree of homology in the amino acid sequences. The crystal structures of *S. aureus* PC 1 have similarities with tertiary structures of two other class A beta-lactamases (Kelly et al., 1986; Samraoui et al., 1986; Herzberg and Moult, 1987); therefore, there might not necessarily be any structural correlations between nucleotide and amino-acid sequences.

The PSE-2 shows a high degree of homology with the OXA-2 beta-lactamase but no homology with TEM-1 except near the active site serine. It is interesting that OXA-1, OXA-2 and PSE-2 share common sequences; they do, nevertheless, differ in their substrate profile (Huovinen, Huovinen and Jacoby, 1988a). These nucleotide sequencing results supported the hypothesis first proposed in the late

1960s by Naomi Datta (Medeiros, 1989) that the TEM-1 beta-lactamase evolved from the beta-lactamase of *K. pneumoniae*, as there is great homology between TEM-1 and chromosomally encoded beta-lactamase of *K. pneumoniae* LEN (Arakawa et al., 1986). There is also significant homology between these enzymes and the SHV-1 enzyme which is encoded both by the chromosome and plasmids of *K. pneumoniae*. The outcome of these results suggest that these enzymes (SHV-1, TEM-1 and LEN) all have a common genetic origin.

Thus the application of these studies (immunological, DNA-hybridisation, nucleotide and amino-acid sequencing) in taxonomy of the beta-lactamases will completely reorganise the existing and popular classification systems, which has been based on the biochemical properties of the enzymes (Matthew et al., 1975). Over 50 plasmid-encoded beta-lactamases have been identified and classified by their biochemical properties. Classification of these enzymes on the basis of genetics and molecular biology will probably reclassify some beta-lactamases into groups of biochemically unrelated enzymes. The new system proposed by Bush (1988a) which incorporates both biochemical and molecular aspects may be universally adopted and should prove more accurate for beta-lactamase classification, especially amongst the chromosomal enzymes.

1.3.3C Chromosomal Beta-lactamases

These beta-lactamases are present in nearly all Gram-negative bacteria (Tables 1a, 1b) even though the amount of enzyme endogenously present may be small (Sykes and Matthew, 1976). They can be either constitutive or inducible or stably derepressed. Induction can only occur in certain species such as *Pseudomonas aeruginosa, Enterobacter* spp., *Citrobacter* spp., *Serratia* spp., indole-positive

Table 1a

Properties of Various Chromosomal Cephalosporinases Produced by Specific Strains of Bacteria[a]

Strain	Relative rate of hydrolysis[b]										K_i(μM)		MW (10^3)	pI	Class[c]	Ref[d]
	Cer	Cep	Cxm	Ctx	Cfx	Mox	Az	Imi	Pen	Clox	Clox	Clav				
Cephalosporinases																
Enterobacter cloacae GN7471	100	190	<1	<1	<1	<1			10		0.02	>100	44	8.4	I/C	
Pseudomonas aeruginosa GN10362	100	450	<1	<1	<1	<1			30		0.006	>100	37	8.7	I/C	
Citrobacter freundii GN7391	100	130	<1	<1	<1	<1			5		0.006	>100	37	8.6	I/C	
Serratia marcescens GN10857	100	90	<1	<1	<1	<1			5		0.001	>100	38	8.9	I/C	
Escherichia coli GN5482	100	270	<1	<1	<1	<1			60		0.007	>100	39	8.7	I/C	
Oxyiminocephalosporinases																
Proteus vulgaris GN7919	100	180	1140	80	<1	<1	20	<1	20		1.3	1.1	30	8.8	I	
Pseudomonas capacia GN11164	100	320	240	170	<1	<1	20	<1	160		3.4	1.7	24	9.3	I	
Bacteroides fragilis GN11477	100	50	40	20	<1	<1	4	<1	5		0.4	0.2	32	5.2	I	
Penicillinase																
Alkaligenes faecalis GN14061[e]	<1	<1	<1	<1	<1		<1	<1	100	70	–	>100	29	5.9		
Broad spectrum enzymes																
a) Metalloenzymes																
Pseudomonas maltophilia GN12873	100		500	20		1	<1	400	1670		–	>100	32	6.9	E?	
Flavobacterium odoratum GN14053	100	690	2750	1100	80	80	<1	1060	210		–	>100	26	5.8		
Legionella gormanii ATCC33297	100	160	90	50	10	90	<1	5	7	20	–	>100	25	10.5		
Bacteroides fragilis G237	100	110	70	80	50	260	<1	470	260	320	–	>100	26	4.8		
b) *Klebsiella* enzymes																
Klebsiella oxytoca GN10650	100	120	20	5	<1	1	10		200	20	–	0.5	27	5.3		
Klebsiella pneumoniae SC10436	100	50	20	5	<1	1	60	<1	280	30		0.2[f]	27	6.5	IV/A	

a = Strains were selected on the basis of the largest amount of data available;
b = Hydrolysis of cephaloridine placed at 100. All other rates are relative to cephaloridine. A value of <1 indicates that no hydrolysis was observed under the conditions of the test;
c = Richmond & Sykes class indicated by Roman numeral, molecular class indicated by letter
d = Reproduced from Sanders (1989);
e = For relative rate of hydrolysis, hydrolysis of penicillin G is 100
f = ID_{50}

Table 1b

Chromosomal Beta-lactamases in Bacteria not mentioned in Table 2a

Bacteria spp.	Type of chromosomal Beta-lactamases*					Inhibition by
	Cep'ase[a]	Pen'ase[b]	Me[c]	Ind[d]	Const[e]	Clav[f]
Morganella	+	–	–	+	–	–
Shigella	+	–	–	–	+	–
Salmonella	+	–	–	–	+	–
Legionella pneumophilia	+	+	–	–	+	+
Branhamella catarrhalis	+	–	–	–	+	+
Acromobacter	+	–	–	–	+	–
Flavobacterium odoratum	–	–	+	–	+	–
Fusobacterium nucleotum	–	+	–	–	+	+
Campylobacter jejuni	NK	NK	NK	–	+	NK
Providencia	+	–	–	+	–	–
Aeromonas hydrophila	+	–	–	+	–	+

a = Cephalosprinase; b = Penicillinase; c = Metalloenzyme
d = Inducible; e = Constitutive; f = Clavulanic acid
NK = not known
* = Reference, Wiedemann, Kleibe and Kresken (1989)

Proteus spp *Providencia*, *Yersinia* spps., on exposure to certain beta-lactams. (Acar and Minozzi, 1986; Livermore, 1987b; Nayler, 1987; Bush, 1988a). These enzymes are classified as class 1 by the scheme of Richmond and Sykes (1973) and comprise the most important cephalosporinases. They can be produced constitutively and copiously as a result of a stable mutation in a beta-lactamase regulatory gene leading to derepression (Lindberg and Normark, 1986). This kind of mutation is quite common among beta-lactamase inducible species at a high frequency (Gwynn and Rolinson, 1983; Wiedemann, 1986).

The method by which resistant mutants emerge is different in those strains where the genes for beta-lactamase production are inducible as in *Enterobacter cloacae, Citrobacter freundii*, *Proteus vulgaris*, *Serratia marcescens* and *Pseudomonas* spp. The genetics behind the resistance problems have been found in *E. cloacae.* Cloning experiments revealed that at least three genes are involved in the mechanism of induction: the amp C gene, which codes for the beta-lactamase; the amp R gene, which activates the amp C gene in the presence of an inducer; and the amp D gene, which is a negative regulator for amp C gene expression (Peter, Korfmann and Wiedemann, 1988). In *E.coli*, *Shigella* spp., *Proteus mirabilis* the cephalosporinase 1 is produced constitutively in tiny basal amounts (Livermore, 1987a). The resistant mutants of *E.coli* strains had been selected and the background of the mutation to beta-lactamase was overproduction of the chromosomal gene which was caused by promoter mutations, attenuator mutations or gene amplification (Normark et al., 1977; Normark and Burman, 1977; Bergstrom and Normark, 1979). On the other hand, *Klebsiella* and *Salmonella* spps. produce constitutive enzymes of broad-spectrum and non-class 1 cephalosporinase beta-lactamases respectively (Livermore, 1987; Wiedemann, Kliebe and Kresken, 1989).

Different beta-lactam antibiotics act as different types of inducers (strong/weak/labile/nonlabile). This creates the possibility of the emergence of resistant strains during clinical therapy and, indeed, may be the commonest and most important cause of emerging resistance to newer beta-lactams (Livermore, 1987a; Bush, 1988a). There have been recent reports of *Pseudomonas aeruginosa* (Livermore and Yang, 1987) and of Enterobacteriaceae, such as *Citrobacter* spp. (Gootz, Jackson and Sherris, 1984), *Enterobacter cloacae* (Sanders and Sanders, 1983) and *Serratia* spp. (Sanders and Sanders, 1988) in which cephalosporins, monobactams and extended spectrum penicillins are resisted by inducible beta-lactamases. These mutants have been implicated in the emergence of multiple drug resistance during therapy (Collatz et al., 1984; Sanders and Sanders, 1985, 1986, 1988; Milatovic and Braveny, 1987). Induction can also be influenced by different factors under laboratory circumstances, such as culture media, duration of induction, concentration of inducer and stability of inducer to the induced enzyme. Induction data can also be interpreted in different ways depending upon the concentration chosen for the induction studies (Gootz and Sanders, 1983; Cullmann, Dalhoff and Dick, 1984; Dalhoff and Cullmann, 1984).

In contrast to the inevitable transient nature of beta-lactamase induction, selection of stable, derepressed mutants with resistance to a variety of newer beta-lactam antibiotics can occur in both clinical strains of *Enterobacter cloacae* and *Pseudomonas aeruginosa* (Quinn et al., 1986; Quinn, DiVincenzo and Foster, 1987) and laboratory strains (Gootz, Sanders and Goering, 1982; Livermore, 1987a). Recently, it has been found that clinical *Enterobacter cloacae* mutants can produce two different but closely related beta-lactamases with different pIs. They have been called type-A and type-B, and the biochemical distinction of these two enzymes was carried out by Then et al (1988). On the other hand, molecular studies

such as heterogeneity in the amp R and amp C gene interaction, genetic control of beta-lactam production, impact of the amp D gene and its product on beta-lactamase production in Enterobacter cloacae (Goering et al, 1988; Korfmann and Wiedemann, 1988; Peter Korfmann and Wiedemann, 1988) have recently been studied.

In Gram-positive bacteria, chromosomal beta-lactamase production has been found in staphylococci, streptococci and enterococci (Wiedemann, Kliebe and Kresken, 1989). In the Bacillus genus (such as Bacillus cereus and Bacillus licheniformis) (Abraham and Waley, 1979; Collins, 1979) and in the genus Clostridium (Clostridium butyricum, Clostridium clostridiiforme and Clostridium ramosum) (Nord and Olsson-Liljequist, 1984) only inducible penicillinases have been detected so far. Fortunately, no beta-lactamases have been found in clinically important Clostridium difficile and Clostridium perfringens (Nord and Olsson-Liljequist, 1984). However, Mycobacterium spp. have been found to produce a chromosomal enzyme which has affinity for both penicillin and cephalosporins (Kasik, 1979).

1.3.3D Plasmid-Mediated Beta-Lactamases

Bacterial plasmids are extrachromosomal molecules of circular double-stranded DNA. Those that mediate antibiotic resistance were first discovered in Japan in 1957 (review: Tayler, 1989). Most resistance determinants of clinical relevance are encoded by plasmids which replicate independently from the chromosome. Most clinical plasmids can transfer to other bacterial cells and even to other species by their own

transfer system (conjugation). Smaller plasmids (<16 Mdal or 24 kb) cannot transfer but they can usually be mobilised by a transferable factor or plasmid of larger size (Wiedemann, 1981). R-plasmids allow rapid mobility of resistance genes enabling an efficient spread through bacterial populations. Twelve plasmid-determined beta-lactamases are known to be encoded by transposons (Table 2). These are genetic elements that can transfer from one plasmid to another and between plasmids and the bacterial chromosome (Medeiros, 1989). The existence of beta-lactamase genes on plasmids and transposons will ensure that a beta-lactamase originally confined to one group of bacteria will sooner or later appear in most other groups of bacteria.

Until now more than 50 different plasmid-mediated beta-lactamases (Table 3) have been identified among Gram-negative bacteria. TEM-1 is the most predominant enzyme and is found in nearly all Enterobacteriaceae (Simpson, Harper and O'Callaghan, 1980; Roy et al, 1983, 1985; Simpson et al, 1986; Huovinen, Huovinen and Jacoby, 1988b), Pseudomonas aeruginosa (Jouvenot, Bonin and Michel-Briand, 1983; Huovinen, Huovinen and Jacoby, 1988a), Yersinia enterocolitica (Matthew, 1979), Vibrio cholerae (Matthew, 1979), Acinetobacter spp. (Joly-Guillo et al, 1988), H. influenzae (Matthew, 1979; Machka et al, 1988), N. gonorrhoeae (Roberts, Elwell and Falkow, 1977), and in N. meningitidis (Dillon, Pauze and Yeung, 1983). The TEM-2 beta-lactamase is the next commonest enzyme although it has not been found in the two latter species (Wiedemann, Kliebe and Kresken, 1989).

The wide variety of OXA- and PSE-enzymes are also quite common. In the group of OXA-enzymes the most common is OXA-1 which is distributed in many

Table 2

Beta-lactamase Transposons

Transposon	Beta-lactamase	Phenotype	Size (kb)	Reference[b]
Tn-3	TEM-1	Ap	5	
Tn-4	TEM-1	Ap sm Sp Su	24	
Tn-AB	TEM-1	Ap Sm	14.3	
Tn-1699	TEM-1	Ap Gm Km	9.3	
Tn-1700	TEM-1	Ap Gm Km Tm	9.3	
Tn-1	TEM-2	Ap	5	
-[a]	SHV-1	Ap	14.3	
Tn-1412	LCR-1	Ap Gm Km Sm Sp Su Tm	19	
Tn-2603	OXA-1	Am Sm Sp Su Hg	20	
Tn-2410	OXA-2	Ap Su Hg	18.5	
Tn-1411	OXA-3	Ap Cm Gm Km Sm Su Tm	15	
Tn-1409	OXA-4	Ap Cm Sm Sp Su Tp	14.6	
Tn-1406	OXA-5	Ap Gm Km Su Tm Hg	17.2	
Tn-1401	PSE-1	Ap Sm Sp Su Hg	12	
Tn-1403	PSE-1	Ap Cm Sm Sp	18	
Tn-1404	PSE-2	Ap Gm Km Sm Sp Su Tm	9.6	
Tn-2521	PSE-4	Ap Sm Sp Su	6.8	
Tn-1405	PSE-4	Ap Sm Sp Su	8	
Tn-1408	CARB-3	Ap Cm Sm Sp Su	25.2	

a = Tn number not assigned
b = Reproduced from Medeiros, 1989

The other plasmid-mediated beta-lactamases encoded by transposons are:

HMS-1; LCR-1; OXA-6; OXA-7; PSE-3; CARB-4; AER-1; SHV-2 (Amyes, 1989).

Table 3

Biochemical Properties of the Plasmid-encoded Beta-lactamases in Gram-negative Bacteria

Beta-lactamase	Relative rates of hydrolysis or V_{max} (Pen = 100%)											Inhibition by				pI	MW ($x10^3$)
	Amp	Carb	Oxa	Meth	Clox	Cer	Cep	Cmd	Ctx	Caz	Az	Clav	Clox	pCMB	Nacl		
Broad spectrum enzymes																	
TEM-1[a]	72	12	4	1	<0.2	60	13	20	<1			+	+	-	-	5.4	22
TEM-2[a]	107	10	5	0	0	74	20		0			+	+	-	-	5.6	23.5
SHV-1[a]	212	8	0	<2	<2	56	8						+	+/-	-	7.6	22
HMS-1[a]	253	14	<2	<2	<2	183	3						+	+	-	5.2	21
TLE-1[a]	67	13	4	5	6	52	15	24				+	+		-	5.55	19.8
TLE-2[g]	140	13							6							6.5	19
ROB-1[a]	107	19			<0.2	37	4.5					+	+		-	8.1	
LCR-1[a]	145	4		20	3	55	24					+	+	-		5.85	44
NPS-1[a]	223	18	40	<0.1		3							-	-		6.5	25
OHIO-1[a]	140	11	<0.5	<0.5	<0.5	79	8	15				+	+		-	7.0	22
SAR-2[h]	101.3	48	64	100.2	ND	27	ND	ND	20	ND		-	+	ND	ND	8.3	36
LXA-1[i]	155	40	<1	<1	<1	118	45	ND	<1	ND		+	+	ND	ND	6.7	24
Oxacillin-hydrolysing enzymes																	
OXA-1[a]	419	65	271	342	119	112	43	ND	22	NM		-	-	+/-	+	7.5	23.3
OXA-2[a]	236	27	581	31	157	108	61	32	1			-	-	-	+	7.7	43.9
OXA-3[a]	178	10	336	29	350	44	10		0				-		+	7.1	41.2
OXA-4[a]	325	57	188	357	121	110	20		63			-	-		+	7.45	23
OXA-5[a]	188	40	210	109	258	89	175	35	49			-	-		+	7.62	27
OXA-6[a]	596	46	1048	585	301	149	24	82	28			-	+		-	7.8	40
OXA-7[a]	545	48	702	424	494	136	51	47	31			-	-		+	7.65	25.3
GN 11499	357	43			271	71	57					+		+/-		6.9	41.5
OXA-E1[e]	756	107.7				49.6	13	39.4	18.4	NH		-	-	-	-	7.5	
Carbenicillin-hydrolysing enzymes																	
PSE-1[a](CARB-2)	84	100	9	1.1	0	27	0	10	27				-	+		5.7	28.5
PSE-2[a]	173	87	273	286	902	50	11		16					+	+	6.1	12.4
PSE-3[a]	101	253			3	10			<1				-	-		6.9	12
PSE-4[a](CARB-1)	88	150	8	16	<2	40	4		1							5.7	32
CARB-3[a]	100	147	12.5	0.6	0.5	44	0.5									5.75	31
CARB-4[a]	130	79	1	2	<1	15	2					+	-	+	-	4.3	22
CARB-5[d]																6.3	
BRO-1[a]	103	78		131	5	23	24	31								5.6	
AER-1[a]	38	98	0.9	0.3	0	26	77	18	20							5.9	22
N-3[a]	113	113			<2	<12	<2									6.0	24
																(5.73)	
N-29[a]	124	128			<2	6	<2									6.9	22
																(6.83)	
SAR-1[e]	63	122		0	0	21		53				+	+			4.9	33.7
Extended spectrum Beta-lactamases																	
*TEM-3[b](CTX-1)	71	14.5					107		445	40	8.5					6.3	24
*TEM-4[c]	50	12					120		232	10	<1					5.9	24
*TEM-5[b](CAZ-1)	78	60					380		150	490	116					5.55	
*TEM-6[c]																5.9	
*TEM-7[c]	93	20					16		1.9	1.7						5.41	
+TEM-9[f](RHH1)						100			18.0	52	60			+		5.5	
+TEM-10[f]	170	47			21	100	24		2.1	90	13	+		+		5.57	29
*SHV-2[b]	140	19	0				106		70	6.5	1					7.6	
*SHV-3[c]	200	19					110		67	4	<1					7.0	
*SHV-4[b](CAZ-5)	195	35					200		115	52	4					7.75	
*SHV-5[b](CAZ-4)	242	31					180		134	49	2					8.2	
*CAZ-2[b]	238	75.2					165		640	261	207					5.9	
																(6.0)	
*CAZ-3[b]	89						80		18.4	10.2	2.3					5.2	
																(5.3)	
+CAZ-1o[j]						100		57	1.0	1.0	<0.5					5.6	
+CAX-h1[j]						100		21	2.3	36	<0.5					6.5	
UN[k]											-					5.7	
																(5.9)	
+MJ-1[l]	72	14	32		15	95	80		8		-					5.35	
+MJ-2[l]	8	13	<0.2		<0.2	<0.2	.9		10		-					5.55	
PMG-25[m]																5.55	
FEC-1[n]	17					100	198	125	23	0.13	-	+				8.2	48
**TEM-E1[o]	100	29.6				42.1			1.48	0.31	-					5.4	22
**TEM-E2[p]	100	23				87			0.84	0.87	-					5.3	23
**TEM-E3[t] (NMH)	100	22				50			2.9	39						5.6	25.5
**TEM-E3[t] (SLH)	100	34				38			1.6	29						5.6	23.5
Amp C type																	
CEP1[q]																8.0	37.5
CEP2[r]		48				108	114	73								8.1	36.2

NM = not measurable; * = amoxycillin was used instead of ampicillin for substrate profile; ** = ampicillin was taken as 100%; + = cephaloridine was taken as 100%; a = Medeiros, 1989; b = Sirot et al., 1989; c = Philippon, Labia & Jacoby, 1989; d = Amyes, 1989; e = Reid & Amyes, 1986; f = Quinn et al., 1989; g = Reid et al., 1987; h = Nandivada & Amyes, 1987; i = Yang, Jacoby & Livermore, 1988; j = Vuye, Verschraegen & Clayes, 1989; k = Corkill, Hart & Shears, 1989; l = Deschaseux et al., 1988; m = Jacoby et al., 1989; n = Matsumoto et al., 1988; o = Payne, Marriott& Amyes, 1989; p = Payne, Marriott & Amyes, 1990; q = Bobrowski et al., 1976; r = Levesque et al., 1982; s = OXA-E1 is the reclassifiction of the enzyme OXA-1 like enzyme described in the text (tables 33, 34); t = Payne et al., 1989.

Enterobacteriaceae (Matthew, 1979). OXA-2 has been isolated from *E.coli*, *P. mirabilis*, *Serratia*, *Salmonella* and even *Bordetella bronchiseptica.* OXA-4, OXA-5, OXA-6 and OXA-7 are relatively rare enzymes (Medeiros, Cohenford and Jacoby, 1985). OXA-3 has been found mainly in *Klebsiella* spp. (Simpson, Harper and O'Callaghan, 1980; Roy et al., 1983). The OXA enzymes, as their name suggests, have the specialised capability of hydrolysing the penicillinase resistant isoxazolyl penicillins such as oxacillin in addition to penicillins (Dale and Smith, 1974). PSE enzymes, which were originally found in *Pseudomonas* and thought to be confined to that genus have recently been found in enterobacterial strains (Hedges and Matthew, 1979; Medeiros, Hedges and Jacoby, 1982; Levy et al., 1985; Simpson et al., 1986; Reid et al., 1988a). These enzymes have a high affinity for carbenicillin. The PSE-2 beta-lactamase resembles the OXA enzymes in having a high relative activity against oxacillin (Medeiros, 1989). The most common PSE enzyme, PSE-4, has recently been identified in *K. pneumonia* and *E. cloaceae* (Reid et al., 1988a).

The enzyme SHV-1 is less common than the TEM-1 beta-lactamase though it has the same broad spectrum. However, the plasmid-borne beta-lactamase SHV-1 was recently found to be capable of modification to produce SHV-2 (Kliebe et al., 1985). The modification of SHV-1 to SHV-2 was of crucial importance because SHV-2 was identified in clinical strains of *K. pneumoniae* and *Serratia marcescens* in 1983 (Knothe et al., 1983). The SHV-1 enzyme had been known for more than 10 years basically as an enzyme predominantly associated with a penicillinase activity and limited cephalosporinase activity. SHV-2 is mainly a cephalosporinase capable of hydrolysing third generation cephalosporins and monobactams. It is the first enzyme to be discovered with such an extended spectrum to the newer and active cephalosporins. Since then, other extended spectrum beta-lactamases, derivatives of

both TEM (Bauernfeind and Horl, 1987; Brun-Buisson et al., 1987; Paul et al., 1987; Sirot et al., 1987; Spencer et al., 1987; Gutmann et al., 1988; Petit et al., 1988) and SHV (Bure et al., 1988; Jarlier et al., 1988; Gutmann et al., 1989) enzymes have evolved in clinical isolates of *E.coli*, *E. cloacae*, *Citrobacter*, *Serratia*, and *Klebsiella* spps. Each enzyme has the capability to hydrolyse one or more third generation cephalosporins and confer resistance to these drugs. In addition, a series of other, individual plasmid-mediated beta-lactamases (Table 3) have been identified and classified under broad-spectrum type enzymes (Table 3).

1.4 Bacterial Resistance to Beta-lactams

The extent of antibiotic resistance mediated by a particular beta-lactamase in a population of bacteria is dependent on various factors. Firstly, the efficiency of the beta-lactamase in hydrolysing an antibiotic depends on its rates of hydrolysis (V_{max}), conventionally expressed as a ratio relative to the rate of hydrolysis benzylpenicillin, ampicillin or cephaloridine. Secondly, the level of resistance might be related to the enzyme's affinity for the antibiotic (K_m), a value often difficult to obtain with substrates which are hydrolysed weakly (Livermore et al., 1986). A third factor might be the amount of beta-lactamase produced which can affect the levels of susceptibility (Livermore et al., 1986; Medeiros, 1989). For example, the TEM-1 beta-lactamase activity of clinical isolates of *E. coli* which can vary 100-fold (Simpson et al., 1986; Medeiros, 1989). The elevated production of beta-lactamase has been associated with the enhanced resistance to cephamandole and to some third generation cephalosporins in an isolate of *E.coli* (Jacoby and Sutton, 1985). Enhanced levels of TEM-1 cannot be correlated to increased resistance to ampicillin (Reid-PhD thesis).

Within the bacterial cell there are two ways in which the beta-lactamases can contribute to antibiotic resistance. Firstly, in Gram-negative bacteria, there is a model exemplified by TEM-1 producing *H. influenzae*, the beta-lactamase remains trapped in the periplasmic space and there is no barrier to antibiotic penetration (Medeiros and O'Brien, 1975). In this case, the effect of inoculum size determines the minimum inhibitory concentration, and, indeed, it was found possible to treat, the infection caused by *H. influenzae* with ampicillin when the inoculum of infecting bacteria was low (Moxon, Medeiros and O'Brien, 1977; Murphy and Todd, 1979). A high level of resistance can occur when promoter mutations occur which results in greater expression of beta-lactamase activity (Chen and Clowes, 1987).

A second model is the localisation of beta-lactamase (e.g. TEM-1 producing *E.coli* strain) between the barrier to antibiotic penetration (outer membrane) and the antibiotic targets (penicillin binding proteins on the cytoplasmic membrane). In this position the enzyme can sequentially destroy antibiotic molecules as they make their way through the barrier. Consequently, single bacterial cells can acquire high levels of resistance, unlike the previous example (Medeiros, Kent and O'Brien, 1974; Sykes and Matthew, 1976).

Other types of resistance could include "trapping" of beta-lactams by beta-lactamases in non-covalent complexes. This suggests the removal of active antibiotic from the periplasm (Then and Angehrn, 1982; Sanders and Sanders, 1986). The trapping is a process which leads, at least temporarily, to the mutual inactivation of one molecule of antibiotic and one molecule of enzyme (Nayler, 1987). The operation of this process has been postulated under circumstances in which beta-lactamase has high affinity (low K_i) for a drug but little tendency to form a covalent acyl enzyme.

The potential for trapping of this type has been disputed on the grounds that some theoretically trappable substrates retain activity against enzyme-hyperproducing strains (Livermore, 1985). Trapping without any hydrolysis, could only lead to a high level of resistance if the quantity of beta-lactamase was very great, the organism was poorly permeable and the beta-lactam was poorly diffusible. In addition, it is also suggested that a beta-lactamase which is capable only of reversibly binding beta-lactams might promote the drug accumulation rather than resistance (Livermore, 1987a). Thus non-covalent trapping remains conceivable but has not been demonstrated convincingly (Nayler, 1987).

In Gram-positive bacteria, plasmid-mediated beta-lactamases are predominant in *Staphylococcus aureus*. In *S. aureus*, about 70% show resistance to beta-lactam antibiotics (Wiedemann, Kliebe and Kresken, 1989), . . produce exoenzymes which are immunologically distinguishable (A-D) (Richmond, 1965) and are of importance both in community and hospital-acquired infections. The beta-lactam resistance genes can be located on different transposons and therefore can be integrated into the bacterial chromosome (Lyon and Skurray, 1987). Almost all resistant coagulase-negative staphylococcal strains are found to produce plasmid-mediated beta-lactamases (Rosdahl, Jarlov and Knudsen, 1986). Most of them have a molecular weight of 28,000 and have more affinity for penicillins than cephalosporins. The enzymes can be inducible in some strains (Dyke, 1979). Constitutive synthesis occurs only occasionally in nosocomial isolates (Rosdahl, 1973).

Streptococcus uberies and *S. faecalis* also produce beta-lactamases; and *S. faecalis* produces plasmid-mediated beta-lactamase (Matthew and Harris, 1976; Murray and Mederiski-Samaroj, 1983; Medeiros, 1984). Recently, hybridisation analysis has

shown that this gene was closely related to those of *S. aureus* (Murray et al., 1986); Nevertheless, this enzyme is relatively rare and has been found in only a few strains (Patterson, Masecar and Zervos, 1988).

1.5 Development and Spread of Plasmid-mediated Beta-lactamases

The earliest emergence of penicillin resistance was in *S. aureus* (Kirby,1944). The percentage of penicillin-resistant *S. aureus* strains has remained unchanged over the years; the average has been about 70% since 1953 (Kirby and Ahern, 1953; Wiedemann, Kliebe and Kresken, 1989). Other common bacteria isolated from community and hospital-acquired infections, such as *H. influenzae*, *E. coli*, *S. aureus*, *Enterobacter* spps and *Ps. aeruginosa* have all been found to have the capability of possessing beta-lactamase determined resistance. Only *S. pneumoniae* remains incapable of producing a beta-lactamase-determined resistance (Wiedemann, Kliebe and Kresken, 1989).

In *E. coli*, the development of penicillin resistance mediated by plasmid-mediated beta-lactamase occurred shortly after the introduction of aminopenicillins (Anderson and Datta, 1965). The percentage of ampicillin resistant *E. coli* strains stayed almost static, at 21.9% without any significant change during the period 1975-1984 (Wiedemann, Kliebe and Kresken, 1989). The TEM-1 beta-lactamase was the most prevalent beta-lactamase, and this did not change following the emergence of newly developed resistance to the recently introduced cephalosporins. *Klebsiella* strains were found to produce SHV-1 as the predominant enzyme, the next commonest enzyme was TEM-1. Ampicillin resistance in this species is species-specific. This is due to the localisation of the SHV-1 resistance gene on both chromosome and plasmid (Nugent and Hedges, 1979). Similarly, resistance to

cefoxitin and ampicillin is mainly regarded as species-specific in *Enterobacter* spp. Cefotaxime resistant strains have evolved by overproduction of the chromosomal enzyme, but the percentage of resistance has not increased since the introduction of cefotaxime in 1980 (Weinstein, 1985; Sanders and Sanders, 1988; Wiedemann, Kliebe and Kresken, 1989).

The increase in the number of resistant strains among each species usually occurred shortly after the introduction of the respective drug into therapy. After this, the proportion of resistant strains remains static. This is true for all kinds of bacterial infections such as epidemic enteric infections, ~~most~~ both community-acquired and nosocomial infections; however, *N. gonorrhoeae* and *H. influenzae* seem to be the exceptions. It is quite obvious that the development of resistance is a continuous process as there are rapidly growing number of beta-lactamases causing failures during the therapy among clinical isolates (Bush, 1988a).

1.6 Epidemiology of Worldwide Resistance to Beta-lactam Antibiotics

The main reason for the development of resistance is the widespread use of antibiotics that fosters the selection of the resistant organisms which predominates at a local level may then spread worldwide. The introduction of the broad-spectrum penicillins and cephalosporins in the 1960s in Europe and USA rapidly led to the increased prevalence of ampicillin resistance among Gram-negative bacteria, especially *E.coli*, *Salmonella*, *Klebsiella* and *Haemophilus* spps (Medeiros, 1987). The resistance mechanisms to beta-lactam drugs were largely determined by plasmid-encoded beta-lactamases among Gram-negative bacilli; and, as genes on plasmids were freely transferable, they spread the resistance determinant rapidly through the bacterial population (Anderson and Datta, 1965; Medeiros, 1989).

Indeed, the TEM-1 beta-lactamase, common in enterobacteria, was first discovered in strains of *H. influenzae* in 1974 in the USA (Thornsberry and McDougal, 1982). Even though it was rare at first in this species, it reached predominance in most regions (Thornsberry and McDougal, 1982). By 1976, TEM-1 appeared in a few strains of *N. gonorrhoeae* in England and, subsequently, in the USA (Perine et al., 1977). The incidence of beta-lactam resistance rapidly increased (1979 to 1982) by about 10-fold in the States and similar increases occurred in other countries (Medeiros, 1989).

The introduction of beta-lactamase resistant, third-generation cephalosporins led to the occurrence of mutations on plasmid-encoded beta-lactamases. These mutated genes were transferable. This is exemplified by the evolution of SHV-2 in *Klebsiella* isolates in Frankfurt, W. Germany. The SHV-2 enzyme conferring resistance to cefotaxime and other newer cephalosporins. It had arisen from a mutation in SHV-1 which involved two genetic steps in resistance gene and one amino-acid change in the active site of the enzyme (Kliebe et al., 1985; Barthelemy, 1988).

Further evolution of SHV-2 on the continent of Europe has led to the emergence of the more potent novel cephalosporinases, SHV-3, SHV-4 (CAZ-5) and SHV-5. These SHV derivatives resulted from the heavy selection pressures of third generation cephalosporin usage in Europe (Gutmann et al., 1988; Jarlier et al., 1988; Petit et al., 1988). These enzymes were identified in the isolates of *K. pneumoniae* and *E. coli*, some of which had caused outbreaks of nosocomial infections mainly from urinary tract infections. All these SHV- derivatives have possessed very close structural similarity despite the heterogeneity of their hydrolytic activities (Vedel, et al., 1989). The SHV-3 differs from SHV-1 in two amino-acid residues and it is

assumed that SHV-3 was a result of the substitution of ser-213 for gly (as in SHV-2) and the replacement of arg-180 by leu which caused a decreased pI from 7.6 to 7.0.

The blaSHV-3 gene is highly homologous (92%) with the chromosomal gene encoding for LEN-1 beta-lactamase of *K. pneumoniae* (Nicolas et al., 1989). On the other hand SHV-4 (CAZ-5) differed from SHV-1 by five amino-acid substitutions. The unusually high activity of SHV-4 (CAZ-5) towards ceftazidime was manifested by the substitution of a lysine for glutamic acid at position 214 of the mature protein (Peduzzi et al., 1989). There is a dispute whether both CAZ-4 and SHV-5 are the same and so biochemical comparisons and genetic studies of the two enzymes will be necessary to resolve this (Sirot et al., 1989). Analogous similarities were observed between the CAZ-4 and SHV-5 and it has been assumed that they might be the same enzyme (Sirot et al., 1989). Of these new SHV-enzymes, only SHV-2 has been found in more than one location i.e. in Chile and Greece (Medeiros, 1987). This might reflect that the mutation from SHV-1 to SHV-2 is quite minor compared with the genetic changes from SHV-1 to the other SHV enzymes.

Around 1987, there had been an outbreak of nosocomial cefotaxime resistant isolates of *K. pneumoniae* and other enterobacterial strains. All strains produced a pI 6.3 beta-lactamase (CTX-1), and were mainly derived from urinary tract isolates in several French hospitals (Brun-Buisson et al., 1987; Sirot et al., 1987). In the same year (1987), the other plasmid-determined beta-lactamase CAZ-1 was identified in *K. pneumoniae* strains from patients in a French hospital. This enzyme had more affinity for ceftazidime than other later generation cephalosporins (Sirot, Labia and Thabaut, 1987). These new enzymes CTX-1 and CAZ-1, are now designated as TEM-3 and TEM-5 because of their ability to hybridise with a TEM-gene probe. It has been shown that both these enzymes were TEM-2 derivatives

(Sirot et al., 1988). TEM-3 and TEM-5 enzymes arose by fairly minor point mutation in TEM-1 type penicillin genes (Sougakoff et al., 1988). Only three amino acid substitutions in TEM-1 resulted in TEM-3. For TEM-5, there were again three amino acid substitutions from TEM-1, Involvement of a second residue, lysine, substituted for Glu at position 237 (Philippon, Labia and Jacoby, 1989), seems necessary to justify the unusually high rates of hydrolysis of ceftazidime and aztreonam by this enzyme (Sougakoff et al., 1988). Structure-activity relations suggest that the oxime substituent of these cephalosporins is a major structural factor in the catalytic process observed with the new beta-lactamases TEM-3 (CTX-1), TEM-5 (CAZ-1) including SHV-2 (Labia et al., 1988a). In addition, other TEM-type mutants such as TEM-4 (Paul et al., 1987), TEM-6 (Bauernfeind and Horl, 1987), TEM-7 (Gutmann et al., 1988), TEM-9 ((RHH-1) (Spencer et al., 1987) and TEM-10 (Quinn et al., 1989) have also been identified.

The ceftazidimases CAZ-2 (Chanal et al., 1988) and CAZ-3 (Labia et al., 1988b) were also confirmed as derivatives of TEM beta-lactamase (Sirot et al., 1989) which were isolated from the clinical strains of *K. pneumoniae*, in 1987 in France. The TEM-7 enzyme was produced in a clinical strain of *Citrobacter freundii* M2 isolated from the urine of a hospitalised patient in Paris (Gutmann et al., 1988). The beta-lactamases TEM-4, TEM-6, TEM-9 and TEM-10 were isolated from *E.coli* (TEM-4 and TEM-6) and *K.pneumoniae* (TEM-9 and TEM-10) in Germany, France, England (UK) and USA respectively. Three novel expanded-spectrum beta-lactamases, TEM-E1, TEM-E2 (Payne, Marriott and Amyes, 1989, 1990) and TEM-E3 (Payne et al., 1989) isolated from *E.coli*, *K. oxytoca* and *E.cloacae* isolates from Belgium (TEM-E2) and England (TEM-E1 and TEM-E3) respectively, have been included into third generation cephalosporin (3GC) enzyme groups.

When considering the epidemiology of beta-lactamases, and especially those that

confer resistance to 3GC, the contribution of the inducible chromosomally-determined enzyme should be taken into account (Olson et al., 1983; Sanders, 1983). It has been demonstrated that clinical isolates have become resistant to beta-lactam antibiotics while the patient was receiving appropriate therapy, a phenomenon rarely seen prior to the introduction of 3GC drugs (Sugarman and Pesanti, 1980; Sanders and Sanders, 1988). There have been recent reports which show similar results with the treatment of respiratory tract and soft tissue infections (Dworzack et al., 1987), neutropenia and cystic fibrosis (Bragman et al., 1986; Paull and Morgan, 1986; Pederson et al., 1986). It is possible to select mutants during clinical therapy (Sanders and Sanders, 1988) which overproduce the enzyme and mediate resistance to 3GC antibiotics when infections caused by *Enterobacter*, *Citrobacter*, indole-positive *Proteus*, *Providencia*, *Serratia*, *Morganella* spps and *P. aeruginosa* which produce inducible cephalosporinases (Sykes and Smith, 1979), are treated (Seeberg, Tolxdorff-Neutzling and Wiedemann, 1983; Gootz, Jackson and Sherris, 1984; Livermore and Yang, 1987). Reports on several other Gram-negative bacteria such as *E.coli*, *Klebsiella*, *Salmonella*, *Shigella* and, especially, *Serratia*, *Enterobacter* and *Pseudomonas* spps. which have caused resistance problems to newer cephalosporins have been discussed in a recent review (Sanders and Sanders 1988; Wiedemann, Kliebe and Kresken, 1989). Mutations in the bacterial chromosome i.e. the mechanisms for an overproduction through the inactivation of the amp D gene which usually negatively controls the expression of the beta-lactam gene, is the main resistance mechanism. This can also result in the constitutive overproduction of the chromosomal enzyme (Medeiros, 1987; Sanders and Sanders, 1988; Wiedemann, Kliebe and Kresken, 1989).

1.7 Surveys of Beta-lactam Resistant Gram-negative Bacteria

The incidence and type of antibacterial drug resistance has been studied in different regions and countries. These epidemiological studies are absolutely essential in order to evaluate the spread of drug resistance all over the world. There have been recent reviews on these studies and also epidemiology of beta-lactamases (Amyes, 1989; Wiedemann, Kliebe and Kresken, 1989; Young, Nandivada and Amyes, 1989).

Even though there have been several studies on beta-lactam resistance, very few controlled surveys have been undertaken, especially in tropical countries. Most of these clinical studies concentrated on plasmid-mediated (transferable) resistance in Gram-negative bacteria from different sources, mainly responsible for urinary tract infections (UTI), urinogenital tract infections, gastroenteric infections etc. in different regions of Africa (Senegal and South Africa), Asia (Bangkok, Indonesia and India), Europe (England, Scotland, Germany, Italy, Paris and Spain), South America (Brazil) and USA (Boston and Providence) (Simpson et al., 1980, 1986; Jouvenot, Bonin and Michel-Briand., 1983; Roy et al., 1983, 1985; Grüneberg, 1984; Esposito et al., 1986; Stobberingh, Houben and Boven, 1985; Medeiros and Jacoby, 1986; Nandivada and Amyes, 1987; Shaokat et al., 1987; Huovinen, Huovinen and Jacoby, 1988b; Reid et al., 1988b). From these surveys it has been shown that the TEM-1 beta-lactamase is the most successful with a high incidence all over the world. Among the other common plasmid-mediated beta-lactamases are TEM-2, OXA-1, OXA-2, SHV-1 and PSEs. There are also strains with combinations of TEM-1 with one of these common enzymes and these have been occurred worldwide, though in far fewer organisms than TEM-1 alone. The most common resistant clinical strains isolated are *E.coli* (18.6%), *Klebsiella* spp. (20%), *Pseudomonas* spp. (22.5%), *Proteus* spp. (12.5%), *Enterobacter* spp. (24.7%) and a

low numbers of Citrobacter spp. (22.2%), Salmonellae (6.1%) and Shigellae from faecal specimens (Wiedemann, Kliebe and Kresken, 1989).

Apart from frequently obtained common plasmid-mediated beta-lactamases (TEMs, OXAs, PSEs and SHV), there has also been a new series of enzymes identified in the surveys; for example TLE-2, SAR-1, the broad-spectrum penicillinases BRO-1 and CARB enzymes. The latter carbenicillinases have each been found on only one occasion. These new enzymes may be species-specific and give a particular advantage to those particular organisms under antibiotic challenge. Another beta-lactamase OHIO-1 is a good example of an endemic enzyme confined to a geographic region (Shales et al., 1986). It was isolated from various species of Enterobacteriaceae from two hospitals in Ohio, one in Columbus and the other in Cleveland. The resistance gene for the OHIO-1 beta-lactamase, located on different plasmids, was found to have common genetic origin in all strains producing this enzyme (Kron et al., 1987).

It is obvious from available data that the occurrence of the expanded-spectrum beta-lactamases, the TEM and SHV derivatives are not widespread yet, except in some outbreaks in France and Germany. There have only been isolated cases both in England and West India (Bombay). The first cephalosporinase present in an Indian clinical isolate has just been found (D.J. Payne personal communication). The genetic background still has to be investigated in order to find out the origin of the resistance gene.

1.8 Combination Therapy

Binding of beta-lactam to beta-lactamase does not always result in hydrolysis. In fact, a number of beta-lactam molecules act as potent inhibitors, or inactivators, for a variety of beta-lactamases. Beta-lactamase inhibitors may fall into several distinct classes: 1) metal ion chelators such as EDTA or O-phenanthroline (Sabath and Abraham, 1966); 2) amino-acid modifiers such as p-chloromercuribenzoate or the boronic acids (Jack and Richmond, 1970; Beesley et al., 1983; Carwright and Waley, 1984); 3) active site directed irreversible inhibitors such as the suicide inactivator clavulanic acid (Brown et al., 1976; Fisher et al., 1981); 4) beta-lactam antibiotics that have very low k_{cat} values such as aztreonam, moxalactam and cefoxitin (Bush, Freudenberger and Sykes, 1982; Bush et al., 1985; Bush and Sykes, 1986).

Even though beta-lactamases are major determinants of bacterial resistance, the introduction of new beta-lactamase-resistant cephalosporins has only marginally influenced the epidemiological situation. After the introduction of the third-generation cephalosporins (3GC), there has been a constant proportion (18-20%) of *E. coli* strains which overproduce the chromosomally mediated beta-lactamases. More importantly, the introduction of the 3GC has resulted in the evolution of new plasmid-mediated beta-lactamases capable of destroying these newer beta-lactam agents. An alternative strategy to combat resistance problems has been combination therapy, the combination of beta-lactam and beta-lactamase inhibitor, which has recently been perceived as an alternative strategy to overcome very stable beta-lactamases.

In fact, the therapeutic use of beta-lactam antibiotic combinations that were intended to exploit the beta-lactamase-inhibition principle enjoyed a short vogue in the late

1960s (Greenwood, 1986). It was not until the appearance of clavulanic acid (Reading and Cole, 1977; Neu and Fu, 1978) and sulbactam (English et al., 1978) that the idea became a viable therapeutic prospect. Penicillin-inhibitor combinations have two main advantages in overcoming stable beta-lactamases. Firstly, they have the attraction of providing the option of oral therapy, if necessary with appropriate prodrugs (Greenwood, 1986); and, these combinations are far less expensive than other later generation beta-lactams. These combined antibiotics are exemplified by (1) amoxycillin + clavulanic acid (augmentin); (2) ampicillin + clavulanic acid (sultacillin); and (3) ticarcillin + clavulanic acid (Timentin) which all are in clinical practice at present. Sultacillin was introduced in 1980 (Wise, Andrews and Bedford, 1980), augmentin in 1981 (Jackson et al., 1983) and timentin in 1985 (Roselle et al., 1985).

Clavulanic acid is a potent inhibitor with a beta-lactam structure but has an oxygen in the ring instead of sulphur (Nayler, 1987). It is a naturally available penem. It is active against many of the plasmid-mediated beta-lactamases of Gram-negative bacteria, including the widely distributed TEM- type, SHV-1, OXA-1 and OXA-3 enzymes produced by clinical isolates (Roy et al., 1989; Rubio et al., 1989). Very significantly, these inhibitors (clavulanic acid and sulbactam) also act against the novel extended spectrum enzymes of TEM and SHV derivatives (Jarlier et al., 1988; Sirot et al., 1988).

Some chromosomal beta-lactamases of obligate anaerobes such as many *Bacteroides* spp. and of the class 4 beta-lactamases of *Klebsiellae* are also inhibited by clavulanic acid (Roy et al., 1989; Werner et al., 1989) and sulbactam (Lees et al., 1986; Nord, 1986). They have little activity against the class 1 beta-lactamases that are produced by most Gram-negative aerobes (Livermore et al., 1989). Clavulanic acid

can potentiate labile beta-lactams such as amino- and carboxypenicillins against these stable beta-lactamases (Reading and Slocombe, 1986; Bush, 1988b). The spectrum of activity of timentin also includes staphylococci and *Pseudomonas aeruginosa* (Fuchs et al., 1984). It has been noted that clavulanic acid can antagonise ticarcillin action against some beta-lactamase inducible isolates. It should be emphasised that whilst induction of class 1 beta-lactamases by clavulanate may cause a temporary impairment of the activity of the ticarcillin, the phenomenon is not a potential source of permanent resistance (Livermore, et al., 1989).

Several studies to evaluate the clinical efficacy, safety and pharmacokinetics of clavulanic acid (Adam et al., 1989; Claes, Vandeursen and Baert, 1989; Cox, Meewis and Horton, 1989; Franz et al ., 1989; Fricke et al., 1989; Meier, Adam and Heilmann, 1989; Nord, Bergan and Thorsteinsson, 1989; Obwegeser et al., 1989; Weismeier et al., 1989) and sulbactam (Lees et al., 1986) in the presence of penicillins have been conducted both *in vivo* and *in vitro* for both prophylactic and therapeutic use and have proven to be very effective. These combined drugs can be administered either by oral or parenteral route (Aronoff et al., 1986) for various kinds of infections: gynaecological (Giamarellou et al., 1986); abdominal surgical (Study group of intraabdominal infections, 1986; Menzies et al., 1989); soft tissue, bone and joint (Loffler et al., 1986; Weismeier et al., 1989); cartilage (Meier, Adam and Heimann, 1989); urinary tract (Kim et al., 1986; Naber and Wittenberger, 1986; Fischbach et al., 1989); intra cerebral (Hanninen and Rossi, 1986; Stahl et al, 1986; Franz et al., 1989); acute epiglottitis(Wald et al., 1986) and meningitis (Rodriguez et al., 1986). They are also quite useful as prophylactic agents in surgery (Dieterich H-J et al., 1989; Friese et al., 1989; Hall et al., 1989; Menzies et al., 1989).

The united action of ampicillin and clavulanic acid combinations have

been proven to be compatible with other effective drug combinations such as metronidazole + gentamicin (Hall et al., 1989), metronidazole + cefuroxime (Friese et al., 1989), metronidazole + netilmicin (Schmitt et al., 1989), clindamycin + gentamicin (Fink et al., 1989), cefoxitin (Dieterich H-J et al., 1989), cefotaxime (Fischbach et al., 1989), ciprofloxacin (Schmidt et al., 1989) and mezlocillin (Menzies et al., 1989). For sulbactam, the combination with the beta-lactam is comparable with the efficacy of other drug combinations; gentamicin + clindamycin (Study group of intraabdominal infections, 1986); chloramphenicol + ampicillin (Rodriguez et al., 1986); metronidazole + cefotaxime (Foster et al., 1986); and cefotaxime (Loffler et al., 1986).

Recently, another combined drug (YTR-830H or tazobactam + piperacillin) has been introduced and is now under study (Marunaka et al., 1988). This beta-lactamase inhibitor binds PBP 2 of Gram-negative bacteria and has poor antibacterial activity like the previous ones (Moosdeen, Williams and Yamabe 1988). In addition, another new inhibitor (6-AMPA) has been tested *in vitro* with ampicillin and found to have excellent inhibiting properties with isolated enzymes but is less useful with intact organism (Chin, McElrath and Neu, 1988).

Despite the fact that combined drugs are very effective both *in vivo* and *in vitro* against many beta-lactamase producing bacteria, especially enterobacterial strains (English et al., 1978; Retsema, English and Girard, 1980; Labia, Morand and Peduzzi, 1986; Brook, 1989; Roy et al., 1989; Rubio et al., 1989; Werner et al.,1989), there have been recent reports that beta-lactam/clavulanic acid resistant bacteria which produce the TEM-1 (Martinez et al., 1987, 1988; Sanders et al., 1988; Williams et al., 1988) and SHV-1 (Sanders et al., 1988) enzymes have emerged. The resistance derives from hyperproduction of the beta-lactamase; and, very recently, the

genetic base for this hyperproduction was found in 10 resistant *E. coli* clinical strains isolated in Spain (Martinez *et al*, 1989). It was found, by transformation experiments, that very small plasmids, ranging in size from 2.5 to 7.0 kb, were providing 10 copies per bacterial chromosome. This gave an increased production of the TEM-1 beta-lactamase. It was also observed that a large plasmid (55 kb) was also present in one to two copies per chromosome. Thus it appeared that the large plasmid had yielded smaller plasmids. They had become multi-copy and thus boosted TEM-1 production. There was one other exceptional circumstance in which resistance results from the production of low levels of PSE-1 enzyme in five *E. coli* clinical strains seemed to be responsible for the rapid hydrolysis of ticarcillin rather than inhibition by potassium clavulanate (Sanders *et al*, 1988). This was proven as the susceptibility to potassium clavulanate was reflected by the ability of this inhibitor at higher concentrations to reduce the MIC of ticarcillin against the *E. coli* to clinically achievable concentrations (Sanders *et al*, 1988).

Since these reports, there have not been any published data detailing resistance problems of beta-lactam/clavulanate in clinical isolates. The increasing clinical use of these drugs has resulted in an increasing demand for *in vitro* susceptibility tests with these agents. In addition, there is a lack of agreement as to whether MIC tests with these combinations should be performed in the presence of constant or variable concentrations of the beta-lactamase inhibitor (Dance *et al*., 1989). False conclusions can be drawn if beta-lactamase inhibitor concentrations *in vitro* are considerably different from those encountered *in vivo* as the effect of beta-lactamase inhibitor concentrations on the results of MIC tests varies considerably from one species to another (Fuchs *et al*, 1984; Simonet *et al*, 1989).

The Aims of This Thesis

The main objectes of the first part of this thesis were:

(A). To study the epidemiology of the beta–lactam resistance in South India, among Gram-negative pathogens.

(B). To investigate the biochemical and genetical mechanisms involved in beta-lactam resistance.

The second part of this project was aimed at:

(C). Examination of the development of resistance to beta-lactam/clavulanic acid combinations.

CHAPTER 2

MATERIALS and METHODS

2.1 Bacterial Strains

The Gram-negative clinical isolates studied in this thesis were collected from India during a three month period in 1984, by Dr. Hilary-Kay Young (Young et al., 1986). All strains were maintained at -70^{o}C in nutrient broth containing 10% glycerol v/v (Analar). Details of the individual strains are presented in the appendix.

The standard bacterial strains used in this thesis are in table 4 and standard bacterial plasmids are detailed in table 5. The standard beta-lactamase producing strains are listed in table 6.

2.2 Information Storage and Retrieval

Information concerning each bacterial isolate was recorded on a computer data form and stored on the database program dBASE III (Ashton-Tate, Milton Keynes). Care was taken to ensure that no repeat specimens were included in the study with the help of the database system.

2.3 Materials

2.3.1 Antimicrobial Agents

All antimicrobial agents used during this study were supplied sterile. The antibiotics

Table 4

Standard Bacterial Strains

Bacterial Strain	Markers	Reference
E. coli K12 J62	$his^-pro^-trp^-lac^-$	Bachmann (1972)
E. coli K12 J62-1	$his^-pro^-trp^-lac^-Na^R$	Bachmann (1972)
E. coli K12 J62-2	$his^-pro^-trp^-lac^-Rf^R$	Bachmann (1972)
E. coli K12 J53	met^-pro^-	Bachmann (1972)
E. coli K12 (X^+)		Obtained from K.B. Sharma
E. coli K12 J53-2	$met^-pro^-Rf^R$	Given by Dr H-K. Young
E. coli Mc 1022	Sm^R	Obtained from Dr S.G.B. Amyes

X^+ - This is a plasmid, see page 51

Table 5

Standard Bacterial Plasmids

Plasmid designation	Markers	Mr (kb)	Reference
R1	Ap Cm Km Sm Su $\underline{IncF_2}$	90	Meynell & Datta (1966)
RP4	Ap Km Tc $\underline{IncP_1}$	52	Datta et al. (1971)
R6K	Ap Sm $\underline{Incx}$	38	Kontomichalou et al (1970)
R1010	Ap Cm Sm Sx $\underline{Inc}$ N	54	Nugent & Hedges (1979)
Sa	Cm Km Sm Su Sp $\underline{IncW}$	33	Watanabe et al. (1968)
x^+		61	Sharma et al. (1984)

Table 6

Standard Beta-lactamase Producing Strains

Bacterial strains	Plasmid or transposon	Beta-lactamase produced	Reference
E. coli J53	R1	TEM-1	Matthew et al (1979)
E. coli J53	R6K (Tn2660)	TEM-1	Hedges et al (1974)
E. coli J53	RP4 (Tn1)	TEM-2	Hedges et al (1974)
E. coli J53	PC $FF0_4$	TEM-3	Given by Dr S.G.B. Amyes
E. coli J53	$PCFF1_4$	TEM-5	Given by Dr S.G.B. Amyes
E. coli J53	-	TEM-7	Given by Dr S.G.B. Amyes
E. coli J53	R1010	SHV-1	Petrocheilou et al (1977)
E. coli J53	R455 (Tn2603)	OXA-1	Dale & Smith (1974)
E. coli J53	$R46\text{-}T^S$	OXA-2	Dale & Smith (1974)
E. coli J53	R57b	OXA-3	Medeiros et al (1985)
E. coli 7528	PMG 203	OXA-4	Medeiros et al (1985)
P. aeruginosa	PMG 54	OXA-5	Medeiros et al (1985)
P. aeruginosa	OMG 329	OXA-6	Medeiros et al (1985)
E. coli	PMG 202	OXA-7	Medeiros et al (1985)
P. aeruginosa Pu21	RPL 11	PSE-1	Matthew & Sykes (1977)
P. aeruginosa Pu21	R151	PSE-2	Matthew (1978)
P. aeruginosa Pu21	RMS 149	PSE-3	Sawada et al (1974)
P. aeruginosa Dalgleish	PMG 19	PSE-4	Furth (1975)
E. coli D31	R22 Ka	CEP-1	Bobrowski et al (1976)
Alcaligenes sp.	PLQ3	CEP-2	Levesque et al (1982)
E. cloacae P99		P99	Fleming et al (1963)

and their suppliers are listed in table 7. The non-chemotherapeutic agent nitrocephin (87/312) was provided by Glaxo Group Research Ltd. For assays beta-lactam solutions were made up aseptically adding sterile sodium phosphate buffer (pH 7.0, 25mM). For preparation of the agar plates, beta-lactams were suspended in sterile distilled water. Preparations of sulphamethoxazole and nalidixic acid were made by dissolving the compound in 0.1N NaOH and the solution was then made up aseptically with sterile distilled water. Solutions of rifampicin and chloramphenicol were made by first dissolving these drugs in a minimum volume of absolute ethanol and then the solution was made up with distilled water. Water soluble rifampicin was also used in the latter experiments. Nitrocephin was dissolved with the aid of dimethyl sulphoxide (DMSO) (Analar, BDH Chemicals Ltd., Poole) and made up in sodium phosphate buffer (pH 7.0, 25mM). All other compounds listed were dissolved directly in sterile distilled water. Compounds, with the exception of nitrocephin stock solution (500mg/l) which could be stored at -20°C for up to more than one month in the dark, were prepared when required.

2.3.2 Buffers

The most commonly used buffer during this study was sodium phosphate (pH 7.0, 25mM). This was prepared as described in Data for Biochemical Research (Oxford University Press, 1974) (30.5mls of 0.5M Na_2HPO_4 and 19.5mls of 0.5M $Na_2H_2PO_4$ were mixed and made up to one litre in order to get 25mM sodium phosphate buffer). Other specialist buffers are described in the appropriate section.

Table 7

Antimicrobial Agents

Compound	Supplier
Chemotherapeutic Agents	
Ampicillin	Beecham Research Laboratories, Middlesex
Aztreonam	E.R. Squibb & Sons, Middlesex
Benzyl penicillin	Glaxo Laboratories Ltd., Greenford, Middlesex
Carbenicillin	Beecham Research Laboratories, Middlesex
Cefamandole	Dista Products Ltd., Basingstoke
Cefazolin	Eli Lilly and Co. Ltd., Basingstoke
Cefotaxime	Roussel Laboratories Ltd., Middlesex
Cefotetan	I.C.I. Ltd., Macclesfield
Cefoxitin	Merck, Sharp and Dohme Ltd., Herts
Cefsulodin	Ciba-Geigy Laboratories, Horsham
Ceftazidime	Glaxo Laboratories, Greenford, Middlesex
Cefuroxime	Glaxo Laboratories Ltd., Greenford, Middlesex
Cephaloridine	Glaxo Laboratories Ltd., Greenford, Middlesex
Cephalothin	Eli Lilly and Co. Ltd., Basingstoke
Cephradine	E.R. Squibb & Sons, Middlesex
Cephalexin	Glaxo Laboratories Ltd., Greenford, Middlesex
Chloramphenicol	Parke-Davis, Pontypool, Gwent
Clavulanic acid	Glaxo Laboratories Ltd., Greenford, Middlesex
Cloxacillin	Beecham Research Laboratories, Middlesex
Gentamicin	Roussel Laboratories Ltd., Middlesex
Kanamycin	Bristol Laboratories Ltd., Middlesex
Methicillin	Beecham Research Laboratories, Middlesex
Nalidixic acid	Sterling Winthrop Laboratories, Surrey
Oxytetracycline hydrochloride	Glaxo Laboratories Ltd., Greenford, Middlesex
Oxacillin	Sigma Chemicals Ltd., London
Piperacillin	Lederle Laboratories Division, Hants
Rifampicin	Le Petite, Milan, Italy
Spectinomycin	Upjohn Ltd., Crawley, Sussex
Streptomycin	Glaxo Laboratories Ltd., Greenford, Middlesex
Sulphamethoxazole	Wellcome Foundation Ltd., Kent
Trimethoprim	Wellcome Foundation Ltd., Kent
Non Chemotherapeutic Agents	
Nitrocephin	Glaxo Laboratories Ltd., Greenford, Middlesex
para-chloromercuric benzoic acid	Sigma Chemicals Ltd., London
Sodium chloride	

All antimicrobial concentrations are expressed in terms of the base.

2.4 Media

2.4.1 Complex Media

The complex media used were as follows:

Nutrient Broth No. 2 (CM67) (Oxoid, Basingstoke, Hants)

Isosensitest Broth (CM473) (Oxoid, Basingstoke, Hants)

Diagnostic Sensitivity Test Agar (CM216) (Oxoid, Basingstoke, Hants).

Columbia Agar Base (CM331) (Oxoid, Basingstoke, Hants)

MacConkey Agar (CM76) (Oxoid, Basingstoke, Hants).

2.4.2 Minimal Media

Table 8

Preparation of Double Strength Davis and Mingioli (DM) Basal Medium

K_2HPO_4	14.0g
KH_2PO_4	6.0
Tri-sodiumcitrate	0.9g
$MgSO_47H_20$	0.2g
$(NH_4)_2SO_4$	2.0g

A one litre solution containing the above compounds was made up with distilled water in the order given and then 50ml quantities of base were distributed and

autoclaved at 15psi for 15 minutes.

Media (DM) was diluted to single strength before use by adding supplements and molten agar (Bacteriological Agar No 1). The supplement solutions for incorporation in the minimal medium are given in table 9.

2.4.3 Preparation of Plates

2.4.3A Plates Containing DM Minimal Media

Plates containing minimal media were prepared by adding supplements (table 9) aseptically to 50mls double-strength DM base. Antimicrobial drugs were added aseptically where necessary to give the required concentrations. Finally, 1.4mls of a 20% glucose solution were added and the volume made up to 60mls by the addition of sterile distilled water. This was mixed and added to 40mls of molten Bacteriological Agar No 1 (1.5g/100 mls) (Oxoid). After rolling gently to mix, the plates were poured and once set, dried inverted at 55^{o}C for 10-15 minutes. In addition, plates (with no antibiotics) were also made up with and without one of the supplements (auxotrophic requirements of the standard recipient strain) in order to confirm the positive conjugation results. All plates were used within 7-10 days of manufacture.

2.4.3B Complex Media Plates

Agar plates with complex media were made up by following the instructions given by the manufacturers. These solutions were sterilised at 15psi for 15 minutes and then cooled to 55^{o}C. Plates containing antimicrobial agents were made by adding the

Table 9

Supplement Solutions

Solution	Source	Strength prepared	Final Concentration	Mode of Sterilisation
D-glucose	BDH	200 mg/L	2.8 mg/ml	Aucoclaving/ filtration
L-histidine	BDH	5 mg/L	50 mg/L	Steaming
L-methionine	Sigma	5 mg/L	50 mg/L	Steaming
L-proline	BDH	5 mg/L	50 mg/L	Steaming
L-tryptophan	Sigma	2.5 mg/L	50 mg/L	Steaming

required concentration of antibiotic solution to the molten Oxoid Diagnostic Sensitivity Test Agar (DSTA) maintained at 55°C. Poured plates were left for setting and then dried inverted at 55°C for about 10-15 minutes. Similarly, Nutrient and MacConkey agar plates were poured with or without antibiotics as required according to the manufacturer's instructions.

2.5 Methods

2.5.1 Identification of Bacterial Species

All enterobacterial pathogens isolated from the patients, were classified to the genus level by standard biochemical characterisation and catalogued by Dr. Hilary-Kay Young.

2.5.2 Viable Counts

Bacterial cultures were diluted to a series of concentrations in single strength DM base. The appropriate diluted suspensions were vortexed (Rotary Mixer, Gallenkamp) and 0.1ml amounts spread with a sterile glass spreader onto solid media.

All plates were incubated inverted at 37°C for 18 to 24 hours and for 66 hours in the case of DM medium.

2.5.3 Sensitivity of Ampicillin and Cephaloridine Resistant Strains

An overnight nutrient broth cultures of each organism was diluted to 10^{-4} by serial

dilutions in single strength DM base medium with no supplements. This diluted suspension (10^5cfu/ml) was placed on to the surface of DSTA plates containing ampicillin or cephaloridine at 10mg/l.

2.5.4 Susceptibility Tests against Antimicrobial Agents

The susceptibility of each isolate was also determined against a selection of antibacterial drugs (table 7), other than ampicillin and cephaloridine, by inoculating similar dilutions (10^{-4}) onto Oxoid DSTA plates containing 10mg/l of drug, except for sulphamethoxazole and spectinomycin where 100mg/l was used and rifampicin where 25mg/l was used. A series of beta-lactam antibiotics also were tested against the strains at different fixed concentrations (Results, tables 14, 28, 35).

Each plate was inoculated with one negative control either *E.coli* J62 or *E.coli* J62.2. (Rif^r) as required. A DSTA plate containing no antibiotic was also inoculated as a positive control. Plates were inverted and incubated for 18 hours at 37°C. Presence of growth at the point of inoculation indicated resistance.

2.5.5 Determination of Minimum Inhibitory Concentrations (MICs) on Solid Media

The MICs of ampicillin and cephaloridine (or other beta-lactams where required) for each organism was tested by diluting an overnight broth culture 10,000-fold in single strength DM and 2µl (10^5cfu) of this was inoculated as before onto the surface of DSTA plates containing antimicrobial agents. The MICs of ampicillin in the

presence of a fixed concentration of clavulanic acid (8 mg/l) was also measured. Drugs were incorporated into the plates, two-fold concentrations ranging from 1 to 1250 mg/l. The MICs were expressed as the lowest concentration of drug permitting no visible growth. The negative control strain was included as before to ensure the reproducibility of sensitivity testing results.

2.5.6 MICs in Liquid Media

The MICs of oxacillin were tested by the broth dilution method in Iso-Sensitest broth (ISTB) (Oxoid). A series of test tubes containing ISTB with decreasing two-fold concentrations (broth dilutions) ranging from 20 to 0.625 mg/l of the antibiotic were inoculated with 2 μl of 10^{-4} dilution of an overnight culture (approximately 10^5 cfu/ml). These inoculated tubes were vortexed briefly to make sure the culture was mixed evenly in the nutrient broth and incubated statically for 18 hours at 37°C. After incubation, the concentration at which ISTB test tube showed no visible growth was taken as MIC.

2.5.7 Mutation Studies

Overnight cultures (4.5 ml) of standard bacteria in nutrient broth were centrifuged (4,500 rpm, 20 minutes) to harvest the cells. They were resuspended in the same amount of single strength DM base and washed (4,500 rpm, 20 minutes). The pellet was resuspended again in 4.5 ml of single strength DM base. Hundred microlitres quantities of appropriate diluted suspensions were spread onto the surface of Oxoid DSTA plates containing different concentrations of the required antibiotics on which resistant mutants were selected.

One hundred microlitres of 10^{-4} and 10^{-6} dilutions of these strains were plated onto

MacConkey agar plates without any antibiotics as positive controls. All plates were incubated at 37°C for 24 hours or 72 hours. The count of colony forming unit on the MacConkey agar plates was noted to indicate the original bacterial concentration of the nutrient broth. Colonies grown on the DSTA plates containing antibiotics were also counted in order to evaluate the mutational frequency. The mutational frequency to a particular antibiotic was taken as the ratio of the number of mutants to the original concentration of bacteria in the culture.

2.5.8 Plasmid DNA Transfer

Conjugation experiments were performed by inoculating both donor and recipient cells from fresh MacConkey agar plates separately into 4.5mls of Nutrient Broth No. 2 (Oxoid). Cultures were incubated statically at 37°C. Following incubation, 0.1ml of the donor was mixed with 1ml of the recipient strain in 4.5ml of fresh pre-warmed nutrient broth (Amyes and Gould, 1984). This mixture was then incubated at 37°C for 18-24 hours. After an overnight incubation this mating mixture was mixed on a Whirlmixer to separate the donor and the recipient cells which were then collected by centrifugation at 4,500rpm for 20 minutes (Heraeus Christ Bactifuge). The pellet was washed with 2 volumes (9.9ml) of single strength DM base without supplements and again the pellet was collected (4,500rpm for 20 minutes). The pellet was resuspended in 5.6ml (or 0.56ml i.e. 10-fold increased concentration when required) of DM base without supplements (equivalent to the volume of the original mating mixture). This resuspended mixture (10µl) was streaked out on the surface of selective DM agar plates which would allow only for the growth of a plasmid-containing recipient strain. Controls were made by inoculating the washed donor and recipient cells onto the same selective plates. The plates were incubated for 24-72 hours. Resultant

transconjugant colonies were purified by streaking out on identical DM selective plates. The purified transconjugants were checked for their auxotrophic requirements on DM medium without antibiotics in them.

2.5.9 Plasmid Mobilisation

In bacteria where no self-transmissible plasmids could be demonstrated, plasmid DNA was mobilised with the transfer factor X^+ (Sharma, Sharma and Prakash 1984). A 0.1ml amount of an overnight culture of the donor strain (*E.coli*) containing the transfer factor X^+ was added to 1ml of recipient in 4.5ml of pre-warmed nutrient broth. Conjugation was allowed for 24 hours at 37°C by incubating statically. Following the incubation, 1ml of this mixture was mixed with 1ml of an 18 hour broth culture of the recipient *E.coli* strain J62.2 in 4.5ml of pre-warmed nutrient broth. After further incubation at 37°C for 24-48 hours, transconjugants were selected on suitable media containing rifampicin and the selective antibiotics. The resultant transconjugants were purified on the same selective plates. The purified transconjugants were tested for possession of the gene from the original culture by plasmid isolation and testing for the resistance markers present in the original clinical strain.

2.5.10 Transformation of Plasmid DNA

Plasmids of interest which were found to be neither self-transferable nor mobilisable were subjected to transformation experiment (Saunders, Docherty and Humphreys, 1984).

2.5.10A Preparation of Competent Cells

A 1ml of an overnight culture of *E.coli* MC 1022 grown statically for 18 hours at 37^{o}C was added to a 100 ml of pre-warmed nutrient broth in a 250ml conical flask. This culture was incubated at 37^{o}C with aeration (Gallenkamp orbital shaker at 160 rpm) until the bacterial density reached 0.15-0.2 OD at A660 with a Bausch and Lomb spectrophotometer. The culture was then chilled on ice for 5-10 minutes. The cells were harvested at 12,000g for 5 minutes at 4^{o}C. The pellet was resuspended in 25mls of 10mM calcium chloride solution and washed by centrifugation (12,000g for 5minutes at 4^{o}C). The pellet was resuspended in 5ml of 75mM calcium chloride which contained 0.5% w/v glucose and 3-(N-morpholino) propane sulfonic acid (MOPS) at pH 6.5. The final product was a suspension of competent cells containing 2.5-5.0x10^{9}. The competent cells were kept stored at -70^{o}c until they were required.

2.5.10B Transformation of Extracted Plasmid DNA into Competent Cells

The plasmid DNA to be transformed was extracted by the method of Takahashi and Nagano (1984) and analysed by agarose gel electrophoresis to estimate the plasmid DNA content (see below). Once sufficient plasmid DNA was obtained, it was used for the transformation. A 0.2ml amount of competent cells in a sterile Eppendorf (polypropylene) tube was added to the plasmid DNA (0.01-10µg) and the mixture was made up to 0.5ml with 75mM calcium chloride solution containing 0.5w/v glucose and 10mM MOPS at pH6.5. This mixture was kept on ice for 45 minutes. The tubes were then transferred to a 42^{o}C water bath for 10 minutes. At the end of 10 minutes, the tubes were taken out and 0.5ml of nutrient broth was added to it. This was followed by incubation at 37^{o}C for 2 hours.

After incubation, the transformation mixture was diluted and a 0.1ml amount of appropriate dilutions were spread onto the surface of nutrient agar plates containing antimicrobial agents to select for plasmid transformants. Control plates of nutrient agar with no antibiotics were also inoculated by the mixture of transformation to quantify the number of viable cells surviving the procedure. Concomitantly, MacConkey agar plates were also used as control plates to detect contaminants in the transformation mixture.

2.5.11 Isolation of Plasmid DNA

A small scale method was followed for the isolation of plasmid DNA throughout the thesis. This method was described by Takahashi and Nagano (1984). Cells were grown with shaking in 4.5mls nutrient broth overnight at 37°C and harvested by centrifugation (4,500rpm, 20 minutes, Heraeus Christ Bactifuge). The method suggested by the authors was then followed exactly.

All extracted plasmid DNA solutions were either analysed directly by agarose gel electrophoresis or were stored at 4°C after brief heating at 66°C for 5 minutes.

2.5.12 Plasmid DNA Preparation for Restriction Endonuclease Digest

Preparation of plasmid DNA for restriction endonuclease digest was performed as described by Takahashi and Nagano (1984) with a minor modification as follows

To the extracted plasmid DNA pellet was added 350µl of 10mM Tris-acetate, 2mM disodium EDTA, pH8.0 (TE) buffer and 50µl of acetate buffer (91mM

sodium acetate, 10mM Tris-acetate, 2mM disodium EDTA, pH 8.0), and these solutions were mixed. The DNA solution was ethanol precipitated with 2 volumes of cold (-20°C) absolute ethanol for 20-30 minutes on ice or at -20°C and collected by centrifugation (2874g, 5 minutes, 4°C, MSE Microcentaur). The pellet was dried in a vacuum dessicator (30-60 minutes). The pellet was dissolved in 50µls of sterile distilled water and a DNAase-free RNAase solution (Sigma) was added to the DNA solution to give a final concentration of 50mg/l or 1mg/l of the RNAase. This mixture was incubated at 37°C for 15 minutes.

Ten microlitres of the plasmid DNA solution was removed to a sterile Eppendorf tube. To this were added 2µls of the appropriate restriction enzyme buffer (10x strength, supplied by NBL Enzymes Ltd., Northumberland) 7µls of sterile distilled water followed by 5-10u of the required restriction endonuclease (*Hind* 111 or *ECO* R1). The tube was incubated at 37°C for 1-3 hours or overnight and the reaction was terminated by cooling to 4°C and adding 7µls of loading buffer (0.1% SDS, 0.05% bromophenol blue, 25% sucrose and 5mM sodium acetate (Sigma) or 0.1% bromophenol blue plus 50% glycerol in distilled water). The sample was analysed by 0.7% agarose gel electrophoresis in parallel with *Hind* III (Murray and Murray, 1975) or other appropriate enzyme-digested lambda DNA as molecular weight markers.

2.5.13 Agarose Gel Electrophoresis

Plasmid DNA was electrophoresed by horizontal slab gel electrophoresis by the method of Meyers et al. (1976). A 30µls volume of plasmid DNA was mixed with 10µls of loading buffer and placed into the wells of a horizontal slab gel (14 by 25 x 0.5cm) consisting of 0.5% or 0.7% agarose (Sigma) in Tris-acetate buffer (40mM

Tris-acetate, 2 mM disodium EDTA, pH 8.0). Similarly, the 27 μl of restriction endonuclease digested DNA samples were loaded into a gel of 0.7% or 1% agarose.

The DNA samples were electrophoresed initially at about 200 V to allow the DNA to enter the gel from the wells and then both plasmid DNA and restriction endonuclease-digested DNA were electrophoresed overnight at 60 V. The agarose gels were run submerged in Tris-acetate buffer (40 mM Tris-acetate, 2 mM disodium EDTA, pH 8.0). The gels were stained with a solution of ethidium bromide (0.5 mg/l) either in distilled water or in the Tris-acetate buffer for a minimum of 30 minutes. Plasmid DNA was visualised over a long-wave ultra-violet light source (330 nm) (Ultra-violet Products Inc., Cambridge) after destaining the gel with distilled water. The distance travelled by DNA through the gel is inversely proportional to the logarithm of its molecular size. The sizes of the sample DNA were measured from the sizes of the standard plasmid DNA which were run concurrently with the samples. The stained agarose gels could be kept submerged in the ethidium bromide solution for up to 10 days without any significant diffusion of the plasmids into the gel.

Photographs of the DNA agarose gel were taken with a Polaroid camera with an orange filter. The gels were exposed to the film for 3 minutes (f8 and shutter speed B).

2.5.14A Beta-lactamase Studies

2.5.14A1 Spot Test for Beta-lactamase Activity with Nitrocephin

Nitrocephin spot test was performed on separated single colonies of bacteria with a

nitrocephin solution (50 mg/L) (O'Callaghan et al., 1972) for beta-lactamase activity. The intact substrate is yellow but becomes red when the beta-lactam bond is broken. A colour change of the nitrocephin from yellow to red within 5 minutes was taken as positive reaction. Beta-lactamase production in individual colonies was also identified by incorporating nitrocephin (50 mg/l) in the agar. Bacterial colonies with high production of beta-lactamase were distinguished by discolouration of the nitrocephin present in the plates.

2.5.14B Preparation of Beta-lactamases

2.5.14B1 Extraction of Crude Beta-lactamases on Large Scale

The method followed to extract beta-lactamases was described by Simpson, Harper and O'Callaghan (1980). One litre volumes of nutrient broth were inoculated and incubated overnight with shaking at 37°C. Cells were harvested at 6,000 rpm for 15 minutes at 4°C (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Instruments). The pellet was washed in 120 ml of 25 mM, sodium phosphate buffer (pH 7.0) and the pellet was again collected (6,000 rpm, at 4°C for 15 minutes). The pellet was resuspended in 5 or 10 ml of 25 mM sodium phosphate buffer (pH 7.0). This suspension was disrupted in small volumes by 2 cycles of ultrasonication with constant cooling (6-8 μm, 30 seconds, MSE Soniprep 150, MSE Instruments, Crawley). Cell debris was removed by centrifugation at 16,000 rpm for 40-60 minutes at 4°C (Sorvall). The supernatant, crude enzyme preparation, was stored at -20°C until required.

For standard beta-lactamase preparations either 100 ml or one litre cultures were used for the enzyme extraction. Hundred millilitre cultures were harvested and washed

and finally resuspended in 1 or 2mls of phosphate buffer in order to get the required concentration. They were stored at -20°C in small aliquots for sequential use in order to avoid the protein loss.

2.5.14B2. Extraction of Crude Beta-lactamases on Small Scale

Beta-lactamases in small amounts were extracted by the method described by Livermore, Maskell and Williams (1984). Either 2 or 5ml nutrient agar slopes were inoculated with the appropriate beta-lactamase producing strain and incubated for 18 hours at 37°C. Cells from the surface of the slopes were removed by addition of 1ml of sodium phosphate buffer (25mM, pH7.0) and thoroughly resuspended. The cells were then sonicated (6-8μm, 15 seconds x 2, Soniprep). The lysate was cleared by centrifugation (MSE Microcentaur centrifuge, 11,500g 20 minutes, 4°C) and the supernatant was stored at -20°C until required.

Small amounts of beta-lactamases were also prepared from 10mls of nutrient broth by harvesting (4,500rpm, 20 minutes) the cells and washing them in the phosphate buffer. This was followed by collecting the pellet and resuspending it in the same buffer ready for sonication (6-8μm, 15 seconds, 2 cycles Soniprep). The sonicated culture was centrifuged (11,500g, 20 minutes, 4°C) and cleared supernatant was collected and stored at -20°C.

2.5.14C Induction Studies on Beta-lactamase Production

Induction studies were carried out on the cefuroxime resistant *Salmonellae*.

Nutrient broth (4.5ml) was inoculated with a single colony from a fresh MacConkey plate and incubated overnight at 37°C. One hundred mls of pre-warmed nutrient broth was

inoculated with 1 ml of this overnight culture and shaken for 2 hours at 37°C. Cefuroxime was then added to the growing culture at one-quarter of the MIC and incubation was continued for a further 2-3 hours. At the end of the incubation period, the beta-lactamase was extracted as previously described for large-scale preparation of crude enzyme extracts. These enzyme extracts were measured for their protein concentration and specific activities. These were compared with the uninduced control strain.

2.5.14D Assessment of Beta-lactamase Activity of Crude Enzyme Preparations

The presence of beta-lactamase activity was indicated by a colour change of nitrocephin and the length of time this took (Simpson, Harper and O'Callaghan, 1980). This assessment was performed by mixing crude enzyme samples (30 μl) to 100 μl of nitrocephin solution (50 mg/l) in a microtitre tray (Cooke Microtitre System, sterline). Time taken for reaction was noted for each enzyme preparation. The reaction time (in seconds) was converted into amount (in μl) of the respective enzyme to be loaded on the gel. Previous experiments in this laboratory have shown this procedure delivers the correct quantity of the enzyme onto the gel (Reid, 1986).

2.5.14E Concentration of Beta-lactamases

One litre of overnight culture (nutrient broth) was harvested (6,000 rpm, 15 minutes, 4°C) and the cells were resuspended in sodium phosphate buffer (25 mM, pH 7.0) and washed at 6,000 rpm for 10 minutes at 4°C. The pellet was resuspended in 5 ml of the same buffer and sonicated for 2 cycles (6-8 μm, 30 seconds). The sonicated extract was separated by high-speed centrifugation (18,000 rpm, 60 minutes, 4°C).

The volume of the supernatant was measured and its specific activity was estimated (specific activity = enzyme activity/protein concentration). Then ammonium sulphate

was slowly added to 50% saturation (i.e 29.1g ammonium sulphate for 100ml of enzyme preparation) and left to precipitate by constant stirring for 30 minutes. This mixture was centrifuged (6,000 rpm, 30 minutes, 4°C) to collect the pellet. The pellet was resuspended in 0.8 ml of sodium phosphate buffer (25mM, pH 7.0). The protein concentration of this suspension was measured and its specific activity also estimated. This concentrated enzyme preparation was used for further assays.

2.5.14F Analytical Isoelectric Focusing

Isoelectric focusing of beta-lactamases was carried out according to the method described by Matthew et al. (1975). The crude beta-lactamase extracts were focused on thin layer polyacrylamide gels containing carrier ampholines supported by a glass plate. Details of the preparation of the IEF gels are given in Matthew et al. (1975). The composition of the polyacrylamide gel mixture is shown in table 10.

2.5.14F1 Application of the Beta-lactamase Samples on the IEF Gels

The amount of beta-lactamase loaded on the IEF gel was equalised with regard to the enzyme activity (see section 2.5.14D). Samples of the beta-lactamase enzymes (maximum of 100µls) were applied on the surface of the gel near the anode. Isoelectric focusing was carried out at 1 watt (maximum voltage 500V) for around 18 hours (LKB 10-2000V Power Pack). At the end of each run, the pH of the gel was read with a miniature flat-ended combined glass electrode (Pye-Unicam 403, 30M8E07) at 0.5 or 1.0cm intervals from the cathode to the anode. After pH measurements, the gel was stained for beta-lactamase activity and photographed.

Table 10

Composition of Polyacrylamide Gel for Isoelectric Focusing (IEF)

Material	Supplier	Volume used	Final concentration
5% tetramethyl-ethylenediamine (TEMED) in distilled water	Sigma	0.2 ml	0.25 mg/L
40% ampholines w/v (various pH ranges)	LKB	2.0 ml	2% w/v
Acrylamide (100 g) plus methylene bisacrylamide (2.7 g) in water (300 mls)	BDH	9.0 ml	acrylamide 70 g/L biacrylamide 2 g/L
Distilled Water		25.0 ml	
Riboflavin (20 mg/L)	Sigma	4.0 ml	2 mg/L

2.5.14F2 Visualisation of Beta-lactamases on IEF Gel with Nitrocephin Stain

With the aid of nitrocephin, the chromogenic cephalosporin, beta-lactamase activity was detected on the gels. The IEF gel was over-laid with a sheet of Whatman No. 54 paper which had been dipped in a solution of nitrocephin (500mg/l). Care was taken to avoid allowing any air bubbles to be trapped between the gel and the staining paper. The focused bands of beta-lactamase activity appear red on a yellow background.

2.5.14F3 Photography of Analytical IEF Gels

The gradual revelation of the beta-lactamase bands occurred on the focused gel after treatment with the nitrocephin; however, by the time the weakest bands were visible, the strongest bands had diffused. In order to obtain a complete record, a series of photographs were taken. The gel was photographed with a polaroid camera with transmitted light and a Wratten 58 green filter (f8 and shutter speed 1/8 of second).

2.5.14F4 Beta-lactamase Identification on the Analytical IEF Gels

A maximum of 12 beta-lactamase enzyme samples could be loaded on each gel plate. A primary identification of all test enzyme extracts was performed on polyacrylamide gel containing a broad range ampholine (3.5-10.0) and compared with the standard beta-lactamases (TEM-1, TEM-2 and SHV-1 or OXA-2) of different isoelectric points (pIs). Subsequently, both the beta-lactamases of interest and the ambiguous or indistinct enzymes were checked for identity by running side-by-side with the suitable standard enzymes (table 6) of similar pIs. This was usually

performed on gels containing an appropriate mixtures of ampholines to provide a narrower pH range than had been used previously in order to increase sensitivity.

2.5.14G Assays of Beta-lactamase Activity

2.5.14G1. Hydroxylamine Assay System

The hydroxylamine assay system was as described by Smith (1976). This method was followed during this thesis for the substrates like oxacillin and methicillin without any modification.

2.5.14G2 Spectrophotometric Assay of Beta-lactamase Activity

Beta-lactam compounds generally give characteristic absorption spectra in the ultra-violet (UV) region of the spectrum. A detectable shift in the ultraviolet absorption spectrum of penicillins and cephalosporins occurs upon hydrolysis of the cyclic bond in the beta-lactam ring. The wavelength at which maximum decrease in absorption occurs following hydrolysis is termed λ_{max} (lambda maximum). This is determined by comparing intact and fully hydrolysed substrate. The rate of substrate hydrolysis is assessed by measuring the rate of decrease in optical density (OD) at lambda maximum.

The basic method employed in this thesis, for spectrophotometric assays was that of O'Callaghan, Muggleton and Ross (1968). During this project the assays were performed on a Perkin-Elmer Lamda 2 uv/vis spectrophotometer which had a thermostatically controlled cell carrier with a chart recorder.

Different molar concentrations of penicillin and cephalosporins were used in the

assays. Penicillin substrates were prepared at 10^{-2}M and cephalosporin substrates were made up at 10^{-3}M in 25mM phosphate buffer (pH 7.0). Test cuvettes (containing 0.3ml substrate and 2.6mls sodium phosphate buffer (25mM, pH 7.0)) and blank cuvettes (containing 2.9mls buffer) were prepared and equilibrated at 37°C. Beta-lactamase enzyme preparation (0.1ml) was then added to each cuvette and monitoring of the decrease in OD at λ_{max}(lambda) was begun immediately. The decrease in the optical density in the initial linear part of the reaction curve was taken to obtain a value for change in OD per minute.

calculation: $$R = \frac{\Delta OD \times N \times \text{enzyme dilution}}{OD_1 \times \text{time}}$$

where
R = µmoles of substrate hydrolysed minute^{-1}ml^{-1} of enzyme
ΔOD = change in optical density
N = µmoles substrate in cuvette (0.3 for cephalosporins, 3.0 for penicillins)
OD_1 = optical density of intact substrate

Protein concentrations of crude beta-lactamase enzymes were estimated as described in a further section and the specific activities of enzymes were expressed per mg protein.

2.5.14G3 Estimation of the Protein Concentration

Protein estimations of beta-lactamase enzymes and outer membrane proteins were carried out by diluting the samples 1 in 1000 in sodium phosphate buffer by the method described by Waddell (1956). The protein concentrations (mg/ml in the original preparation) of the diluted samples were read at two different wavelengths (215 and 225nm) and calculated as follows: (OD at 215nm - OD at 225nm) x correction factor. This method was fully described by Hesslewood (1973).

It was found to be a more accurate and sensitive method than that the more widespread method of Lowry et al (1951).

2.5.14G4. Specific Activities of Beta-lactamases

Specific activities of the required enzymes were measured as follows:

$$\text{Specific activity} = \frac{\text{Enzymic activity against nitrocephin}}{\text{protein concentration}}$$

Total enzymic activity = Enzymic activity x total volume of enzyme.

2.5.14G5 Michaelis Menton Kinetics Determination

The K_m and V_{max} values were obtained by measuring the rate of hydrolysis at limiting substrate concentrations and plotting the reciprocal of the substrate concentration against the reciprocal of the rate by the Lineweaver-Burk method (1934). The intercept on the abscissa gives the negative of the reciprocal of the Michaelis constant (K_m), the K_m value being the substrate concentration at which half the maximal velocity occurs (Dixon and Webb, 1958).

The V_{max} values were normalised with respect to benzyl penicillin, i.e.:

$$\text{Relative } V_{max} = \frac{(V_{max} \text{ per } \mu l \text{ of enzyme) of substrate}}{(V_{max} \text{ per } \mu l \text{ of enzyme) of benzyl penicillin}} \times 100$$

2.5.14G6 Inhibition Studies on Beta-lactamase Activities

The effect of beta-lactamase inhibitors on the enzymic activity was examined by spectrophotometric assay system as described above (O'Callaghan, Muggleton and Ross, 1969). Inhibition profiles of the enzymes were carried out with nitrocephin (0.1mM) as substrate. The potential inhibitor was added at the start of the assay to both the test and blank cuvettes at various concentrations ranging from 10mM to 100nM. The effect of the inhibitors was expressed as the concentration required to inhibit the 50% activity of the beta-lactamase. The value of the inhibitor concentration was termed as ID_{50}.

The inhibitor constants (K_i) of the enzyme were calculated by repeating the assays in limiting substrate concentrations in the presence of required enzyme inhibitor (clavulanic acid). Again double reciprocal plots of $1/S^*$ against $1/V^*$ were plotted. Clavulanic acid inhibition of beta-lactamases is competitive and the reciprocal plot intercepts the ordinate at the same point as the K_m line. However, the plots in the presence of inhibitor intercept the abscissa near the origin than the lines obtained without inhibition. If the distance to the origin from the intercept is -1/Kp then:

$$K_i = \frac{i}{K_p/K_m - 1}$$

Where "i" is the concentration of the inhibitor (Dixon and Webb, 1958).

2.5.14H Molecular Weight Determination of Beta-lactamase Enzymes

The method followed was described by Andrews (1964). The proteins ovalbumin, α-chymotrypsinogen and cytochrome C (all Sigma) were all dissolved at 12mg/ml in sterile distilled water and 1ml was applied to the Sephadex G-75 column (section, 2.5.14H1). The position of the peaks of the protein markers was established by

* 1/S represents the reciprocal of the substrate concentration and 1/V respresents the reciprocal of the velocity of the reaction

Table 14

Sensitivity Testing of 284 Isolates of *Enterobacteriaceae* to Cephalosporins Not Shown in Table 1

Beta-lactam drug	Antibiotic concentration (mg/L)	Number of resistant strains					
		E. coli (n=138)	*Klebsiella* spp. (n=75)	*Proteus* spp. (n=42)	*Enterobacter* spp. (n=16)	Others (n=13)	Total (n=284)
Cephradine[a]	<8	34	25	7	1	5	72
	>8	95	38	18	8	5	164
	>100	9	12	17	7	3	48
Cefazolin[a]	<8	104	48	21	2	6	181
	>8	28	12	7	6	4	57
	>100	6	15	14	8	3	46
Cefamandole[b]	<8	106	52	34	12	9	213
	>8	32	23	8	4	4	71
Cefoxitin[b]	<8	125	52	34	4	9	224
	>8	13	23	8	12	4	60
Cefotetan[b]	<8	131	63	34	12	12	252
	>8	7	12	8	4	1	32
Ceftriaxone[c]	<4	133	69	42	15	13	272
	>4	1	4	0	1	0	6
	>32	4	2	0	0	0	6

[a] First generation Cephalosporin; [b] Second generation Cephalosporin; [c] third generation Cephalosporin

studied in further detail in order to investigate the resistance mechanisms involved.

3.2 Beta-lactam Resistance in *E.coli* Population

A significant proportion (77%) of the *E.coli* were ampicillin resistant and over half of them were highly resistant to the drug (table 13). The incidence of cephaloridine resistance was also high (57%) amongst these strains, although none of them had high MICs to cephaloridine (>1280 mg/l) (table 13). In addition, five *E.coli* isolates were able to resist all the beta-lactam antibiotics tested in this survey.

Even though the combination of ampicillin and clavulanic acid showed a dramatic effect on the MICs of ampicillin (table 13), some *E.coli* strains (36) were moderately resistant (MIC 40-160mg/l). Only one strain possessed increased resistance to ampicillin (MIC 60mg/l) when the inhibitor was present (MIC for ampicillin, 160mg/l). This might result either from reduced permeability of the bacterial cell wall or, more likely, antagonism between the beta-lactam and the beta-lactamase inhibitor in that particular organism.

The ampicillin-resistant *E.coli* were examined further for the transferability of the resistance genes.

3.2.1 Self-transferable and Mobilisable Beta-lactam Resistance in *E.coli*

Each resistant strain was tested for its ability to transfer the resistance determinant. It was mixed in nutrient broth with an *E.coli* J62.2 recipient as described in the Materials and Methods. The conjugation results showed that about 53% (56/106) of the ampicillin-resistant clinical isolates of *E. coli* possessed auto-transferable resistance

plasmids carrying beta-lactam resistance genes. The results showed that 21.7% (23/106) of the strains transferred cephaloridine resistance. Ampicillin and cephaloridine determinants were independently transferred by 14 isolates. For further analysis there was a total of 28 transconjugants (14 ampicillin and 14 cephaloridine resistant transconjugants) (table 15). Thirty-three clinical isolates possessed self-transferable resistance to ampicillin, but not to cephaloridine. Similarly, there were nine transconjugants selected on cephaloridine which were not resistant to ampicillin (10mg/l). Therefore, 70 transconjugants were taken for further study. Most of the ampicillin resistant transconjugants were moderately resistant to ampicillin (MIC <1280 mg/l), although three transconjugants strains exhibited high level resistance (MIC >1280 mg/l).

The remaining *E.coli* clinical isolates which did not appear to have self-transferable resistance genes were retested. Overnight broth cultures of clinical strains and *E.coli* K-12 (X^+) were mixed in a ratio of 1 to 10 and incubated for 24 hours for conjugation. Following the incubation, 1ml of this mixture was mixed with 1ml of the recipient *E.coli* J62.2 for further incubation.

A further 15 *E.coli* isolates showed positive mobilisation of resistance genes in the presence of the transfer factor X^+. Eight isolates transferred resistances to both ampicillin and cephaloridine and six were found to have mobilisable resistance only to ampicillin. A single isolate was able to transfer a cephaloridine R-plasmid into the standard recipient strain, without the co-transfer of ampicillin resistance (table 15).

Attempts made either to transfer or to mobilise the resistances to the second (cefuroxime) and third generation (ceftazidime and cefotaxime) cephalosporins from those *E.coli* strains which were resistant to them failed. However, these strains

Table 15

Transfer of Ampicillin and Cephaloridine Resistances in E. coli Isolates (138)

Total number of ampicillin resistant strains = 106/138

Number of Donor Strains	Selecting antibiotic		Number of Transconjugants
	Amp[a]	Cer[b]	
Conjugation results			
14	+	+	28
33	+	-	33
9	-	+	9
		Total transconjugants	70
Mobilisation results			
8	+	+	16
6	+	-	6
1	-	+	1
		Total transconjugants	23

[a] Amp, Ampicillin (10 mg/L)

[b] Cer, Cephaloridine (10 mg/L)

This experiment was performed on two separate occasions and the result shown is representative of that found on each occasion.

transferred ampicillin resistance. This implied that the resistances to the later generation cephalosporins are probably chromosomally-mediated.

3.2.2 Characterisation of R-plasmids in the *E.coli* Transconjugants

Each transconjugant from *E.coli* was examined for its plasmid content and the plasmids were characterised by their size and resistance profile. Plasmid DNA was extracted from each transconjugant and analysed by 0.5% agarose gel electrophoresis along with the standard plasmids as described by Takahashi and Nagano (1984) and the plasmid sizes were estimated from the standard curve (Materials and Methods). The proportion of beta-lactam-resistant *E.coli* carrying R-plasmids had been high (67%). These results revealed that a large number of different plasmid types (37) were present in the *E.coli* population as no plasmid was found on more than five occasions. From this it can be deduced that very few plasmid types were responsible for direct cross-infection in the population. The different plasmids are shown in table 16. If the same plasmid was found in the recipient after separate selection with ampicillin and cephaloridine, it was only counted once in the table 16. There were a further 18 different plasmids identified among the mobilisable R-plasmids (table 17).

The sizes of plasmids in most of the transconjugants were large, a few of them were greater than 100 kb and some were greater than 150kb. On 13 occasions (8 for the auto-transferable plasmids and 5 for the mobilisable plasmids) no plasmid DNA could be detected, which probably reflects their large size. Most of the plasmids of these transconjugants were shown to have resistance genes to more than five non-beta-lactam antibiotics and almost all of them were resistant to aminoglycosides.

Table 16

Characteristics of R-plasmids from Isolates of *E. coli*

No.	Resistance pattern	Size (kb)
Self-transmissible (Obtained from 56 isolates)		
5	Ap Ce Cm Km Sm Sx Tc Tp	53.0
5	Ap Ce Cm Sm Sx Tc Tp	52.8
5	Ap Sx	-
3	Ap Ce Cm Gm Km Sm Sp Sx Tc Tp	93.7
3	Ap Ce Cm Km Sm Sx Tc Tp	90.0
3	Ap Ce Km Sm Sx Tp	94.7
2	Ap Ce Cm Gm Km Sm Sp Sx Tp	69.6
2	Ap Ce Gm Km Sm Sx Tp	81.5
1	Ap Ce Cm Gm Km Sm Sp Sx Tc Tp	123.3
1	Ap Ce Gm Km Sm Sp Sx Tc Tp	-
1	Ap Ce Cm Gm Km Sm Sp Sx Tp	-
1	Ap Ce Cm Gm Km Sm Sp Sx Tc	50.3
1	Ap Ce Cm Gm Km Sm Sp Sx Tp	45.3
1	Ap Ce Cm Km Sm Sp Sx Tc Tp	18.9
1	Ap Ce Cm Gm Km Sm Sx Tc Tp	62.0
1	Ap Ce Cm Gm Km Sm Sp Sx Tp	91.5
1	Ap Ce Cm Km Sm Sp Sx Tc	51.4
1	Ap Ce Gm Km Sm Sp Tc	49.1
1	Ap Ce Km Sm Sx Tc Tp	232.0
1	Ap Ce Cm Sm Sx Tc Tp	76.1
1	Ap Ce Cm Sm Sx Tc Tp	112.5
1	Ap Ce Cm Km Sm Sx Tc	89.5
1	Ap Ce Cm Km Sm Sx Tc	43.8
1	Ap Ce Cm Km Sm Sx Tc	104.9
1	Ap Ce Cm Sm Sx Tp	55.1
1	Ap Ce Km Sm Sx Tp	72.7
1	Ap Ce Cm Sx Tc	51.4
1	Ap Ce Cm Sx Tp	268.3
1	Ap Ce Cm Sx Tp	52.2
1	Ap Gm Sm Sx Tp	76.7
1	Ap Ce Sx Tc	109.9
1	Ap Ce Sx Tp	-
1	Ap Ce Sx Tp	56.4
1	Ap Ce Sm Sx	89.8
1	Ap Sm Sx Tp	72.6
1	Ap Ce Km Sx	74.4
1	Ap Ce Sx	83.4
1	Ap Ce Sx	22.5

This result was confirmed on two separate occasions.

Table 17

Characteristics of R-plasmids Mobilised from Isolates of E. coli

Size	Resistance pattern	size (kb)
Mobilised from 15 isolates		
4	Ap Ce Cm Gm Km Sm Sp Sx Tc Tp	38.3
2	Ap Ce Cm Gm Km Sm Sp Sx Tc Tp	81.75
2	Ap Ce Sp Sx Tc	-
1	Ap Ce Cm Gm Km Sm Sp Sx Tc Tp	57.6
1	Ap Ce Cm Km Sm Sp Sx Tc Tp	33.0
1	Ap Ce Cm Gm Km Sm Sp Sx Tc	91.5
1	Ap Ce Gm Km Sm Sp Sx Tc Tp	78.0
1	Ap Ce Gm Km Sm Sp Sx Tc	73.5
1	Ap Ce Cm Gm Km Sm Sx Tp	33.6
1	Ap Ce Cm Km Sm Sp Sx Tc	97.5
1	Ap Ce Gm Km Sp Sx Tc Tp	82.5
1	Ap Ce Cm Sp Sx Tc Tp	103.5
1	Ap Ce Sm Sx Tc	27.0
1	Ap Ce Sp Sx Tc	82.5
1	Ap Ce Sm Sx Tc	-
1	Ap Ce Sm Sp Sx	-
1	Ap Ce Sp Sx	-
1	Ap Ce Sx Tc	61.5
Total transconjugants obtained - 23		

This result was confirmed on two separate occasions.

3.2.3 Non-beta-lactam Resistance among the Urinary Pathogens and the Transconjugants of *E.coli*

Almost every ampicillin-resistant clinical isolate (214/230: 93%) and virtually every transconjugant (92/93: 99%) of the *E.coli* isolates were sulphamethoxazole resistant (table 18). Most sulphamethoxazole resistant strains were also resistant to trimethoprim, as this drug has been exclusively co-administered with sulphamethoxazole in India for the treatment of urinary tract infections. The next most common resistance determinant associated with ampicillin resistance was streptomycin which might result from the treatment of tuberculosis or leprosy infections, as these infections are common in most areas of India amongst the poorer populations. The resistance genes for gentamicin and spectinomycin were associated with ampicillin resistance genes in less than 50% (80/230, gentamicin and 83/230, spectinomycin) of the clinical isolates; 54% (50/93) of the R-plasmids from *E.coli* were spectinomycin resistant; and less than 50% (32/93) of the R-plasmids were gentamicin resistant.

3.2.4 Distribution of Beta-lactamases in Ampicillin-Resistant *E.coli* and in their Transconjugants

3.2.4A Beta-lactamases in *E.coli* Transconjugants

The beta-lactamases produced by the transconjugants of *E.coli* obtained from both mating and mobilisation experiments were identified and categorised by isoelectric focusing of crude enzyme extracts by comparison of their migration against standard beta-lactamases. The vast majority of transconjugant strains (71.9%) encoded the TEM-1 beta-lactamase in spite of the diversity of plasmid types on which the gene

Table 18

Types and Proportion of Non-beta-lactamase Resistances among Ampicillin Resistant Urinary Pathogens Including Clinical Strains of E. coli, and Their Transconjugants

Antibiotics	Percentages of resistant strains		
	Urinary Pathogens	E. coli	Transconjugants of E. coli
Ap	100	100	100
Ce	80	75	91
Cm	77	77	66
Gm	35	21	34
Km	43.5	53	64.5
Sm	87	96	78.5
Sp	36	18	54
Sx	93	92	99
Tc	85	97	73
Tp	77	78	68
Tp + Sx	76	76	66.7

Total number of urinary pathogens tested (including E. coli), 230; total number of E. coli clinical strains tested, 106; total number of transconjugants of E. coli tested, 93; (transfer and mobilisation positive).

This result represents the average of two separate determinations

was carried. Amongst the remaining types of beta-lactamases identified, five previously well characterised enzymes were also represented, although in far fewer strains. These beta-lactamases were TEM-2, OXA-1, OXA-2, SHV-1 and PSE-1 beta-lactamases (table 19). In a few strains two or more enzymes were encoded. In addition, TEM-1 like enzymes and also a novel enzyme with a high pI were observed in the transconjugants and their discoveries are discussed in the sections 3.2.4A1, 3.2.4A2 and 3.2.4A3.

The transconjugants obtained by mobilisation had plasmids encoding only two types of beta-lactamases, TEM-1 and OXA-1. Once again, the most prevalent beta-lactamase was TEM-1 as it was produced by 20 of a total of 23 transconjugants (table 19). OXA-1 was found in only 3 of the 23 transconjugants.

3.2.4A1 Hyperproduction of TEM-1 Beta-lactamase

The specific activity range of TEM-1 in all but three of the strains was between 1 and 35μM. However, in three *E.coli* transconjugants, the TEM-1 beta-lactamases were hyperproduced (TEM-1^{++}). Their hyperproduction was revealed by measuring their specific activities which were much higher than that for the normal TEM-1 enzyme. In strain 1 the specific activity was 102.2μM, in strain 2 the specific activity was 271.4μM and in strain 3 it was 191.7μM. All three of these specific activities were at least 5-10 fold more than the average TEM-1 enzyme expression of a strain containing plasmid R1. The expression of these beta-lactamases on the polyacrylamide gel was so prominent that they were considerably darker with more satellite bands (fig 1). These hyperproduced enzymes were determined by large plasmids, all greater than 100kb. The biochemical properties of these enzymes were the same as those of the

measuring the optical density of the fractions at 278nm by the method of Waddell (1956). Beta-lactamase enzyme samples were then applied to the calibrated column and the molecular sizesof the proteins were determined from the standard curve (i.e. log molecular weight verses fraction Nos of the peaks of the protein markers).

2.5.14H1 Preparation of Sephadex G-75 Column: Gel Filtration

Sephadex G-75 (Pharmacia, Uppsala, Sweden) was swollen in a hot water bath at 100^{o}C for 3 hours in sodium phosphate buffer (25mM, pH 7.0). The concentration of the Sephadex was about 84mg/ml and a total amount of 300ml of the suspension was made up. The slurry was allowed to cool and then poured carefully into an LKB (Bromma, Sweden) gel filtration column (2cm^{2} x 90) avoiding any air bubbles getting into the column. When the column was full, the top was connected and flow started in a downward direction with a peristaltic pump. The flow rate was adjusted to between 8 and 12mls per hour and the column was washed with sodium phosphate buffer (25mM, pH 7.0) for 48 hours.

2.5.14H2 Separation by Sephadex Gel Filtration

The concentrated bacterial sonicates were used for separation. These samples (1ml) were applied slowly through the sample applicator (LKB) and eluted with phosphate buffer. Fractions were collected by an LKB ultrorac fraction collector. Elution was continued (overnight) until a volume of buffer equivalent to the total column volume had passed through (180mls). The fractions containing the beta-lactamase activity were determined first by testing samples from each fraction with a nitrocephin solution (50mg/l). The peak of activity was quantified by assaying at fractions shown to possess beta-lactamase activity. The fraction containing the peak

activity was compared on the standard curve to establish the molecular weight of the particular sample (section, 2.5.14H)

The column was washed with sodium phosphate buffer for about 12 hours between each run. In addition, column was kept clean from any contamination by adding sodium azide as a preservative in the running buffer when it was not in use.

2.5.15 Isolation of Outer Membrane Proteins

One hundred mls of an overnight nutrient broth culture, grown with aeration, was harvested (6,000g, 10minutes, Sorvall) to obtain cells from which outer membrane proteins (OMPs) could be prepared. The cells were washed once in sodium phosphate buffer (25mM, pH 7.0) and the pellet was resuspended in 10mls of the same buffer. The cells were disrupted by sonication (8μm, 2 minutes, soniprep) with constant cooling. This was followed by centrifugation (2,500g, 10 minutes, 4°C, Sorvall) to remove large debris from the lysate. The supernatant containing the OMPs was decanted and n-lauryl sarcosine (Sigma) was added to give a final concentration of 1-2% w/v. This was incubated at room temperature for 30 minutes. Sarcosyl-insoluble OMPs were sedimented by centrifugation (100,000g, 60 minutes, 4°C) (Sorvall,Ultra-Centrifuge (Du Pont) OTD 65B). The clear pellet was then resuspended in 0.5 or 1.0ml of sodium phosphate buffer (25mM, pH 7.0) and the protein concentration was measured. The protein concentration was adjusted (minimum 20-30ng/μl, loaded up to 2μg/μl) according to the specifications given by the manufacturers of the Phast System (The Phast System, Pharmcia, LKB Biotechnology). The samples were subjected to the Phast System mini SDS-polyacrylamide (discontinuous buffer system) gel electrophoresis. Molecular weight markers (Pharmacia, LKB, 17-0446-01) were included on each gel (range 14,400 to

94,000), provided by Electrophoresis Calibration Kit.

2.5.15A The Phast Gel Electrophoresis (SDS-Polyacrylamide) of Outer Membrane Proteins

The Phast System Gradient gels (8-25 i.e. 6,000-300,000) were used to separate the outer membrane proteins, in discontinuous buffer system. The composition of ready made gradient gels and buffer (SDS) strips were as follows: The gels had continuous 8 to 25% gradient zone with 2% crosslinking. The gels were approximately 0.45 mm thick and the buffer system in the gels was 0.112M acetate (leading ion) and 0.112M Tris, pH 6.4. The gradient gels enhance the band sharpness of the proteins.

The buffer system in Phast Gel SDS Buffer Strips was 0.20M tricine (trailing ion), 0.20M and 0.55% SDS (analytical grade), pH 8.1. The buffer strips were made of 2% agarose IEF.

The molecular weight marker proteins provided by the Pharmacia MW Calibration Kit (17-0446-01), were within the range of 14,400-94,000.

2.5.15B Sample Treatment

The sample treatment followed the procedure given by the Phast System manufacturers. To the suspension of outer membrane proteins in sodium phosphate buffer (25mM, pH 7.0) was added sample buffer (final concentrations of 10mM Tris/Hcl, 1mM EDTA, pH 8.0), SDS to 2.5% and beta-mercaptoethanol to 5.0%. This mixture was boiled for 5 minutes. Bromophenol blue (0.1%, 2.2µl) (loading buffer) was then added to the treated samples at the final

concentration of 0.01%. Protein markers were directly suspended in the sample buffer, SDS and beta-mercaptoethanol solution according to the instructions of the manufacturers and treated in the same way as the samples.

2.5.15C Loading the Samples and Running the Gels

The gradient (8-25) gel was placed gently on the flat surface of the Phast System Separation Unit. Then the sample applicator with the samples (2µl or 4µl) was placed on the surface of the gel. The running of the gel (samples) was immediately started according to the running conditions which were devised following the instructions given by the manufactures and preprogrammed by myself (table 11). The samples were then applied to the gel automatically. The time taken for the separation of the protein samples and markers was about 30-40 minutes.

Table 11

The Gel Running Conditions

Sample application. Down at	1Vh				
Sample application. Up at	10Vh				
Separation (1st Step)	250V	10.0mM	30W	15°C	65Vh1
Separation (2nd Step)	50V	0.1mM	0.5W	15°C	0Vh2

2.5.15D Developing the Phast System Gel

The gradient gel (8-25) was stained and destained by Coomassie blue according to the instructions in the Development Unit of the Phast System. The development

procedure took about 40 minutes. The solutions used were listed in table 12 below. The stained gels were photographed and could also be preserved by using the preserving solution.

Table 12

Solutions Used for Developing the Phast Gels

(A) Stain	:	0.1% Phast Gel Blue R solution or 0.1% Coomassie* blue solution in 30% methanol and 10% acetic acid in distilled water (100ml)
(B) Destain	:	30% and 10% acetic acid in distilled water (3:1:6) (100ml)
(C) Preserving solution	:	10% glycerol and acetic acid in water (100ml)

The patterns of the OMPs were compared with the standard sample and molecular mass of the proteins were estimated from the calibrated curve of the protein markers used.

* Coomassie blue, rather than silver stain, was used to stain the gel because it is specific for proteins and does not detect the other components of the cell surface.

CHAPTER 3

RESULTS

3.1 Survey of Beta-lactam Resistant Urinary Pathogens Isolated in South India

3.1.1 Introduction

There have been very few controlled studies on the incidence of beta-lactam resistance among enterobacterial strains isolated in tropical countries. In 1984, an intensive field study for bacterial resistance in hospital isolates was undertaken in South India. Urinary pathogens (284) responsible for significant bacteriuria were collected and catalogued at the Christian Medical College Hospital at Vellore. These 284 clinical enterobacterial isolates were originally tested for their trimethoprim resistance (minimum inhibitory concentrations >10 mg/l) which was found to be as high as 64% (Young et al., 1986).

These Indian pathogens had been widely exposed to common penicillins such as ampicillin and amoxycillin but had hardly been challenged by cephalosporins. The same Indian isolates (284) have now been further investigated for their beta-lactam resistance employing ampicillin and cephaloridine as resistance markers (break point of 10 mg/l). The results showed that the proportion of ampicillin resistant strains was 230/284 and the the proportion of cephaloridine resistance was 185/284 (table 13).

There was a very high incidence of ampicillin resistance (80.9%) and cephaloridine resistance (65.1%). *E.coli* accounted for 48.6% of the strains of Enterobacteriaceae studied. The remaining strains in this study were mainly *Klebsiella* (26.4%) and *Proteus* (14.7%) species. A minor proportion of *Enterobacter*

Table 13

Susceptibility of 284 Isolates of *Enterobacteriaceae* to Beta-lactam Antibiotics

B-lactam Drug	MIC Range (mg/l)	Number of resistant isolates					
		E. coli (n=138)	*Klebsiella* spp. (n = 75)	*Proteus* spp. (n = 42)	*Enterobacter* spp. (n = 16)	Other (n = 13)	Total (n = 284)
Ampicillin	≤10	32	3	12	2	5	54
	>10-1280	52	29	18	9	1	109
	>1280	54	43	12	5	7	121
Ampicillin & Clavulanic acid[a]	≤10	88	54	19	2	5	168
	>10-1280	50	17	22	14	8	111
	>1280	0	4	1	0	0	5
Cephaloridine	≤10	59	23	12	1	4	99
	>10-1280	79	44	30	12	9	174
	>1280	0	8	0	3	0	11
Cefuroxime	≤8	123	58	30	12	11	234
	>8	15	17	12	4	2	50
Ceftazidime	≤4	133	70	40	16	12	271
	>4	5	5	2	0	1	13
Cefotaxime	≤8	133	68	40	15	13	269
	>8	5	7	2	1	0	15
Aztreonam	≤4	132	70	40	16	11	269
	>4	6	5	2	0	2	15

[a] MICs of ampicillin in the presence of subinhibitory concentrations of clavulanic acid (8 mg/l).

spp (5.6%) and other species were also included in this study.

3.1.2 Minimum Inhibitory Concentrations of Ampicillin and Cephaloridine for Urinary Pathogens

The ampicillin-resistant urinary pathogens were retested for their MICs of ampicillin at a series of doubling concentrations from 10mg/l upwards (10-1280 mg/l). There was a very high incidence of high-level ampicillin resistance (MIC >1280mg/l) (121/230 strains: 52.6%) (table 13). High level ampicillin resistance was found in 42.6% of all the enterobacterial strains (284) tested . The large proportion of highly-resistant strains was not a result of particular bacterial species, which are inherently resistant to ampicillin. The proportion of highly resistant strains among individual species (*E.coli* (39%), *Klebsiella* spp (57%) and *Proteus* spp (29%)) showed that there was no predominance in any one species of highly resistant strains. For cephaloridine, the break-points showed that a significant percentage of the population (185 strains, 65%) were resistant, but the MIC results showed that virtually none of them were able to resist the drug at the high concentration level (1280 mg/l) (table 13). A few *Klebsiella* (8 strains) and *Enterobacter* (3 strains) spp were highly resistant to cephaloridine (table 13).

On the other hand, there was a widespread resistance (250/284: 88%) to piperacillin (8mg/l) and half (126/250; 50%) were resistant to the higher concentration (32 mg/l) of the drug.

3.1.3 Effect of Clavulanic Acid in Association with Ampicillin

Clavulanic acid is a potent inhibitor of many of the plasmid-mediated beta-

lactamases of Gram-negative bacteria, especially broad-spectrum penicillinases. When all the ampicillin resistant strains were tested against ampicillin in the presence of a subinhibitory concentration of clavulanic acid (8 mg/1), the MICs of ampicillin were greatly reduced in over 95% of the highly resistant bacteria (Table 13). This indicated the involvement of beta-lactamases in the resistance mechanisms of these strains. Some bacteria remained highly resistant to the drug combination (Table 13). This was exemplified particularly by certain strains amongst the Klebsiella and Proteus spp.

3.1.4 Sensitivity Testing to Cephalosporins

Susceptibility tests to the first generation (cephradine and cefazolin) and second generation (cephamandole, cefoxitin) cephalosporins showed that about 25% of the strains were resistant to each drug, except for cephradine where 50% were resistant (Table 14). The majority of the population tested had increased susceptibility to two other second generation cephalosporins (cefuroxime and cefotetan) as only 18% an 3.5% were able to grow in the presence of each drug respectively, at the concentrations tested (8 mg/1 for cefuroxime and cefotetan) (Tables 13 and 14 respectively).

Only a few strains were able to resist the third generation cephalosporins ceftazidime (13 strains), cefotaxime (15 strains) and the monobactam aztreonam (15 strains) (Table 13). The proportions of the beta-lactam resistant strains amongst each individual species were varied irrespective of their inherent resistances to beta-lactam antibiotics (Tables 13, 14). E. coli strains are naturally sensitive to beta-lactam antibiotics examined. This was not the case in this survey as there was a high incidence of ampicillin resistance within the E. coli population. These strains were

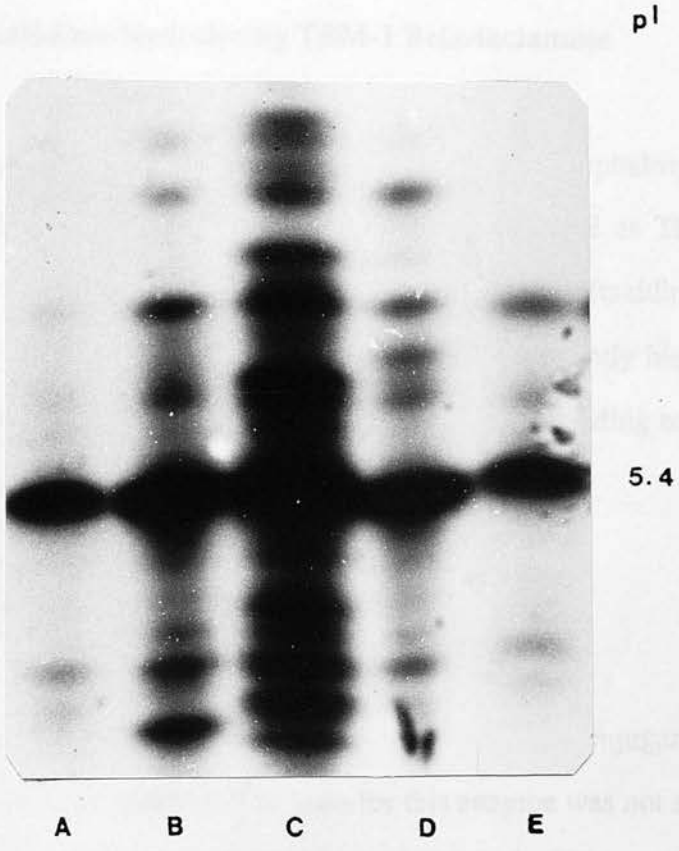
Table 19

Distribution of "Plasmid-associated" B-lactamases among Ampicillin Resistant *E. coli* Strains and Transconjugants

Strains	Number and proportion of B-lactamases														
	TEM-1	TEM-2	OXA-1	OXA-2	SHV-1	PSE-1	TEM-1 + OXA-1	TEM-1 + PSE-1	PSE-1 + PSE-2	TEM-1++	TEM(CAZ^R)	CARB-6	SAR-2	None	Total
Transconjugants															
Auto transferable															
Number	41	2	4	1	1	1	1	0	0	3	1	1	1	0	57
%	(71.9)	(3.5)	(7.1)	(1.7)	(1.7)	(1.7)	(1.7)	(0)	(0)	(5.2)	(1.7)	(1.7)	(1.7)	(0)	(100)
Mobilisable															
Number	13	0	3	0	0	0	7	0	0	0	0	0	0	0	23
%	(56.5)	(0)	(13)	(0)	(0)	(0)	(30.4)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(100)
E. coli clinical strains*															
Number	12	0	8	1	0	1	8	1	1	0	0	0	0	3	35
%	(34.2)	(0)	(22.8)	(2.8)	(0)	(2.8)	(22.8)	(2.8)	(2.8)	(0)	(0)	(0)	(0)	(8.5)	(100)

*Clinical isolates from which no autotransferable or mobilisable resistance could be detected.

The results for the previously identified beta-lactamases were repeated on one further occasion. Confirmation of the new beta-lactamases was performed a minimum of three times.



This experiment was performed on four separate occasions and the result shown is representative of that found on each occasion.

Figure 1

Isoelectric Focusing Pattern of TEM-1 Beta-lactamase of E. coli K-12 (R1) and TEM-1^{++} Beta-lactamases of Three E. coli J62-2 Transconjugants of E. coli Clinical Isolates.

Track A - E. coli K-12 (TEM-1)
Track B - LN19 (TEM-1^{++})
Track C - LN35 (TEM-1^{++})
Track D - LN37 (TEM-1^{++})
Track E - E. coli K-12 (TEM-1)

normal TEM-1 beta-lactamase (table 20) and their activities were inhibited by clavulanic acid (ID_{50} and K_Is fig 2a, 2b). It is particularly noteworthy that the host strains that produced these TEM-1^{++} were resistant to the combination of ampicillin and clavulanic acid (MIC 40+8 mg/l amp+clav).

3.2.4A2 Ceftazidime Hydrolysing TEM-1 Beta-lactamase

A beta-lactamase which hydrolysed the third generation cephalosporin, ceftazidime, was also identified. This enzyme had exactly the same pI as TEM-1 (pI 5.4) and some similar satellite banding. The rate of hydrolysis of ceftazidime for this enzyme (. TEM (Caz^r)) was low (0.03%); but it was significantly higher than a control TEM-1 enzyme (unmeasurable). Despite this, the corresponding host strain remained sensitive to ceftazidime.

3.2.4A3 A Novel Enzyme of High pI 7.4

A novel enzyme of pI 7.4 was identified in an *E.coli* transconjugant strain which had been selected on cephaloridine. The gene for this enzyme was not stable and required the presence of a beta-lactam antibiotic to maintain it. This enzyme had very poor activity against nitrocephin but was more active against carbenicillin on the basis of its substrate profile than the TEM-1 enzyme had been. It did not co-migrate with either OXA-1 or OXA-2 beta-lactamases nor with any enzyme from the SHV group, all of which have pIs in this range. This enzyme was classified as CARB-6 based on its preferential carbenicillin hydrolysis (table 21).

Another novel enzyme with a high pI of 8.3, designated SAR-2, was also identified. This enzyme has been fully characterised and is described in section 3.3.

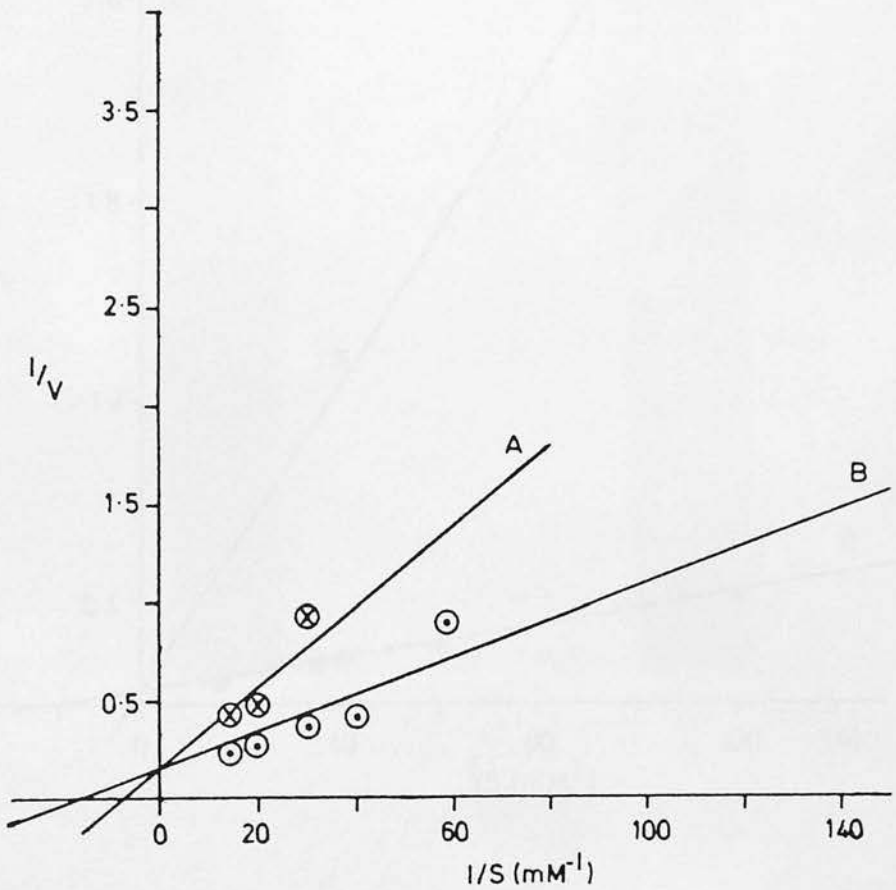
Table 20

Biochemical Properties of TEM-1^{++} and the Standard TEM-1 Beta-lactamases

	Relative rates of hydrolysis[a,b]						Inhibition[d] by clavulanic acid	
	Pen	Amp	Carb	Oxa[c]	Meth[c]	Cer	ID_{50} value (M)	KI value (μM)
TEM-1	100	72	12	4	1.0	60.0	9.5×10^{-8}	1.6
LN19	100	104.5	12	3	16.8	14.0	8.5×10^{-7}	0.84
LN35	100	93	14	0.97	0.6	11.9	0.8×10^{-7}	1.67
LN37	100	85.4	10	3	1.46	16.0	3.4×10^{-7}	ND

a = Spectrophotometric assay method
b = Expressed as a percentage of the value for benzyl penicillin
c = Hydroxylamine assay method
d = Nitrocephin (0.1 mM) as substrate
ND = Not done

This result represents the average of three separate determinations



This experiment was performed on three separate occasions and the result shown is representative of that found on each occasion.

Figure 2a

Lineweaver-Burk Plot of the Strain LN19 Producing TEM-1^{++} Beta-lactamase.

A = with clavulanic acid
B = without clavulanic acid (substrate nitrocephin)

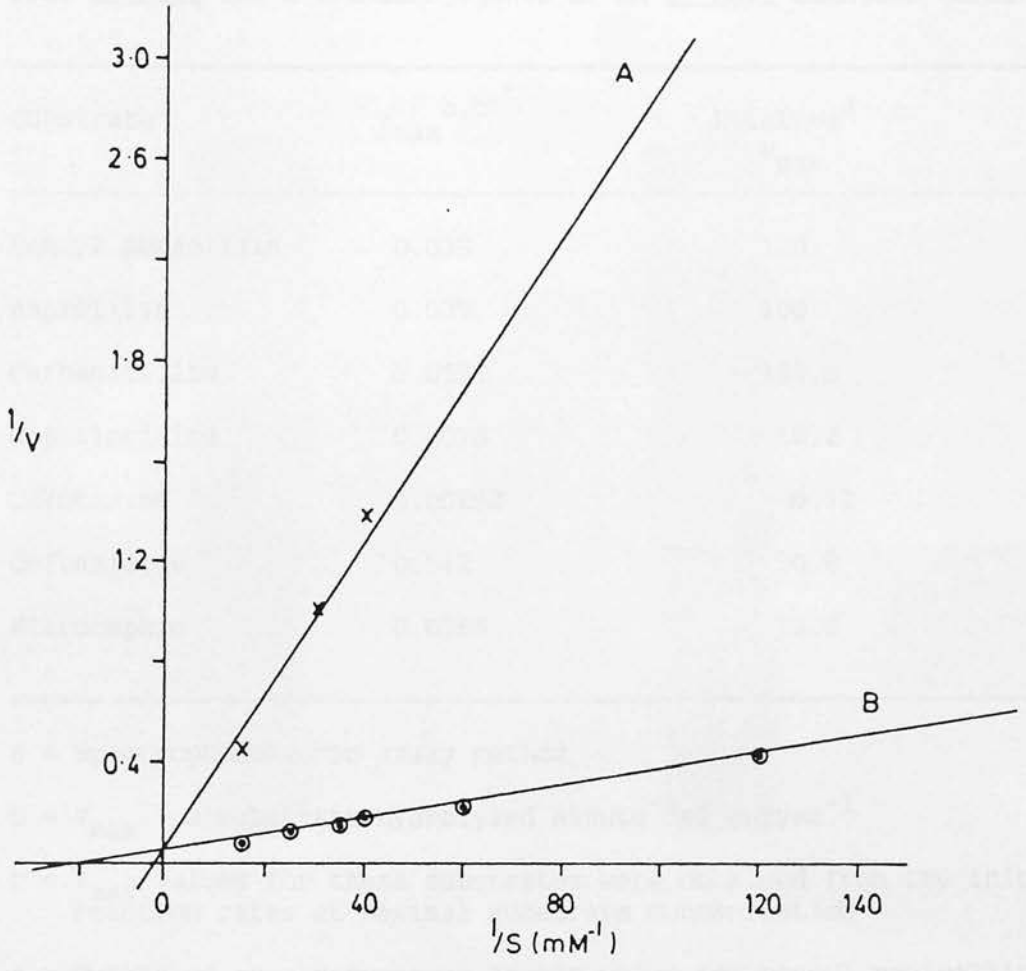


Figure 2b

Lineweaver-Burk Plot of The Strain LN35 Producing TEM-1^{++} Beta-lactamase.

A = with clavulanic acid
B = without clavulanic acid (substrate nitrocephin)

This experiment was performed on three separate occasions and the result shown is representative of that found on each occasion.

Table 21

Rates of Hydrolysis[a] of the Novel Beta-lactamase (CARB-6) Isolated from E. coli J62-2 Transconjugants of An E. coli Clinical Strain

Substrate	V_{max}[b,c]	Relative V_{max}[d]
Benzyl penicillin	0.039	100
Ampicillin	0.039	100
Carbenicillin	0.0597	153.8
Cephaloridine	0.0075	19.2
Cefotaxime	0.00262	6.72
Ceftazidime	0.012	30.8
Nitrocephin	0.0053	13.6

a = Spectrophotometric assay method

b = V_{max}, μm substrate hydrolysed $minute^{-1}ml$ $enzyme^{-1}$

c = V_{max} values for these substrates were obtained from the initial reaction rates at maximal substrate concentration

d = Expressed as a percentage of the value for benzyl penicillin

This result represents the average of two separate determinations

3.2.4B Beta-lactamases in the Rest of the *E.coli* Clinical Isolates

The rest of the clinical *E.coli* isolates (35) in which transferable plasmid resistance could not be identified were also screened for beta-lactamase production. Again, the TEM-1 enzyme was present in the majority of isolates (60%) (table 19), which suggested that the plasmid gene might have transposed onto the bacterial chromosome. Four other "plasmid-mediated" beta-lactamases were also identified, these were OXA-1, OXA-2, PSE-1 and PSE-2 (table 19). It is an infrequent event to find the PSE beta-lactamases in *E.coli* and it was surprising to see both PSE-1 and PSE-2 in one of the *E.coli* clinical isolates tested. In five isolates which were found to be resistant to second and third generation cephalosporins (sections 3.2 and 3.2.1), no new beta-lactamases were detected and it was presumed that the resistance to those drugs might be derived from other resistance mechanisms such as altered outer membrane porins or penicillin binding proteins.

3.3 SAR-2: Characterisation of A Novel Plasmid-mediated Beta-lactamase

3.3.1 Introduction

In *E.coli* strains, many different types of plasmid-determined beta-lactamases have been found. The commonest is TEM-1 enzyme and the rest are TEM-2, TLE-1, OXA-1, 2 novel OXA enzymes, PSE-1, PSE-2, PSE-4, SHV-1 and now these include extended-spectrum beta-lactamases (cephalosporinases) the TEM beta-lactamase derivatives (Medeiros, 1984, 1989; Bush, 1989).

In the survey of beta-lactam resistance among urinary pathogens (Gram-negative rods) isolated in South India (results, section 3.2), a high proportion of *E.*

coli (77%) was found to be resistant to ampicillin. The majority of the ampicillin resistance genes were freely transferable to the standard recipient strain and most of these genes encoded the ubiquitous TEM-1 beta-lactamase. Amongst these transconjugants, one strain was discovered to produce a novel high pI beta-lactamase.

3.3.2 Results

One clinical strain, *E.coli* 146, was highly resistant to ampicillin (MIC >1280 mg/l) but only moderately to cephaloridine (MIC 20mg/l). The other antibacterial drugs to which this strain was resistant were trimethoprim, streptomycin, tetracycline and nalidixic acid (measured at 10mg/l) as well as sulphamethoxazole (measured at 100 mg/l).

3.3.3 Conjugation Experiments

When strain *E.coli* 146 was used as a donor in a conjugation experiment, cephaloridine resistance (10mg/l) could be transferred to the standard recipient (*E.coli* J62.2) which has only a chromosomally-mediated rifampicin resistance.

When selection was made for ampicillin resistance with the same recipient strain, no transfer could be detected.

3.3.4 Plasmid Analysis of *E.coli* 146 and its Transconjugant

Both the original clinical strain *E.coli* 146 and the transconjugant were examined for their plasmid content and their resistance profiles. Both the original and transconjugant contain a single large plasmid of approximately 118kb. The

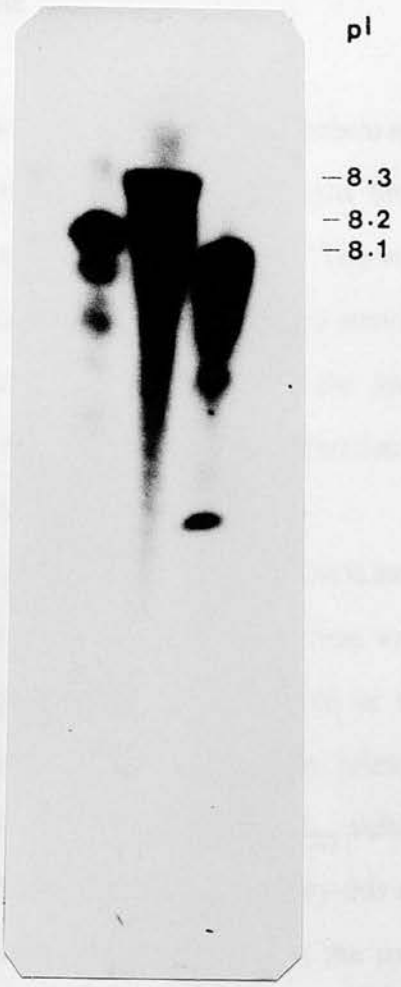
expression of these plasmids was very poor on the agarose gel (Materials and Methods) and, therefore, plasmids were detectable as faint bands. The plasmid in the transconjugant was designated as pUK734. The transconjugant with plasmid pUK734, acquired resistances to sulphamethoxazole (MIC >100 mg/l) and tetracycline (MIC >10 mg/l).

3.3.5 Isoelectric Focusing

Sonicated extracts were prepared (Materials and Methods) from both *E.coli* 146 and the transconjugant *E.coli* J62.2 (pUK 734). The beta-lactamases were examined by analytical IEF employing a broad-range ampholine (pH 3.5-10). Both extracts produced a beta-lactamase with a high pI. The extracts of the cephaloridine-resistant transconjugant was re-examined by IEF with a 1:1 mixture of pH 7-9 and 3.5-10 ampholines to increase the resolution at higher pHs. The beta-lactamase band did not co-focus with any other known beta-lactamase and had a higher pI (8.3) than either the CEP-1 and CEP-2 beta-lactamases (fig 3); therefore the enzyme was considered as novel and was designated as SAR-2. It was found by IEF that the original clinical isolate also produced the TEM-1 enzyme, in addition to the novel enzyme SAR-2. The novel enzyme was weakly expressed in the original clinical isolate. These findings implied that the gene determining the TEM-1 enzyme in the *E.coli* 146 was either chromosomal, or it might be located on a different plasmid of a large size which was not easily extractable intact.

3.3.6 Molecular Weight Determination

The crude extract from the cephaloridine resistant transconjugant was applied to a Sephadex G-75 column calibrated with the standard proteins ovalbumin, α-



This experiment was performed on three separate occasions and the result shown is representative of that found on each occasion.

Figure 3

Isoelectric Pattern of SAR-2, A Novel Beta-lactamase Encoded by Plasmid pUK 734, Compared with the CEP-1 and CEP-2 Beta-lactamases.

Track A - CEP-1
Track B - SAR-2 (*E. coli* J62-2 transconjugant)
Track C - CEP-2

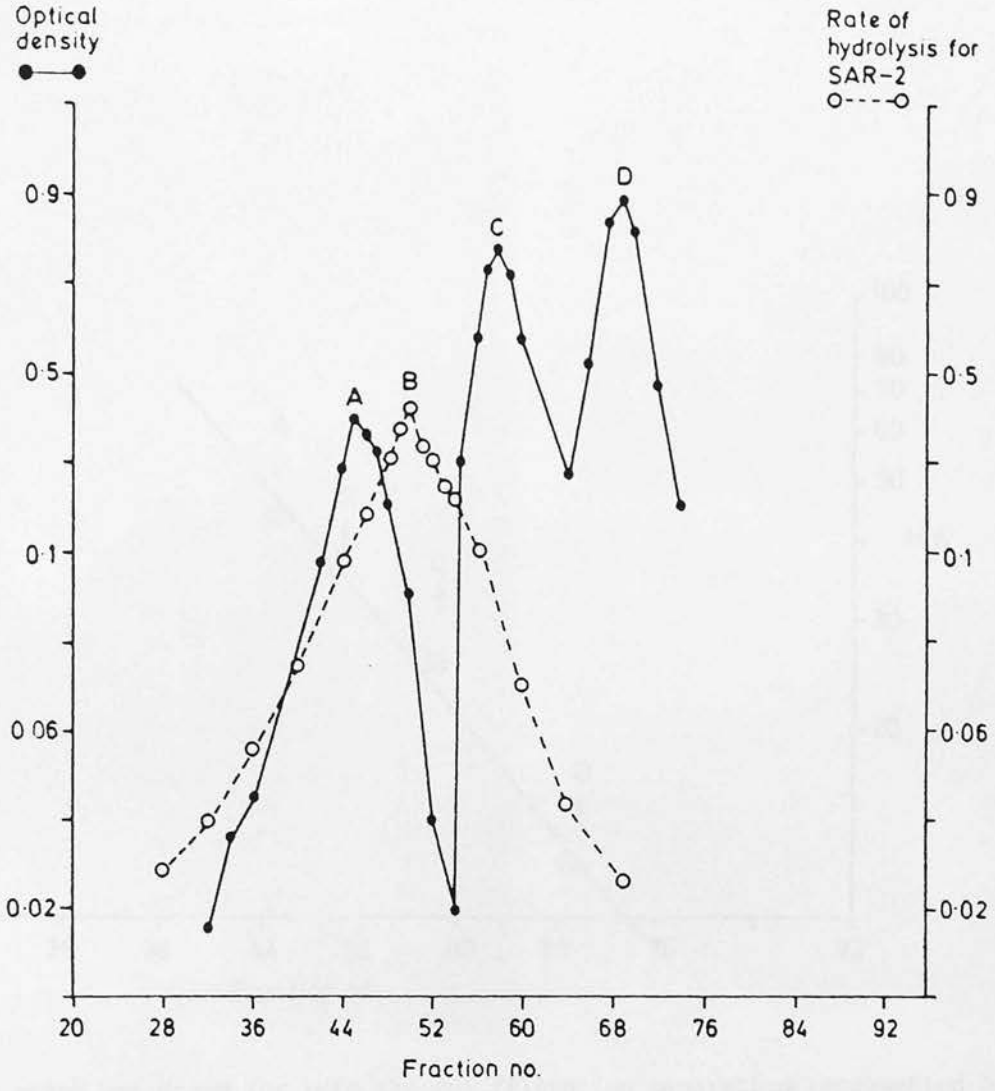
chymotrypsinogen and cytochrome C (fig 4). In comparison with known molecular weight markers the size of the SAR-2 beta-lactamase was 36000 (fig 5).

3.3.7 Substrate Profile

This novel enzyme was concentrated by ammonium sulphate precipitation as detailed in Materials and Methods. One litre overnight broth culture was harvested and washed in sodium phosphate buffer (pH 7.0). The washed pellets were resuspended in small (5mls) quantity of the same buffer and sonicated extract was separated by a high speed centrifugation. After measuring the specific activity of the enzyme, ammonium sulphate was added to 50% saturation to the extract. This mixture was stirred constantly for 30 minutes then centrifuged to collect the pellet. The pellet was resuspended in 0.8ml of the same phosphate buffer and again the specific activity of this suspension was estimated. This enzyme was assayed in the presence of fixed concentrations of various beta-lactams (Simpson, Harper and O'Callaghan, 1980) and the rates related to the hydrolysis of benzyl penicillin. It was impossible to measure the K_m values of beta-lactam antibiotics as these substrates were hydrolysed very slowly by this novel enzyme; therefore V_{max} values for the substrates were obtained from the initial reaction rates at maximal substrate concentrations. Besides benzyl penicillin, the SAR-2 beta-lactamase hydrolysed ampicillin, carbenicillin, oxacillin, methicillin, cefotaxime, nitrocephin and cephaloridine (table 22). The enzyme showed a greater ability to hydrolyse oxacillin and methicillin than most broad-spectrum beta-lactamases.

3.3.8 Inhibition Studies

When assayed for the hydrolysis of nitrocephin, the SAR-2 beta-lactamase was

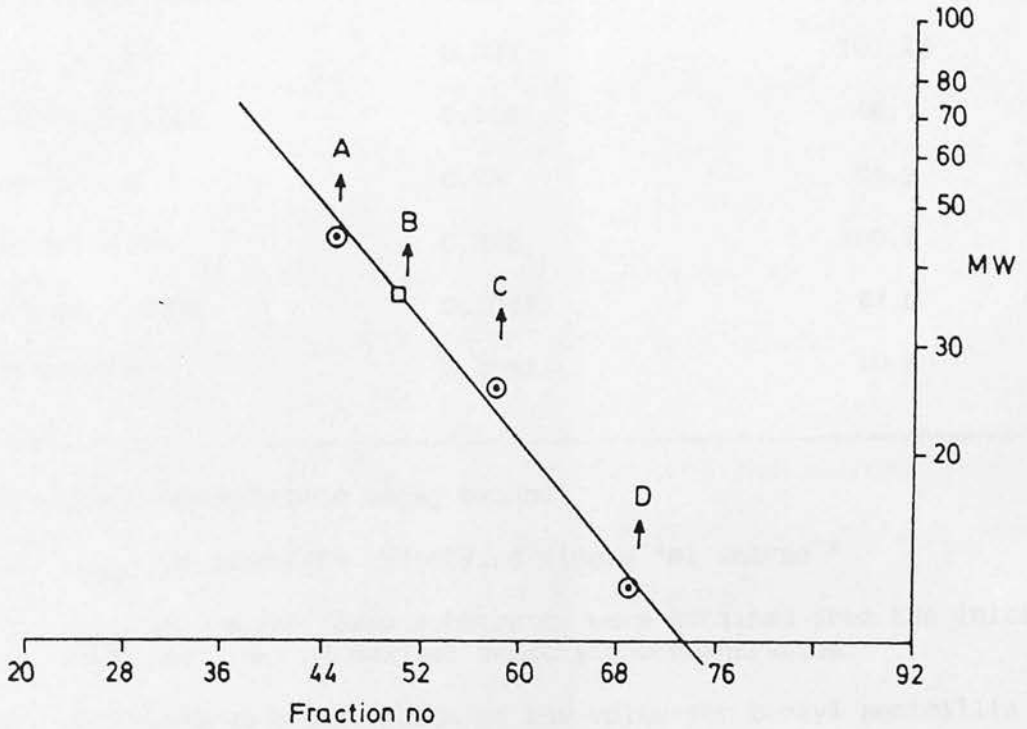


This experiment was performed on two separate occasions and the result shown is representative of that found on each occasion.

Figure 4

Elution Profile of The SAR-2 Beta-lactamase Separated by Sephadex G-75 Gel Filtration

A = ovalbumin (MW 45,000)
B = SAR-2 beta-lactamase (MW 36,000)
C = α-chymotrypsinogen (MW 25,500)
D = Cytochrome-C (MW 12,384)



This graph was drawn for both the gel filtration separations represented by figure 4. The values shown in this figure are for the separation detailed in figure 4. The molecular sizes from the two runs were comparable.

Figure 5

Molecular Weight Determination of the Novel Beta-lactamase (SAR-2) as Measured by Gel Filtration on Sephadex G-75

A = ovalbumin (MW 45,000)
B = SAR-2 (MW 36,000)
C = α-Chymotrypsinogen (MW 25,500)
D = Cytochrome-C (MW 12,384)

Table 22

Relative Rates of Hydrolysis[a] of SAR-2 Beta-lactamase Encoded by pUK 734 in *E. coli* J62-2 Transconjugant

Substrate	V_{max}[b,c]	Relative[d] V_{max}
Benzylpenicillin	0.39	100.0
Ampicillin	0.395	101.25
Carbenicillin	0.186	48.1
Oxacillin	0.25	64.2
Methicillin	0.395	100.2
Cephaloridine	0.1053	27.0
Cefotaxime	0.0762	19.6

a = Spectrophotometric assay method

b = V_{max}, μM substrate hydrolysed $minute^{-1} ml\ enzyme^{-1}$

c = V_{max} values for these substrates were obtained from the initial reaction rates at maximal substrate concentration.

d = Expressed as a percentage of the value for benzyl penicillin

This result represents the average of two separate determinations

highly inhibited by cloxacillin (ID_{50} $<10^{-9}$M). However, it was not sensitive to inhibition by clavulanic acid (ID_{50} $>10^{-4}$) or para-chloro mercuribenzoic acid (ID_{50} $>10^{-4}$) (table 23).

3.3.9 Comparison between the Novel Enzyme and Other Plasmid-mediated Beta-lactamases

The SAR-2 has a higher pI than all previously described plasmid-mediated beta-lactamases and can easily be distinguished from them by isoelectric focusing. Its molecular mass of 36000 suggests that the SAR-2 enzyme is similar to the CEP-2 beta-lactamase found in *Achromobacter* (Levesque et al., 1982); however, SAR-2 has a different substrate profile from CEP-2 and focuses above it. The SAR-2 beta-lactamase has greater affinity for penicillins than CEP-2 which would classify it as a "broad-spectrum" enzyme. On the other hand, it also hydrolyses oxacillin and methicillin more efficiently than any of the other "broad-spectrum" plasmid-mediated beta-lactamases. It is not as efficient as other oxacillin-hydrolysing (OXA) enzymes at hydrolysing these two substrates.

3.4 Beta-lactam Resistance among *Klebsiella* spp.

The second largest population of the enterobacterial strains (284) studied in this survey after *E.coli* (results, section 3.2) were *Klebsiella* spp. (26.4%). Ampicillin resistance in *Klebsiella* spp. is species-specific (Sawai, Yamagishi and Mitsuhashi, 1973; Sykes and Matthew, 1976; Sirot et al., 1988). . These strains isolated from bacteriuria were studied for their transferable resistance to beta-lactam antibiotics.

Table 23

Inhibition Profile of the Novel Beta-lactamase (SAR-2)

Potential Inhibitor	[a] ID_{50} Value (M)
Clavulanic acid	$> 1 \times 10^{-4}$
Cloxacillin	$< 1 \times 10^{-9}$
pCMB[b]	$> 1 \times 10^{-4}$

a = Nitrocephin, 0.1 mM as substrate

b = para-chloro-mercuribenzoic acid

This result represents the average of two separate determinations

3.4.1 Resistance to Ampicillin and Cephalosporins

Ampicillin resistance in *Klebsiella* spp. (75) was almost universal (96%), and more than half of them (43) were highly resistant to the drug (1280 mg/l) (table 13). On the other hand, cephaloridine resistance was found in only 52 strains (69%) and high level resistance to this drug was observed in only 8 strains (MIC>1280 mg/l) (table 13). Most of these strains were sensitive to clavulanic acid, as their MICs to ampicillin were drastically reduced in the presence of the beta-lactamase inhibitor (8mg/l) (table 13). This result indicated the possible involvement of clavulanic acid sensitive beta-lactamases in the strains conferring resistance to ampicillin.

When these strains were tested against other cephalosporins (table 13), a small proportion (17 strains) were resistant to cefuroxime (table 13) while a few strains were able to overcome the activity of the third generation cephalosporins such as cefotaxime (7 strains) and ceftazidime (5 strains) (table 13). These latter five strains were also able to resist the monobactam aztreonam (table 13).

The outcome of sensitivity testing to the series of beta-lactam drugs tested revealed that the *Klebsiella* spp. were predominantly resistant to ampicillin rather than to cephalosporins. The ampicillin resistant strains were investigated for transferable beta-lactam resistance.

3.4.2 Plasmid-determined Beta-lactam Resistance among *Klebsiella* spp.

The results obtained showed that about 50% (37/75) were able to transfer their beta-lactam resistance genes freely into the recipient strain *E.coli* J62.2 (table 24). Only 7 strains transferred both ampicillin and cephaloridine resistance genes. Thirty strains

Table 24

Transfer of Ampicillin and Cephaloridine Resistances in Klebsiella isolates (75).

Number of ampicillin resistant strains = 72/75

Conjugation results

Strains	Selecting antibiotic		Transconjugants
	Amp[a]	Cer[b]	
7	+	+	14
30	+	-	30
Total transconjugants			44

a = Ampicillin (10 mg/L)

b = Cephaloridine (10 mg/L)

This experiment was performed on two separate occasions and the result shown is representative of that found on each occasion.

were discovered to have only transferable ampicillin resistance without co-transferable resistance to cephaloridine (table 24). The transconjugant strains of *Klebsiellae* were further investigated for their plasmid content.

3.4.3 Plasmid DNA Characterisation of the Transconjugants

All plasmids of the *Klebsiella* transconjugants (44) were characterised for their resistance genes that they carried. A representative group of 24 were examined for their plasmid DNA sizes. From the information on the resistance patterns, there were 22 different plasmid types in the 44 transconjugant strains. Among the *Klebsiella* transconjugants (24) for which plasmid sizes were determined 11 different plasmid types were distinguished (table 25a) on the basis of their plasmid sizes and resistance profiles. In the second group of *Klebsiella* transconjugants (20) (table 25b), 11 different resistance patterns were observed based only on resistance profile.

In the first group *Klebsiella* transconjugants (24), the plasmid sizes were large (>80kb) and seven plasmids appeared on more than two occasions. Interestingly, in this group, four plasmids had the same molecular size (116kb) but with different resistance genes (table 25a).

In the second group *Klebsiella* transconjugants (20), 4 resistance patterns were exhibited by the transconjugants on more than one occasion (13 strains) (table 25b). These 4 resistance patterns might indicate the presence of more than 4 types of plasmids. In the second group of *Klebsiella* transconjugants, there were seven different resistance profiles conferred on one occasion each by seven different strains which suggested the presence of 7 different plasmid types.

Table 25a

Plasmid DNA Profile of *Klebsiella* Transconjugants (24)[a]

No.	Resistance patterns	Size (Kb)
6	Ap Ce Cm Km Sm Sp Sx Tc Tp	116
4	Ap Cm	87.1
2	Ap Cm Gm Km Sm Sp Tc Tp	251
2	Ap Cm Gm Km Sm Sx Tc Tp	116
2	Ap Cm Sm Sx Tc	116
2	Ap Cm Gm Sx Tp	95.5
2	Ap Cm Gm	83.2
1	Ap Cm Gm Km Sm Sc Tp	113.4
1	Ap Cm Gm Km Tp	87.1
1	Ap Ce Cm Tc	100
1	Ap Cm Tc	116

[a] - Total *Klebsiella* transconjugants = 44

Total transconjugants tested for plasmid sizes = 24

This result was confirmed on two separate occasions.

Table 25b

Plasmid Resistance Profile of the Rest of the Klebsiella Transconjugants (20)[a]

No.	Resistance patterns
4	Ap Cm Km
3	Ap Cm Sm Sx Tp
3	Ap Cm Sm
3	Ap Cm Gm Km Sm Sp Sx Tc Tp
1	Ap Cm Gm Km Sm Sp Tc Tp
1	Ap Cm Gm Km Sm Sp Sx Tp
1	Ap Cm Gm Km Sm Sp Tp
1	Ap Cm Gm Km Sx Tp
1	Ap Cm Km Sm Sx
1	Ap Cm Tc Tp
1	Ap Gm Sm Tc

[a] - Total Klebsiella transconjugants = 44

Total transconjugants tested only for resistance patterns = 20

This result was confirmed on two separate occasions.

Therefore, Klebsiella transconjugants possessed a minimum of 18 plasmid types (tables 25a, 25b). Most of them (24 strains) harboured resistance genes for five or more antibiotics and were found to be resistant to aminoglycosides (tables 25a, 25b).

3.4.4 Plasmid Resistance in the Transconjugants

The proportion of cephaloridine resistance in the clinical isolates of Klebsiella spp. was 72%. This percentage fell to 15.9% in the transconjugants (table 26). On the basis of this result, it appears that cephaloridine resistance in the clinical isolates must generally be non-plasmid mediated. Sulphamethoxazole resistance linked to the ampicillin resistance was less than 50% in the transconjugants; whereas, it had been notable that in their corresponding clinical isolates it was as high as 70.8%. In the urinary pathogens, as a whole, the linkage of sulphamethoxazole and ampicillin resistance was 93%.

Resistances to chloramphenicol, trimethoprim and streptomycin were widely spread (table 26) in all the strains (urinary pathogens, Klebsiella spp.) including in the transconjugants of Klebsiella spp. A remarkable proportion of the ampicillin resistant R-plasmids also coded for chloramphenicol resistance (97.7%). On the other hand, ampicillin resistant strains encoded linked resistance to spectinomycin was less than 50% in all the strains (table 26). The proportion of linked gentamicin and kanamycin resistant genes with ampicillin resistant genes was over 50% in the Klebsiella and their transconjugant strains. These resistances were less significant in enterobacterial (284) strains as a whole. Linkage of ampicillin resistance to trimethoprim on R-plasmids was about 57% whereas this had been well over 60% in the original (284) strains. The combination of sulphamethoxazole and trimethoprim

Table 26

Types and Proportion of Non-beta-lactam Resistances among Ampicillin Resistant Urinary Pathogens Including *Klebsiella* Clinical Isolates and Their Transconjugants

Antibiotics	Percentages of resistant strains		
	Klebsiella spp.	Transconjugants of *Klebsiella* spp.	Urinary pathogens
Ampicillin	100	100	100
Cephaloridine	72	15.9	80
Chloramphenicol	70.8	97.7	77
Gentamicin	51.4	52.3	35
Kanamycin	56.9	54.5	43.5
Streptomycin	75	61.4	87
Spectinomycin	40.3	31.8	36
Sulphamethoxazole	70.8	47.7	93
Tetracycline	66.7	47.7	85
Trimethoprim	68.1	56.8	76
Trimethoprim + Sulphamethoxazole	68.1	38.6	76

Total number of urinary pathogens tested (including *Klebsiella*) = 230

Total number of *Klebsiella* isolated tested = 72

Total number of transconjugants of *Klebsiella* tested = 44

resistance genes on ampicillin resistance R-plasmids was only 38.6% and it was considerably higher in the *Klebsiella* spp. Less than 40% of the plasmids carried resistance genes to tetracycline and this was notably higher in the both *Klebsiella* spp. and the urinary pathogens (table 26).

The ampicillin resistant transconjugants of *Klebsiella* spp. were screened for the production of plasmid-mediated beta-lactamases.

3.4.5 Beta-lactamases Produced in *Klebsiella* Transconjugants

There were only two types of beta-lactamases produced in the *Klebsiella* transconjugants (table 27). The TEM-1 beta-lactamase was predominant as it was appeared in most of the transconjugants (80%). The second beta-lactamase was SHV-1 beta-lactamase which is commonly found in these strains (Roy et al., 1985). The SHV-1 enzyme was identified alone in only 3 transconjugant strains and in 6 strains where it was co-produced with TEM-1 enzyme (table 27). Despite the presence of broad-spectrum beta-lactamases such as TEM-1 and SHV-1, the 85% of transconjugants remained sensitive to cephaloridine (<10 mg/l) (table 26).

3.5 Incidence of Beta-lactam Resistance in *Salmonella* and *Shigella* spps. Isolated from Gastrointestinal Infections

During the survey for collection of urinary pathogens from significant bacteriuria (results, section 3.1), over 200 *Salmonella* and *Shigella* spps., were also isolated from gastrointestinal infections, at the Christian Medical College Hospital, Vellore, South India, in 1984. Even though beta-lactam resistance among *Salmonella* and *Shigella* spps. is not inherent, the prevalence of ampicillin resistance has gradually become

Table 27

Distribution of "Plasmid-encoded" Beta-lactamases among Klebsiella Transconjugants Selected on Ampicillin and Cephaloridine

Transconjugant strains	Number and proportion of beta-lactamases				
	TEM-1	SHV-1	TEM-1 + SHV-1	NB[a]	Total[b]
Number	34	3	6	1	44
%	(77.3)	(6.8)	(13.6)	(2.3)	100

a, total transconjugants selected = 44

b, NB - No beta-lactamase could be detected

This experiment was performed on two separate occasions and the result shown is representative of that found on each occasion.

common among these species. The *Salmonella* and *Shigella* isolates in the present study were examined in detail for the spread of beta-lactam resistance.

3.5.1 Beta-lactam Resistance Profile in salmonellae

Ampicillin resistance in salmonellae was quite significant because as many as 90% of the strains (92/102) were resistant to the drug (table 28); however, less than half of them were highly resistant to ampicillin (>1280 mg/l). Conversely, these strains were very sensitive to the first generation cephalosporin, cephaloridine. Only 7 strains were able to grow in the presence of this antibiotic (>10 mg/l) and none of them highly resistant to the drug (>1280 mg/l). The proportion of ampicillin resistance was decreased when isolates were challenged with clavulanic acid (56% of the strains remained resistant). The beta-lactamase inhibitor was used at the at subinhibitory concentration (8mg/l) in conjunction with ampicillin (table 28). The salmonellae were more resistant to the action of clavulanic acid than they had been to cephaloridine as they retained high MICs (160+8mg/l) of the combination of ampicillin and clavulanic acid. Resistance to this combination may result from the presence of beta-lactamases which were stable to inhibition by clavulanic acid.

Surprisingly, a significant proportion (65.7%) of the strains were resistant to cefuroxime, the second generation cephalosporin (table 28). This was remarkable because of their sensitivity to cephaloridine. Apart from this, all *Salmonella* strains remained sensitive to other second and third generation cephalosporins (table 28) and this included the monobactam, aztreonam.

The ampicillin and cefuroxime resistant strains were taken further to investigate the resistance mechanisms involved.

Table 28

Proportion of Clinical *Salmonella* Isolates (102) Resistant to Beta-lactam Antibiotics

Beta-lactam antibiotic	MIC range (mg/L)	Number of resistant strains
Ampicillin	< 10	10
	> 10-1280	56
	> 1280	36
Ampicillin + Clavulanic acid[a]	< 10	18
	> 10-1280	84
	> 1280	0
Piperacillin	< 8	11
	> 8	22
	> 32	69
Cephradine	< 8	10
	> 8	90
	> 100	2
Cephaloridine	< 10	94
	> 10-1280	8
	> 1280	0
Cefuroxime	< 8	35
	> 8	67
Ceftazidime	< 4	100
	> 4	2
Cefotaxime	< 4	100
	> 4	2
Aztreonam	< 4	100
	> 4	2

a = Minimum inhibitory concentrations of ampicillin in the presence of subinhibitory concentration of clavulanic acid (8 mg/L)

The other cephalosporins employed were Cefazolin (8 and 100 mg/L); Cefamandole (8 mg/L); Cefoxitin (8 mg/L); Cefotetan (4 mg/L) and Ceftriaxone (4 and 32 mg/L). In each case, about 2% of the total population were resistant to them.

3.5.1A Selection of Ampicillin and Cefuroxime Resistant Transconjugants

It was inferred from the results, the degree (15%) of transferability of ampicillin resistance was very low. Of these 92 ampicillin resistant strains, only 14 could transfer by conjugation their resistance genes into the standard recipient (table 29). All transconjugants were selected on ampicillin as none of the original isolates were cephaloridine resistant and, therefore, no cephaloridine resistant transconjugants were obtained when selection was made for it.

Attempts were made to select the cefuroxime resistant transconjugants both by conjugation and by mobilisation with transfer factor X^+. Both these methods failed. It was assumed the prevalence of cefuroxime resistance among these isolates was mediated either by chromosomal beta-lactamases or by reduced outer membrane permeability. Both ampicillin resistant transconjugants and cefuroxime resistant clinical isolates were studied in detail to examine their resistance mechanisms.

3.5.1b Plasmid DNA of the Ampicillin-resistant Transconjugants

Plasmid DNA of the Salmonella transconjugants was characterised by the sizes and the resistance genes which they carried. There were ten different plasmid sizes with different resistance patterns (table 30). Only two types of plasmids were found on more than one occasion. The interesting feature was the presence of the smallest plasmid (11.5 kb) alone in one transconjugant carrying resistance genes for 9 antibiotics. Occurrence of auto-transferable small plasmids (<25 kb) is unusual. There might be a large plasmid in the same transconjugant along with the small one which

Table 29

Transfer of Ampicillin Resistance in Salmonella Isolates (102)

Total ampicillin resistant isolates = 92/102

Total cefuroxime resistant isolates = 67/102

Conjugation results

Strains	Selecting antibiotic		Transconjugants
	Amp[a]	Cxm[b]	
14	+	-	14
Total transconjugants			14

a, Amp = Ampicillin (10 mg/L)

b, Cxm = Cefuroxime (8 mg/L)

This experiment was performed on two separate occasions and the result shown is representative of that found on each occasion.

Table 30

Characteristics of Resistance Plasmids in Salmonella Transconjugants

Number	Resistance pattern	Size (kb)
4	Ap Sm Sx	78
3	Am Cxm* Cm Gm Km Sm Sp Sx Tc Tp	84.8
1	Ap Sm Sx	90.0
1	Ap Cm	57.5
1	Ap Cm	135.0
1	Ap	65.25
1	Ap	160.5
1	Ap Cxm* Cm Km Sm Sp Sx Tc Tp	11.5

* see text

This result represents the average of three separate determinations

could not be isolated and identified by the plasmid extraction and separation techniques available. This hypothesis is supported by the fact that when the resistance genes were transferred from E.coli J62.2 to E.coli J53, only the 11.5kb plasmid could be visualised in the transconjugant. It is extremely unlikely that the small plasmid would have sufficient available DNA to account for the resistance genes and it would certainly be unable to carry sufficient information for auto-transferability. The rest of the plasmid sizes were within the range of 57- 90kb (table 30) except for two plasmids which were 135 and 160 kb respectively.

There were five different resistance genes patterns on the plasmids. Only two plasmids carried resistance genes more than five antibiotics and they conferred resistance to aminoglycosides.

3.5.1C Comparison of Beta-lactam Resistance Levels of Transconjugants and their Respective Original *Salmonella* Strains

In most of the transconjugants, the level of ampicillin resistance was considerably higher than it had been in the original clinical strains. In some, the MICs of ampicillin were the same as their original clinical isolates (table 31). None of the R-plasmids were cephaloridine resistant (MIC <10 mg/l), although some of their original *Salmonella* isolates were resistant to this drug (MIC >10 mg/l). When the transconjugants were tested against cefuroxime, all were sensitive to the drug except for four of them. This comparison was made with the sensitive strain *E.coli* K-12, J62.2 (table 31). High inocula of these cultures ($1x10^9$ cfu) elevated their MICs of cephaloridine (table 31) but not for cefuroxime. However, four ampicillin and cefuroxime resistant transconjugants were found to have a high MIC to cefuroxime when their size of inocula was raised to 10^9 cfu (table 31).

Table 31

Comparison of Beta-lactam Resistance Levels of Salmonella( ) Isolates and Their J62-2 Transconjugants.

Transconjugant strain (no)	MICs[a] (mg/L) Amp 1 x 10^{5c}	Cer 1 x 10^{5c}	Cer 1 x 10^{9c}	Cxm 1 x 10^{5c}	Cxm 1 x 10^{9c}	Beta-lactamase[b]
LN 123	320 (>1280)	4 (20)	>10	2 (>8)	<8	T1 (T + 0)
125	320 (1280)	4 (20)	>10	2 (>8)	<8	T1 (T + 0)
127	320 (1280)	4 (10)	4	2 (<8)	<8	T1 (T1)
128	320 (1280)	2 (20)	2	2 (<8)	<8	T1 (T1)
129	1280 (640)	2 (20)	>10	4 (>8)	>8	0 (0)
130	1280 (>1280)	2 (10)	>10	4 (>8)	>8	0 (0)
131	640 (>1280)	4 (20)	>10	4 (>8)	>8	T1 + 0 (T + 0)
132	1280 (>1280)	4 (20)	>10	4 (>8)	>8	T1 + 0 (T + 0)
133	320 (320)	4 (20)	>10	2 (<8)	<8	T1 (T1)
134	320 (320)	4 (10)	>10	2 (<8)	<8	T1 (T1)
135	320 (320)	4 (10)	>10	2 (<8)	<8	T1 (T1)
136	320 (>1280)	4 (20)	>10	2 (<8)	<8	T1 (T1)
137	320 (>1280)	4 (80)	>10	2 (<8)	<8	T1 (T1)
138	320 (>1280)	4 (80)	>10	2 (<8)	<8	T1 (T1)
J62-2	4	2	2	2		
J62-2[d]	80	4	>10	4	>8	Std OXA-1

a = Minimum inhibitory concentrations
b = Beta-lactamases (T1 = TEM-1; 0 = OXA-1-like)
c = 1 x 10^5 & 1 x 10^9 bacterial density (organisms/ml)
d = standard OXA-1 beta-lactamase producing strain, E. coli J62-2
( ) = parenthesis, MIC and beta-lactamases of corresponding clinical Salmonella isolates

The beta-lactamases produced by the transconjugants were extracted and identified by isoelectric focusing to establish their resistance mechanism.

3.5.1D Plasmid-determined Beta-lactamases in the Transconjugants

Beta-lactamases from the transconjugants were identified on polyacrylamide gel by analytical IEF; and, as expected, the TEM-1 enzyme was by far the most common (10/14) (table 32). In addition, on two occasions, there was a second beta-lactamase in the transconjugants besides TEM-1, and this was found to be an OXA-1 like enzyme. There were two other transconjugants which also produced this OXA-1 like enzyme, but in the absence of the TEM-1.

Only those transconjugants which had plasmids encoding for OXA-1 like enzymes possessed resistance to the drug cefuroxime (tables 31 and 32); therefore, all the cefuroxime-resistant Salmonella clinical isolates were screened for this type of beta-lactamase production in order to establish the resistance mechanism to cefuroxime.

3.5.1E A Detailed Study on Cefuroxime Resistant Salmonella Strains

3.5.1E1 Mobilisation of Cefuroxime Resistance

The plasmid-carriage of the cefuroxime resistance genes were once again tested by conjugation on the plates containing lower concentrations of cefuroxime (2 mg/l) as four of the ampicillin resistant E. coli J62.2 transconjugants of Salmonella strains were found to be cefuroxime resistant (MIC 4 mg/l); but these attempts failed. Consequently, when the mobilising plasmid X^+ (E. coli) was employed to transfer the

Table 32

Types and Proportion of Beta-lactamases in *Salmonella* Transconjugants

Total number of transconjugants (*E. coli* J62-2) = 14

Transconjugant strains	Beta-lactamases			%
	TEM-1	OXA-1	TEM-1 + OXA-1	
10	+	-	-	71.4
2	-	+	-	14.3
2	-	-	+	14.3
14 = Total transconjugants				100.0

This experiment was performed on two separate occasions and the result shown is representative of that found on each occasion.

cefuroxime resistance; but none of the clinical isolates showed any plasmid transfer. Original clinical isolates were investigated to determine whether the source of cefuroxime resistance can be attributable to the type of beta-lactamase production.

3.5.1E2 Identification of OXA-like Enzyme in Cefuroxime Resistant *Salmonella* Clinical Isolates

The sonicated concentrated beta-lactamase extracts of the four *Salmonella* strains were identified by isoelectric focusing on polyacrylamide gel composed of 6-8 and 3.5-10.0 ampholines in a ratio of 1.5:0.5 respectively and compared to the standard OXA-1 beta-lactamase. Both beta-lactamases had the same main bands which co-focused at a pI of 7.4; however, two satellite bands were absent in the OXA-1 like enzyme whereas they were clearly visible in the standard OXA-1 enzyme (fig. 6).

As the standard OXA-1 enzyme is both plasmid and transposon mediated, it is possible that these resistance genes encoding OXA-1 like beta-lactamase could be located on the chromosome. To confirm this hypothesis, two of the cefuroxime resistant *Salmonella* strains were tested by induction studies.

3.5.1E3 Induction of Beta-Lactamases

Induction of beta-lactamases was carried out as described in Materials and Methods. The bacterial cells from 100 ml broth cultures in exponential phase of the growth cycle, grown in the presence or absence of cefuroxime (1/4 mg/l of their MICs) were harvested and disrupted. The disrupted cell debris was discarded and supernatant was collected. The specific activities of

The result shown compares one of the OXA-1 like beta-lactamase taken from one of the Salmonella strains directly with the original OXA-1 enzyme. The banding patterns and pIs of the beta-lactamases from the three other Salmonella strains were identical. All these enzyme extracts were tested by IEF on four occasions.

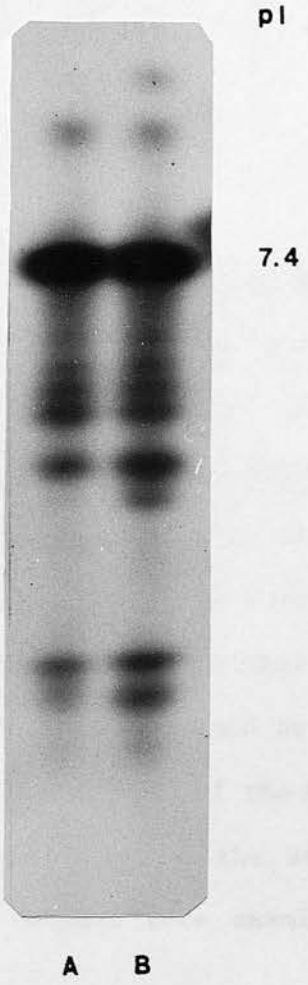


Figure 6

Isoelectric Focusing of OXA-1 and OXA-1-like Beta-lactamases Extracted from a Standard E. coli and a Salmonella Clinical Strains.

Track A - OXA-1 like enzyme Track B - OXA-1 enzyme

these supernatants were measured and it was revealed the OXA-1 like enzymes were not inducible as they had similar specific activities as control OXA-1 like enzyme. According to these results this enzyme might be plasmid-mediated; therefore, all these cefuroxime resistant clinical strains were subjected to plasmid DNA studies.

3.5.1E4 Inspection of Plasmid DNA

Plasmids possessed by these cefuroxime resistant Salmonellae clinical isolates were isolated and electrophoresed in 0.5% agarose gel by the method of Takahashi and Nagano (1984). These results showed that every clinical isolate harboured a minimum of 3 plasmids with a wide range of sizes (>35-88kb). As none of these plasmids was either transferable or mobilisable, in order to determine that OXA-1 like enzyme is indeed plasmid-encoded, an attempt was made to cure the isolates of the plasmid. This experiment failed, leading to the assumption that these OXA-1 like genes might be either plasmid-mediated or, alternatively, could be on the chromosome and produce the enzyme constitutively. The characteristics of the OXA-1 like enzyme from each of the four Salmonella strains were compared with the standard OXA-1 beta-lactamase. The biochemical properties of both enzymes were examined to establish the differences between them.

3.5.1E5 Activity of OXA-1 like Beta-lactamase against Different Substrates and Inhibitors

The rates of hydrolysis of various substrates at fixed concentrations by the OXA-1 like and OXA-1 enzymes were analysed under the same experimental conditions. The results are presented in table 33. The rates were related to the hydrolysis of penicillin G (i.e. 100%). The OXA-1 like and OXA-1 enzymes hydrolysed ampicillin

Table 33

Substrate Profile[a] of OXA-1-like and the Standard OXA-1 Beta-lactamases

	OXA-1-like		OXA-1	
Substrate	V_{max}[b]	Relative[c] V_{max}(%)	V_{max}[b]	Relative[c] V_{max} (%)
Penicillin	0.376	100	1.015	100
Ampicillin	2.845	756.6	5.825	573.9
Carbenicillin	0.405	107.7	2.64	260.0
Cephradine	0.001248	0.33	0.032	3.2
Cephalothin	0.049	13.0	0.0399	5.8
Cephaloridine	0.1865	49.6	0.324	46.9
Cefuroxime	0.047	12.5	0.015	1.5
Cefoxitin	0.007	1.9	NH	-
Cefamandole	0.148	39.4	0.1597	15.7
Cefotaxime	0.0691	18.4	0.0854	12.4
Ceftazidime	NH	NH	NH	NH
Nitrocephin	2.46	654.3	2.54	250.0

a = Spectrophotometric assay method

b = V_{max}, μm substrate hydrolysed $minute^{-1}$ml $enzyme^{-1}$

c = V_{max} values for these substrates were obtained from the initial reaction rates at maximal substrate concentration

d = Expressed as a percentage of the value for benzyl penicillin

NH = No hydrolysis

The velocity constants for the OXA-1 like enzyme are the average of two determinations for one of the Salmonella strains. Concurrent tests with the enzyme preparations from the other three Salmonella strains showed no obvious variation from the results presented.

(755% and 573% respectively), cephaloridine (49.6% and 46.9%) and cefotaxime (18.4% and 12.4%) to similar extents. The OXA-1 like enzyme had a considerably higher activity (8-fold) against cefuroxime than OXA-1. Moreover, the OXA-1 like enzyme could hydrolyse cefoxitin whereas OXA-1 could not (table 33). In addition, the OXA-1 like enzyme had higher activity (>2-fold) than the OXA-1 like enzyme against the substrates such as cephalothin, cephamandole and nitrocephin (table 33). On the other hand, the OXA-1 like enzyme was less effective than OXA-1 against cephradine and carbenicillin (table 33). The relative rates of hydrolysis of oxacillin and methicillin of these enzymes could not be determined spectrophotometrically as the lambda maximum for these antibiotics were very low.

The enzymes shared common inhibitor profiles (table 34) as they were resistant to clavulancic acid (dose for 50% inhibition (ID_{50}>1mM), cloxacillin, p-Chloromercuri-benzoic acid, sodium chloride, cefoxitin, cefotetan and cefuroxime in the presence of nitrocephin as a substrate (table 34).

Cefuroxime resistance in these Salmonella strains was accompanied by the widespread distribution of the novel OXA-1 like enzyme, even though other resistance mechanisms may also play a subsidiary role. This must be true as four of the ampicillin resistant transconjugants had different MICs of cefuroxime (MIC 4mg/1 at 10^5cfu and MIC 8mg/1 at 10^9cfu) than their corresponding clinical isolates (MIC>8mg/1 at 10^5cfu).

Table 34

Inhibition Studies on OXA-1-like and the Standard OXA-1 Beta-lactamases

Potential Inhibitor	ID_{50}[a] (mM)	
	OXA-1-like	OXA-1
Clavulanic acid	> 1.0	> 1.0
Cloxacillin	0.1	0.1
Cefuroxime	> 0.1	> 0.1
Cefoxitin	> 1.0	> 1.0
Cefotetan	> 1.0	> 1.0
Sodium chloride	>10.0	>10.0
p-CMB	> 1.0	> 1.0

a = Nitrocephin (0.1mM) as substrate

pCMB = para-chloromercuribenzoic acid

The inhibitor constants for the OXA-1 like enzyme are the average of two determinations for one of the Salmonella strains. Concurrent tests with the enzyme preparations from the other three Salmonella strains showed no obvious variation from the results presented.

3.5.2 Study of Clinical Isolates of *Shigella* spp.

3.5.2A Introduction

A total of 101 *Shigella* strains were also isolated from gastrointestinal infections at the same time as the *Salmonella* strains (results, section 3.5). These strains were also assessed for their beta-lactam resistance and for the resistance mechanisms involved.

3.5.2B Sensitivity Testing to Beta-lactam Antibiotics

The *Shigella* isolates were examined for their minimum inhibitory concentrations of different beta-lactam antibiotics (table 35). It was found that a remarkable proportion of them (66%) was ampicillin resistant; however, only one strain was highly resistant to the drug (MIC >1280 mg/l). Unlike the *Salmonella* isolates, the shigellae were extremely sensitive to the combination of ampicillin and the beta-lactamase inhibitor clavulanic acid (table 35) as their MICs of ampicillin in most strains dropped to <10 mg/l in the presence of the inhibitor. Only 7 strains were able to resist this combination.

Virtually, none of the strains were able to withstand the antibacterial activity of cephaloridine (10 mg/l) except a single strain (MIC >1280 mg/l). This strain was more resistant to cephaloridine than to ampicillin. The shigellae were sensitive even to drugs (table 35) such as cephradine and piperacillin which were beta-lactamase unstable antibiotics. On the other hand all other species examined so far in this study (urinary pathogens and salmonellae) were very significantly resistant to those antibacterial agents.

Table 35

Number of Clinical Shigella Isolates (101) Resistant to Beta-lactam Antibiotics

Beta-lactam antibiotic	MIC range mg/L	Number of resistant strains	Beta-lactam antibiotic	MIC range mg/L	Number of resistant strains
Ampicillin	≤ 10	34	Cefuroxime[b]	≤ 8	97
	> 10-1280	66		> 8	4
	> 1280	1			
Ampicillin +	≤ 10	94	Cefamandole[b]	≤ 8	100
Clavulanic acid*	> 10-1280	7		> 8	1
	> 1280	0			
Piperacillin	≤ 8	72	Cefoxitin[b]	≤ 8	96
	> 8	26		> 8	5
	> 32	3	Cefotetan[b]	≤ 4	99
Cephaloridine[a]	≤ 10	100		> 4	2
	> 10-1280	1			
	> 1280	0			
Cephradine[a]	≤ 8	88	Cefotaxime[c]	≤ 4	99
	> 8	13		> 4	1
	> 100	0		>32	1
Cefazolin[a]	≤ 8	98	Ceftriaxone[c]	≤ 4	98
	> 8	2		> 4	2
	> 100	1		>32	1
			Ceftazidime[c]	≤ 4	97
				> 4	1
				>32	3
			Aztreonam[d]	≤ 4	97
				> 4	0
				>32	4

* = The subinhibitory concentration of clavulanic acid 8mg/L in the presence of ampicillin
a = First generation cephalosporins; b = Second generation cephalosporins; c = Third generation cephalosporins d = Monobactam

For second and third generation cephalosporins, a few strains had grown in the presence of them (table 35). These results clearly showed that resistance to cephalosporins was very insignificant despite the wide spread ampicillin resistance amongst *Shigella* spp. The ampicillin resistant strains were studied further to locate the genetic location of the ampicillin resistance genes.

3.5.2C Transfer of Ampicillin Resistance from *Shigella* Isolates

The study of transferable beta-lactam resistance in these strains had shown that only 20 (20/67) of ampicillin resistant strains could transfer the ampicillin resistance genes alone (table 36). The percentage of transferable resistance amongst these isolates was 2-fold higher (30%) than in *Salmonella* isolates. The ampicillin resistance genes did not seem to confer any resistance to cephaloridine as no transconjugant could be isolated when the selection was made on cephaloridine (table 36). Indeed the clinical strain that could transfer genes for cephaloridine resistance freely into the standard recipient strain (table 36) was the only strain which conferred resistance to cephaloridine (MIC >1280 mg/l) from the total shigellae population.

3.5.2D Resistance Determinants on R-plasmids of *Shigella* Transconjugants

Characterisation of the plasmids of the *Shigella* transconjugants revealed that all the transconjugant strains possessed ampicillin resistance genes. There were a total of 7 different plasmid types characterised by their plasmid sizes and resistance genes that they acquired (table 37). All the plasmids were about the same size (about 80kb) (table 37), expect one plasmid which was as large as 135.8kb in size. There were 7

Table 36

Transfer of Ampicillin and Cephaloridine Resistances in Shigella Isolates (101).

Total number of ampicillin resistant strains = 67
Total number of cephaloridine resistant strains = 1

Strains with transferable plasmids	Selecting antibiotic		Transconjugants
	Amp[a]	Cer[b]	
20	+	-	20
1	-	+	1
Total transconjugants =			21

a = Ampicillin (10mg/L)
b = Cephaloridine (10mg/L)

This result was confirmed on two separate occasions.

Table 37

Characteristics of Transferable Resistance Plasmid of Shigella Transconjugants

Number	Resistance patterns	Size (kb)
6	Ap cm Sm Sp Sx Tc	83
6	Ap Cm Sm Sx Tc Tp	88.9
3	Ap Cm Gm Km Sm Sp Sx Tc Tp	91.5
2	Ap Cm Sm Sx Tc Tp	135.8
1	Ap Cm Gm Km Sm Sx Tc	88.5
1	Ap Cm Sm Sp Sx Tc Tp	84.0
1	Ap	75.0
1	Ce	-

This result represents the average of two separate determinations

different plasmid resistance patterns, conferring resistance to all common antibiotics. Almost all of them harboured genes for aminoclycosides and each plasmid was resistant to a minimum of five drugs. There was one exception where the plasmid was resistant to ampicillin alone (table 37). Resistance patterns revealed that only one of them was cephaloridine resistant. In this respect, Shigella transconjugants were similar to the transconjugants of Salmonella.

Resistance determinants to chloramphenicol, sulphamethoxazole, streptomycin and tetracycline were remarkably common among the ampicillin resistant transconjugants (95%) (fig 7). R-plasmids coding for ampicillin resistance were also associated with the genes encoding trimethoprim and spectinomycin resistances separately in 50% of the transconjugants. A small proportion (20%) of the R-plasmids possessed linked resistance to gentamicin and kanamycin in each case (fig 7). Only half of the ampicillin resistant transconjugants showed linked resistance to the combination of trimethoprim and sulphamethoxazole. This association between ampicillin resistance genes and the combination of trimethoprim and sulphamethoxazole resistance genes was found to be common, as it had been in isolates tested in the general survey (urinary and gastroenteric pathogens).

3.5.2E Identification of Plasmid-encoded Beta-lactamases of R-plasmids

The identification of beta-lactamases produced in the Shigella transconjugants once again showed the prevalence of the TEM-1 beta-lactamase, which was found in 90% of the transconjugants (table 38). The only other beta-lactamase found in the transconjugants was OXA-1. On two occasions TEM-1 enzyme was co-produced with OXA-1 enzyme. In only one transconjugant OXA-1 beta-lactamase alone was responsible for its host strain's ampicillin resistance. No beta-lactamase

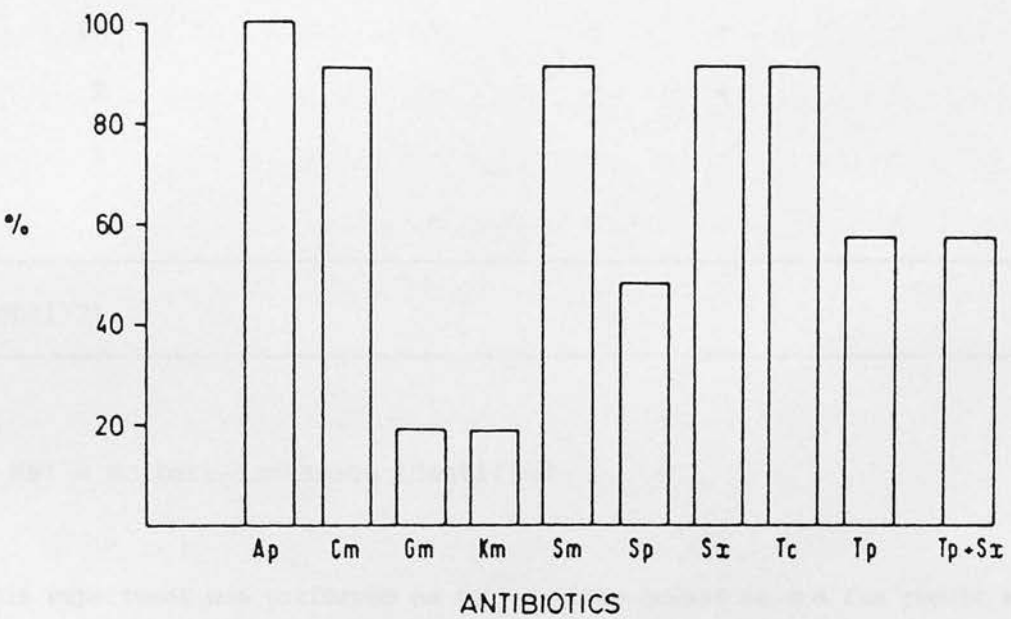


Figure 7

Types and Proportions of Non-beta-lactam Resistance Genes on Ampicillin Resistant Plasmids of Shigella J62-2 Transconjugants.

Ap = 100%; Cm = 90.5%; Gm = 19.0%; Km = 19.0%; Sm = 90.5%; Sp = 47.6%; Sx = 90.5%; TC = 90.5%; Tp = 57.0%; Tp + Sx = 57.0%

Table 38

Plasmid-encoded Beta-lactamases of R-plasmids in Shigella Transconjugants

Total transconjugants obtained = 21

Transconjugant strains	Beta-lactamses				%
	TEM-1	OXA-1	TEM-1 + OXA-1	NBI[a]	
17	+	-	-	-	80.9
2	-	-	+	-	9.5
1	-	+	-	-	4.8
1	-	-	-	+	4.8
Total 21					100.0

[a] NBI = No beta-lactamase identified

This experiment was performed on two separate occasions and the result shown is representative of that found on each occasion.

Table 38

Plasmid-encoded Beta-lactamases of R-plasmids in Shigella Transconjugants

Total transconjugants obtained = 21

Transconjugant strains	Beta-lactamses				%
	TEM-1	OXA-1	TEM-1 + OXA-1	NBI[a]	
17	+	-	-	-	80.9
2	-	-	+	-	9.5
1	-	+	-	-	4.8
1	-	-	-	+	4.8
Total 21					100.0

[a] NBI = No beta-lactamase identified

This experiment was performed on two separate occasions and the result shown is representative of that found on each occasion.

was detectable in the transconjugant selected on cephaloridine (table 38). Although this resistance gene was also transferable from E. coli J62.2 to E. coli J53, no plasmid DNA could be detected in the latter transconjugant. It appears that this transferable cephaloridine resistance might be mediated by a mechanism other than a beta-lactamase.

3.5.2F Minimum Inhibitory Concentrations of Ampicillin for the *Shigella* Transconjugants

The results of these susceptibility tests to ampicillin showed that all the *Shigella* transconjugants possessed approximately the same MIC levels as their corresponding original clinical strains (table 39). The interesting feature was that there was an 8-fold increase in the MIC of ampicillin in four transconjugants and it was doubled on one occasion (table 39). Only one transconjugant occurred which had a reduced MIC level to ampicillin compared to its original clinical isolate. Despite the elevated MICs of ampicillin, after transfer of the respective resistance genes into the standard recipients, all the transconjugants remained cephaloridine sensitive, even though they all produced TEM-1, the broad-spectrum beta-lactamase.

3.6 Mutation Studies on Resistance to the Combination of Ampicillin and Clavulanic Acid

3.6.1 Introduction

Surveys of ampicillin resistant enterobacterial strains from different countries showed some variation in the distribution of beta-lactamases, with TEM-1 always the commonest. The results of this present study have demonstrated that this is true in India as well. The TEM-1 beta-lactamase gene has proved highly successful as a

Table 39

Comparison between Ampicillin Resistance Levels of Shigella Transconjugants and Their Corresponding Clinical Isolates

Number of transconjugants	MICs of Amp[c] for 'T'[a] (mg/L)	MICs of Amp[c] for 'C'[b] (mg/L)
13	160	160
3	1280	160
2	320	320
1	> 1280	160
1	320	160
1	160	320
Total 21 transconjugants		

a, 'T' = transconjugants
b, 'C' = corresponding clinical isolates
c, Amp = Ampicillin

major contributor to the high incidence of ampicillin resistances and it is responsible for the spread of ampicillin resistance world-wide. This beta-lactamase is capable of acquiring mutational modification of its active site in order to meet the challenge of new beta-lactam antibiotics. There are many examples of this, in particular the evolution of extended-spectrum beta-lactamases of the TEM-1 derivatives which can destroy the highly potent later generation cephalosporins.

There are fewer examples of clinical resistance to beta-lactamase inhibitors and, therefore, the objectives of the mutation studies described below were to establish the efficacy of the combination of enzyme labile beta-lactam and beta-lactamase inhibitor (ampicillin and clavulanic acid) and to determine the emergence of possible resistance mechanisms that effect the antibacterial activity of beta-lactam and beta-lactamase inhibitors in *E.coli*.

The TEM-1 enzyme producing plasmid R1 was used for these studies. The R1 plasmid containing *E.coli* strain K-12 was challenged with a mixture of ampicillin (100 mg/l) and clavulanic acid (20 mg/l). In parallel, the same strain harbouring one each of the extended spectrum beta-lactamases (i.e. TEM-3, TEM-5 and TEM-7) were also challenged. This experiment was also performed on the same *E.coli* strain harbouring R1010 plasmid which encodes SHV-1 beta-lactamase. SHV-1 is another common broad-spectrum penicillinase but is, like TEM-1, sensitive to the combination of ampicillin and clavulanic acid.

3.6.2 Mutants of R-1 Plasmid Harbouring and TEM-1 Enzyme Producing *E. coli* Strain

3.6.2A Selection of A Mutant "A" Resistant to Ampicillin and Clavulanic Acid and Mutants "B" Resistant to Clavulanic Acid Alone

Mutants resistant to both the combination (ampicillin and clavulanic acid) and clavulanic acid were obtained on the Oxoid diagnostic sensitivity test agar (DSTA) plates containing the selective antibiotics. The selected mutants were purified on the same selective plates. Mutant "A" was obtained from a R1 plasmid containing, TEM 1 beta-lactamase producing strain *E. coli* K-12 challenged with the combination of ampicillin and clavulanic acid at the concentration levels of 100 and 20 mg/1 respectively. Mutant "A" was re-exposed (fig 8) to clavulanic acid alone at two different concentration levels (64 and 128 mg/1). In this case, two types of "B" mutants were selected: Mutants B1 and B2 were obtained from agar plates containing 64 mg/1 clavulanic acid and mutants B3 and B4 were obtained at 128 mg/1 of clavulanic acid. The mutants "A" and "B" were then rigorously tested for their beta-lactam resistance profiles. The mutation rates are shown in table 39a.

3.6.2A1 Antibiotic Sensitivity Testing for Mutant "A" and Mutants "B"

The minimum inhibitory concentrations of ampicillin, clavulanic acid, combination of ampicillin + clavulanic acid and augmentin (amoxycillin and clavulanic acid) were investigated for these mutants. In addition, all these mutants were tested for their resistance patterns to cefuroxime, the second generation cephalosporin, and third generation cephalosporins such as ceftazidime and cefotaxime (table 40).

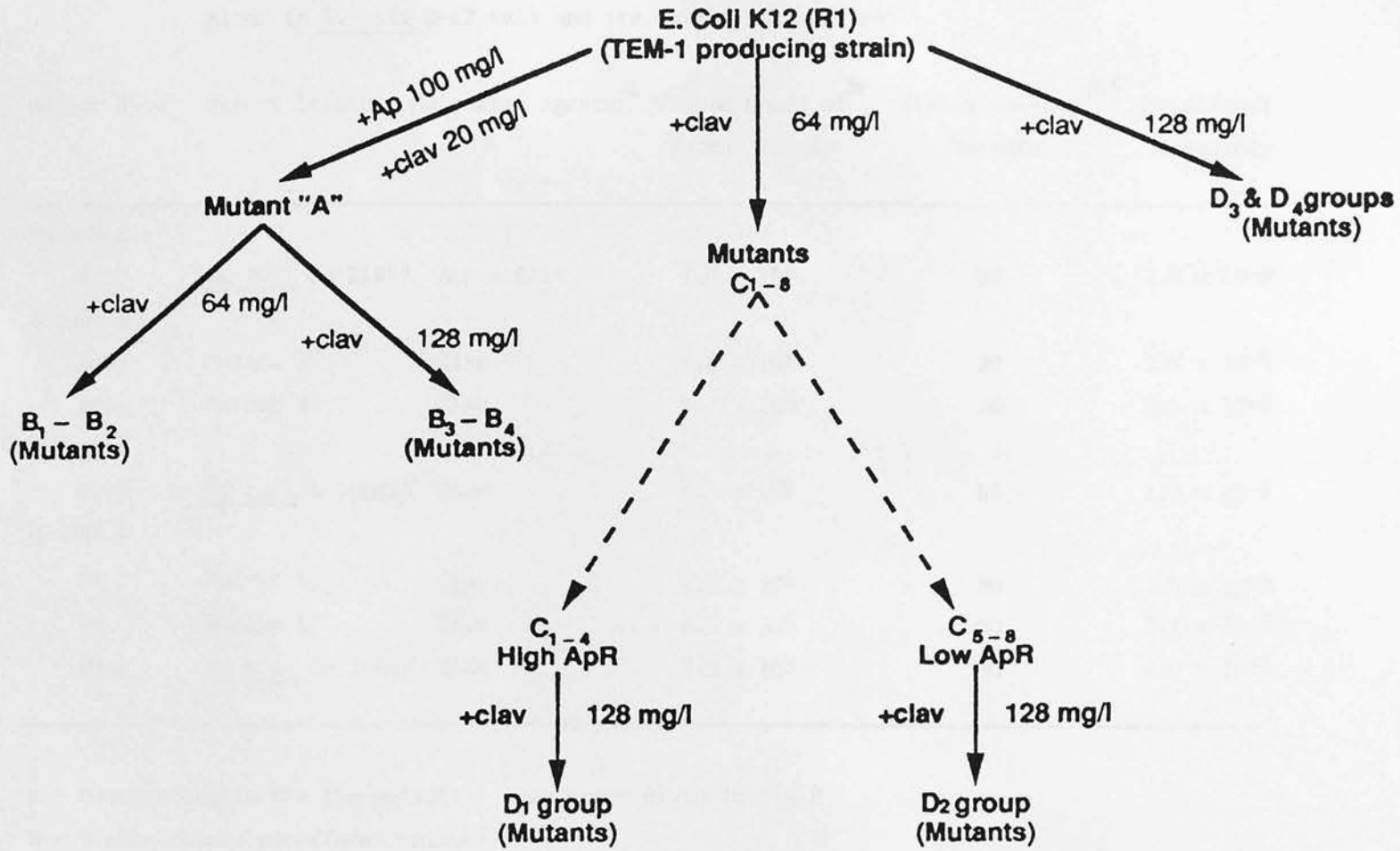


Figure 8. Flow Chart for Mutation Experiment on *E.coli* Strain (R1 Plasmid)

Table 39a. Mutation Rates to Ampicillin/Clavulanic Acid Resistance or Clavulanic Acid Resistance Alone in E. coli K-12 (R1) and its Mutant Derivatives

Mutant Type	Parent Strain	Selective Agents[a]	Viable Count of Parent Strain[b]	Viable Count of Mutants[b,c]	Mutational Frequency
Mutant A					
A	E. coli K-12(R1)	Amp + Clav	7.0×10^8	20	2.8×10^{-8}
Mutant B					
B_{1-2}	Mutant A	Clav	8.6×10^8	20	2.3×10^{-8}
B_{3-4}	Mutant A	Clav	9.7×10^8	20	2.1×10^{-8}
Mutant C					
C_{1-8}	E. coli K-12(R1)	Clav	6.5×10^8	80	1.2×10^{-7}
Mutant D					
D_1	Mutant C_1	Clav	1.0×10^9	20	2.0×10^{-8}
D_2	Mutant C_7	Clav	8.5×10^8	60	7.1×10^{-8}
D_{3-4}	E. coli K-12(R1)	Clav	7.5×10^8	30	4.0×10^{-7}

a - Concentrations for the selective agents are given in fig 8

b - Viable counts are Cfu/ml culture

c - The viable counts for the mutants were obtained from taking the average of the count obtained on three agar plates, each inoculated with 100ul of a washed culture.

See abbreviations

Table 40

Resistance Levels and Patterns of Mutants "A" (Ap/Clav^R) and "B" (Clav^R)

Parent strain	Mutant no.	Minimum inhibitory concentrations (mg/L)					Resistance patterns	FIC indices
		Ap	Clav	Ap + Clav**	Ap + Clav***	Aug (2.5/0.5-160/32)		
E.coli K12 (R1)	-	480	40	< 2.5 + 8	< 2.5 + 32	20 + 4	Ap Cm Sm Sp Sx	0.029
E.coli K12 (R1)	A	2560	40	160 + 8	10 + 32	40 + 8	Ap Cm Sm Sp Sx	0.156
Mutant A	B_{1-2}	>2560	80	160 + 8	-	-	Ap	0.156
Mutant A	B_{3-4}*	>2560	160	160 + 8	-	-	Ap	0.156

* Resistant to Cefuroxime (< 4 mg/L)

Clav = Clavulanic acid (** = 8, *** = 32 mg/L)

FIC = Fractional inhibitory concentrations

FIC Indices - This is the sum of the fraction inhibitory concentrations of the individual drugs measured in the presence of the other at their point of maximum efficacy. If the combined effect of the drugs is additive, the FIC index would be 1.0. Any value of less than 0.7 indicates synergy.

The results showed that mutant "A" had increased resistance a 100-fold to the combination of ampicillin and clavulanic acid (table 40) in comparison with the original *E.coli* K-12 strain. Concurrently, resistances to ampicillin (2560 mg/l) and augmentin (40+8 mg/l of amoxycillin and clavulanic acid) had also increased compared to the parent strain. Mutant "A" was also able to resist ampicillin in the presence of 32 mg/l of clavulanic acid; however the MIC of ampicillin was drastically reduced for the mutant in the presence of the higher concentration level of clavulanic acid (5+32 mg/l of amp+clav).

The other mutants "B" (1-4) obtained from mutant "A" (fig 8) showed an even further increase in the MICs of ampicillin (>2560 mg/l) (table 40) than the parent strain "A". The MICs of clavulanic acid for these mutants were doubled, and, for B1 and B2, there was a 4-fold increase over their parent strain (table 40). These mutants (B1-B4) lost their resistances to ampicillin in the presence of higher concentration level of clavulanic acid (32 mg/l) and to augmentin (table 40).

Interestingly, one of each of these mutants ("A" and "B") had acquired resistance to cefuroxime at the concentration level of 4 mg/l. Apart from this, no mutants had acquired any resistances to any other of the cephalosporins tested (ceftazidime and cefotaxime).

3.6.2A2 Resistances to Non-beta-lactam Antibiotics of the Mutants "A" and "B"

The mutants "A" and "B" were examined for their resistance profile to all other common non-beta-lactam antibacterial agents at fixed concentrations. The results (table 40) showed that the mutant "A" had the same resistance pattern as its parent

strain *E.coli* K-12 strain. The clavulanic acid resistant mutants B1-B4 differed from their parent strain "A"; they lost the resistances to antibiotics such as chloramphenicol, streptomycin, spectinomycin and sulphamethoxazole after exposure to the beta-lactamase inhibitor, clavulanic acid.

3.6.2A3 Examination of Synergy between Ampicillin and Clavulanic Acid as A "Combination" against the Mutants "A" and "B"

The Fractional Inhibitory Concentrations (FICs) of ampicillin (1.25-2560 mg/l) in the presence of clavulanic acid (0.312-320 mg/l), were measured for the mutants "A" and "B" and the original *E.coli* K-12 strain. This was performed to evaluate the synergy between the beta-lactam (ampicillin) and the beta-lactamase inhibitor (clavulanic acid). The results are presented in table 40, and they indicated that there was a decrease in the synergy between ampicillin and clavulanic acid in the mutants. The FIC indices of original strain *E.coli* K-12 strain was 0.029 whereas the values for the mutants ("A" and "B") were 0.156 i.e. about a 6-fold increase in the mutants. The outcome of this experiment suggests that there was a strong synergism between ampicillin and clavulanic acid in the original *E.coli* strain; nevertheless, this synergism declined as the strain became resistant to the combination of ampicillin and clavulanic acid.

3.6.2A4 Exploration of the Resistance Mechanism in Mutant "A"

Firstly, beta-lactamase produced by this mutant was extracted and identified, primarily to see whether it was different from the normal TEM-1 enzyme. The TEM-1 beta-lactamases produced by both the mutant and the parent were identified on polyacrylamide gel by analytical IEF (Materials and Methods) with a 1:1 mixture of

ampholines (pH 4-6 and 3.5-10) to increase the resolution at the lower pH range. Even though the TEM-1 enzyme from the mutant appeared similar to the normal TEM-1 enzyme with respect to its pI and banding on the gel, it differed from the original enzyme. It produced much more prominent bands with nitrocephin (Materials and Methods) and also had increased satellite banding on the gel (fig 9). The darkened bands of TEM-1 beta-lactamase from the mutant "A" suggested that there was a higher level of enzyme production than in the parent strain. However, a detailed study on the biochemical properties of these beta-lactamases were performed to check whether the mutant did indeed produced the same TEM-1 enzyme as the parent strain.

3.6.2A4a Comparison of Biochemical Properties of the Mutant "A" to its parent *E.coli* K-12 Strain

Beta-lactamases of both the mutant and its parent strain were analysed for their hydrolysis capability, protein concentrations and, therefore, their specific activities (Material and Methods) against nitrocephin as a substrate. The specific activities showed that there was about a 12-fold increased activity in the mutant comparing to that of the original TEM-1 enzyme (table 41)

The substrate profile of the enzymes with different beta-lactam antibiotics revealed that the increased specific activity did not alter the relative rates of hydrolysis of TEM-1 beta-lactamase produced in the mutant. Despite the increased specific activity in the mutant, the substrate profile was the same as the original TEM-1 beta-lactamase.

Inhibition studies with clavulanic acid employing nitrocephin as a substrate showed

This experiment was performed on four separate occasions and the result shown is representative of that found on each occasion.

Figure 9

Isoelectric Focusing of Beta-lactamases from R1 Plasmid Containing Standard Strain and its Mutant 'A'.

Track A - E. coli K12 (R1) (TEM-1 enzyme)

Track B - Mutant 'A' (LN182) (Hyperproduced TEM-1)

Table 41

Specific Activities and Inhibition Properties of the Mutant 'A' and Its Parent Strain *E. coli* K-12

Strain	Specific activity[a]	ID_{50} Clav[b]	Km[c] (μM)	Ki[d] (μM)
Parent strain *E. coli* K-12	219.1	9.5×10^{-8}	90	1.6
Mutant 'A'	17.3	1×10^{-7}	66.7	0.36

a, Specific activity = $\frac{\text{Enzymic activity against nitrocephin}}{\text{Protein concentration}}$

(μ moles nitrocephin hydrolysed min^{-1} mg $protein^{-1}$)

b, ID_{50} = Concentration of clavulanic acid required to inhibit 50% of enzymic activity against nitrocephin

c, Km = Michaelis constant (the Km value is the substrate concentration at which half the maximal velocity occurs)

d, Ki = The inhibitor constant for clavulanic acid (inhibition of the enzyme is competitive and the double reciprocal plot intercepts the ordinate at the same point as the Km line, see graphs 10a and 10b)

$$Ki = \frac{i}{\frac{Kp}{Km} - 1}$$

i = concentration of the inhibitors in μM

This result represents the average of three separate determinations

that both the original TEM-1 and the mutant's enzyme were inhibited by clavulanic acid to the same extent. Their ID_{50} and K_i values were similar (table 41 and fig 10a, b) as there were no significant differences. Therefore, the difference in the mutant appears to derive entirely from increased production of the enzyme. The genetic basis for the increased production of the TEM-1 enzyme of the mutant was found out by analysing the plasmid DNA.

3.6.2A4b Genetic Background for the Hyperproduction of the TEM-1 Enzyme in the Mutant "A"

The plasmids in the mutant "A" was extracted and analysed by 0.5% agarose gel electrophoresis as described by Takahashi and Nagano (1984). The plasmid size was determined from a calibration curve and compared to the original R1 plasmid. The characterisation of the plasmid DNA showed that there had been a plasmid rearrangement in the mutant. The plasmid R1 containing strain possessed a single plasmid of 90kb. In the mutant, in addition to the original plasmid R1 (90kb), there were two small plasmids with the sizes of 48 and >10kbases (fig 11). This observation indicates that the mutant had acquired beta-lactamase determinants on small multicopy plasmids in addition to the original large plasmid which had been present in its parent strain. Further experiments were carried out in order to test what effect this rearrangement had on the transferability of resistance to the combination of ampicillin and clavulanic acid from the mutant strain.

3.6.2A4c Transconjugants of the Mutant "A"

The results of the conjugation experiments demonstrated that the acquired resistance to the drug combination in the mutant was not transferable.

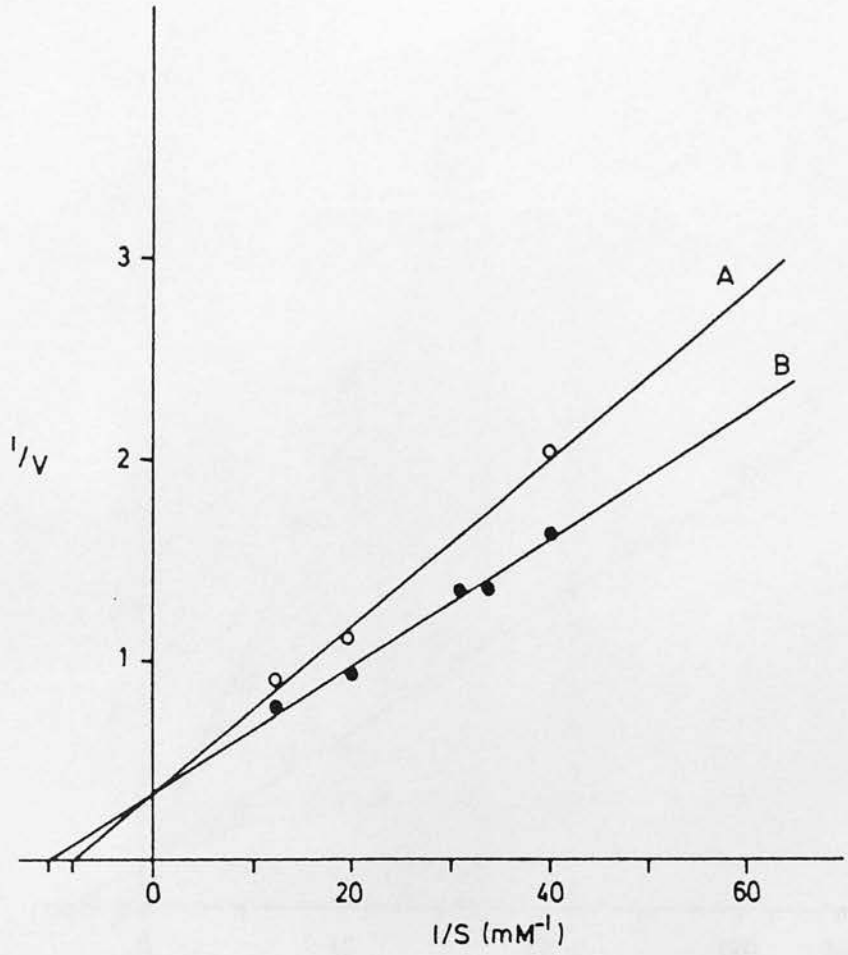


Figure 10a

Lineweaver-Burk Plot of the TEM-1 Beta-lactamase

A = with clavulanic acid
B = without clavulanic acid (substrate nitrocephin)

This experiment was performed on three separate occasions and the result shown is representative of that found on each occasion.

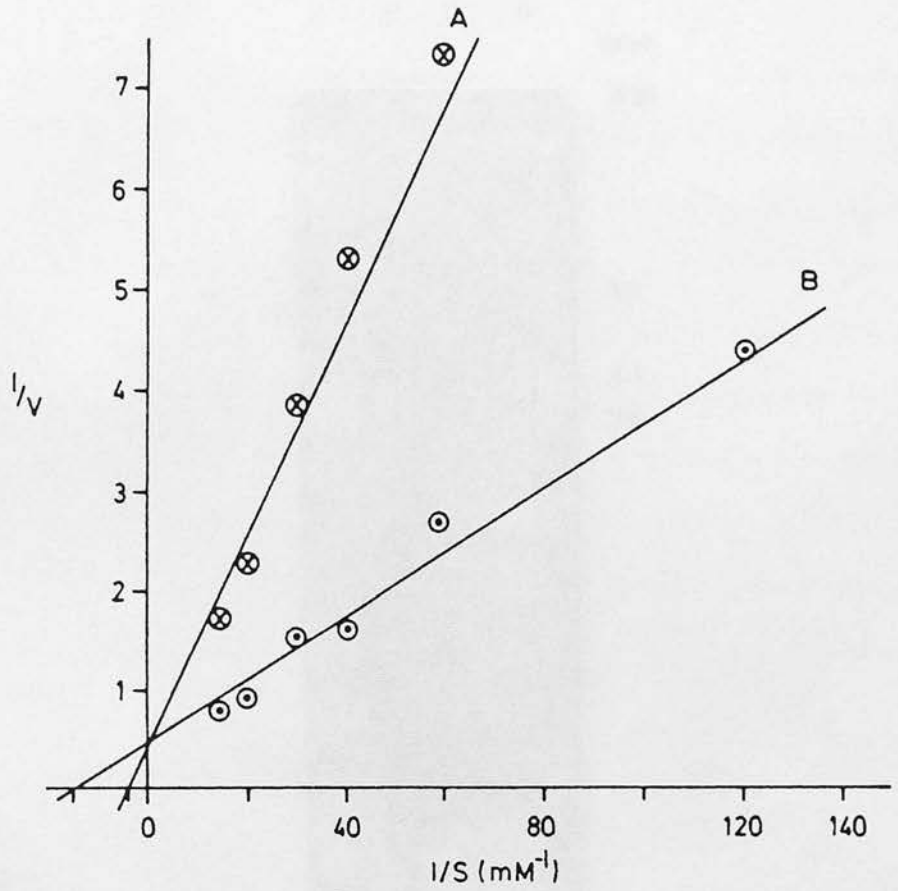
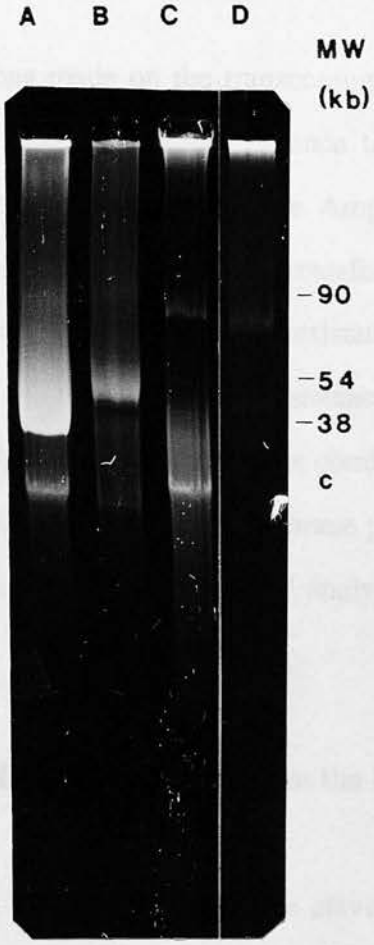


Figure 10b

Lineweaver-Burk Plot of the Mutant 'A' TEM-1 Beta-lactamase.

A = with clavulanic acid
B = without clavulanic acid (substrate nitrocephin)

This experiment was performed on three separate occasions and the result shown is representative of that found on each occasion.



This experiment was performed on five separate occasions and the result shown is representative of that found on each occasion.

Figure 11

DNA Profile of Plasmid R1 and Plasmids from its Mutant 'A'.

Track A - Plasmid R6k
Track B - Plasmid R1010
Track C - Plasmid R1
Track D - Plasmids from the mutant 'A'

'C' - chromosomal DNA

Ampicillin resistance alone was transferred into the standard strain by conjugation. Study of this transconjugant showed that it was exactly as same as the original *E.coli* K-12 strain in all respects (table 42, fig 12). The transconjugant possessed only a single large plasmid (R1) and produced the normal TEM-1 beta-lactamase (fig-12). The other resistance determinants that it possessed were also identical to the R1 plasmid of *E.coli* K12 strain (table 42).

From these observations made on the transconjugant selected on ampicillin, it was clear that the genetic location of the resistance to the combined drug (amp+clav) must be on one of the small plasmids. The Amp+Clav resistance determinant was present in the mutant "A" but it could not transfer into the recipient with the large R1 plasmid, and so the transconjugant was resistant to ampicillin only. There might be an involvement of non-beta-lactamase resistance mechanism such as diminished permeability preventing the entry of antibiotic combination. To establish the possible resistance mechanisms involved, outer membrane proteins of both the mutant *E.coli* strain and original strain were extracted and analysed. The results are presented in section 3.6.2A8.

3.6.2A5 Resistance Mechanisms Involved in the Mutants "B"

A complete study was also performed on the clavulanic acid resistant mutants "B". Beta-lactamases from the mutants were extracted and identified by IEF. The TEM-1 beta-lactamases produced in the mutants "B" were no different from the enzyme produced in their parent strain, mutant "A" (fig 13). This showed clearly that these beta-lactamases, TEM-1, were also hyperproduced in the "B" mutants.

Specific activities of these enzymes were determined and only one mutant

Table 42

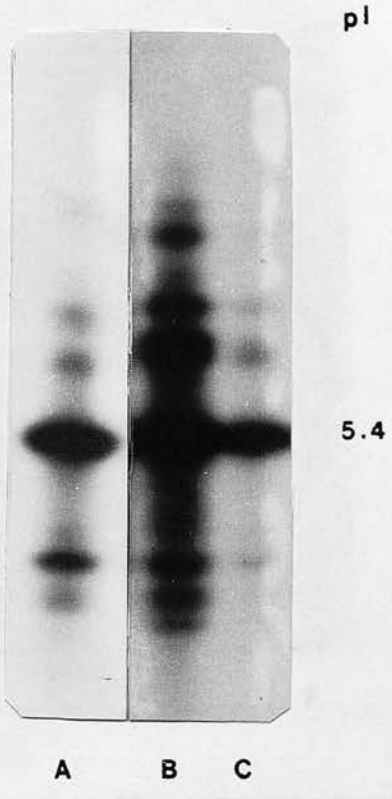
Characteristics of Mutant 'A', *E. coli* J62-2 (R1) and Their J62-2 Transconjugants

Parent strain	Mutant no.	Mics[a]					
		Ap	Clav	Ap + clav[b]	Ap + clav[c]	Aug[d]	Sp.act[e]
E. coli K12 (R1)	-	480	40	< 2.5 + 8	< 2.5 + 32	20+4	17.3
J62-2 trans[f] of *E. coli* K12 (R1)	-	480	40	< 2.5 + 8	< 2.5 + 32	20+4	17.0
E. coli K12 (R1)	'A'	2560	40	160 + 8	10 + 32	40+8	219.1
J62-2 trans[f] of Mutant 'A'	-	490	40	< 2.5 + 8	< 2.5 + 32	20+4	18.3

a = Minimum inhibitory concentrations (mg/L)
b = Ampicillin + clavulanic acid (8 mg/L)
c = Ampicillin + clavulanic acid (32 mg/L)
d = Augmentin (Amoxycillin + clavulanic acid) (2.5/0.5 - 160/32 mg/L)
e = Specific activity (μM nitrocephin hydrolysed/min/mg protein
f = J62-2 transconjugant

All strains shown in this table are resistant to Chloramphenicol, Streptomycin, Spectinomycin, Sulphamethoxazole.

This experiment was performed on two separate occasions and the result shown is representative of that found on each occasion.



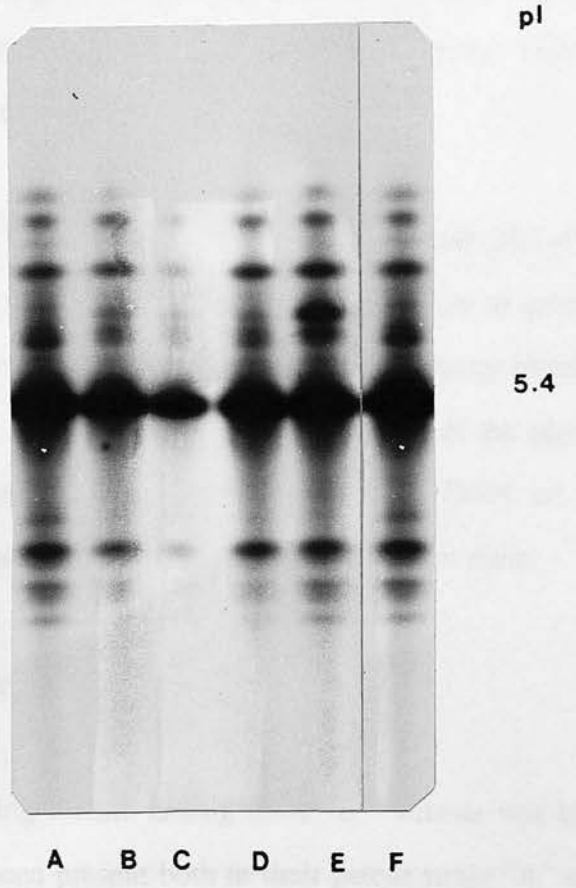
This experiment was performed on five separate occasions and the result shown is representative of that found on each occasion.

Figure 12

Isoelectric Focusing of TEM-1 Beta-lactamases from R1 Plasmid Containing Standard Strain, Mutant 'A' and the Transconjugant of Mutant 'A'.

Track A - *E. coli* K12 (R1) Track B - Mutant 'A' (LN182)

Track C - Transconjugant of mutant 'A' (LN 286)



This experiment was performed on five separate occasions and the result shown is representative of that found on each occasion.

Figure 13

Isoelectric Focusing of TEM-1 Beta-lactamases from R1 Plasmid Containing Standard Strain, Mutant 'A' and Mutants B1 - B4.

Tracks A,B - Mutants B1, B2, (LN281, 282)

Track C - *E. coli* K12 (R1)

Tracks D,E - Mutants B3, B4 (LB284,285)

Track F - Mutant 'A' (LN182)

was found to have increased activity (table 43). There was a variation between the enzymic activities of the mutants "B". Two mutants B2 and B4, selected on 64 and 128 mg/l of clavulanic acid respectively, had the same activity of TEM-1 enzyme as their parent strain. The mutant B3 showed decreased beta-lactamase specific activity while the other mutant, B1, produced TEM-1 enzyme with a 3-fold increased activity in comparison with the parent TEM-1 beta-lactamase from the mutant "A" (table 43).

From these clavulanic acid resistant mutants (B1-4), no resistances could be transferred, not even the resistance determinant to ampicillin. When attempts were made to transfer the resistances with a mobilising plasmid X^+, no transfer was possible. Some rearrangements of the plasmids were taking place. So the following study analysed the plasmid DNA of these mutants in order to determine the presence and types of plasmids in them.

3.6.2A6 Plasmids in the Mutants "B"

The interesting feature among these "B" mutants was loss of the large plasmid R1, which had been present both in their parent strain "A" and in the *E.coli* K-12 strain (fig 14). The "B" mutants retained the other two small plasmids (3.8 and >10kbases), which were also harboured by the mutant "A".

As these plasmids were neither self-transferable nor mobilisable, these small plasmids were transformed into a sensitive strain (*E.coli* MC 1022) to determine the resistance to clavulanic acid was decided on these plasmids. Outer membrane proteins of the mutants were also examined to assess any contribution of non-enzymic resistance mechanism

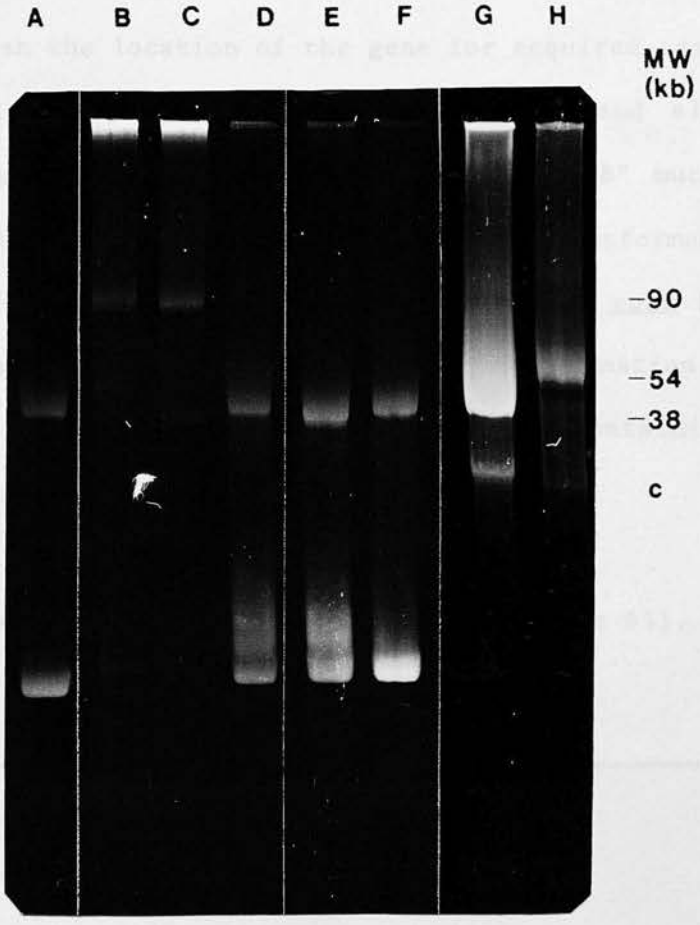
Table 43

Specific Activities of Beta-lactamases Produced by the 'B' Mutants and Their Parent Mutant 'A'

Parent strain	Mutant No.	Specific activities*
E. coli K12 (R1)	A	219.1
A	B1	645.7
A	B2	213.8
A	B3	83.8
A	B4	218.0

* μ moles of nitrocephin hydrolysed/min/mg protein

This result represents the average of two separate determinations



This experiment was performed on four separate occasions and the result shown is representative of that found on each occasion.

Figure 14

DNA Profiles of Plasmids from Mutants B1 - B4, Standard *E. coli* R1 and Mutant 'A'.

Track A - Mutant B1 (LN281)
Track B - Mutant 'A' (LN182)
Track C - Standard *E. coli* (R1)
Track D - Mutant B2 (LN282)
Track E - Mutant B3 (LN284)
Track F - Mutant B4 (LN285)
Track G - Plasmid R6k
Track H - Plasmid R1010
c = chromosomal DNA

in these mutants "B".

3.6.2A7 Transformation of Small Plasmids into a Sensitive Strain

In order to establish the location of the gene for acquired resistances to clavulanic acid, to augmentin and to cefuroxime in one mutant and elevated resistances to ampicillin, the small plasmids present in one of the "B" mutants (Cxm^r MIC 4mg/1) which were nontransferable and nonmobilisable were transformed into a sensitive E. coli MC1022 (sm^r) strain. The competent cells of E. coli MC 1022 were made as described in the Materials and Methods. After transformation, competent cells were spread out on the surface of the MacConkey plates containing suitable selective antibiotics (Ap+Sm and Sm at 10mg/1).

The frequency of the transformation was calculated (table 44).

Table 44

Dilution of the recipient strain (E. coli) MC 122 used for the calculation of the frequency rate	$= 10^{-3}$
Viable count on control plates	= 1000
Count of transformants obtained on selective plates	= 50
Frequency of transformation	$= 5 \times 10^{-2}$

This result was confirmed on two separate occasions.

The results suggested the rate of transformation was very high as 50 colonies out of

1000 control colonies could grow on the selective plates after transformation (table 44).

When these 50 presumed transformants were tested for their MICs for ampicillin and augmentin, all of them were shown to be highly resistant to ampicillin (MIC

›2560mg/l) and augmentin (MIC › 80mg/l of amoxycillin+ 16 mg/l of clavulanic acid) but not to cefuroxime. When these transformants were investigated for their plasmid content none of them was found to contain plasmid DNA. Consequently, the same transformants were checked for the production of beta-lactamase and all were found to produce the enzyme.

On the basis of these results, the small plasmids of the mutants "A" and "B" seem to be responsible for hyperproduction of the TEM-1 beta-lactamase, leading to the acquired resistance to the drug combination and high level MICs to ampicillin. On the other hand, these small plasmids did not seem to possess any resistance genes for cefuroxime and clavulanic acid alone.

The inability to detect plasmid DNA in the transformants might be a result of the incorporation of resistance genes into the chromosomal DNA of the recipient strain E. coli MC 1022. Such incorporation is a rare event and the inability to detect the DNA may have resulted from problems in the plasmid preparation technique. It is unlikely that mutation was the cause because of the high level of resistance and the sensitivity to clavulanic acid combinations.

These mutants "A" and "B" were also examined to establish if there were any modifications in their outer membrane protein offering contribution towards clavulanic acid and cefuroxime (in one mutant) resistances.

3.6.2A8 Outer Membrane Proteins of the Mutants "A" And "B"

In E. coli, porins are one of the most abundant proteins and present in approximately 100,000 copies for cell. They usually have molecular weights between 30,000 and 40,000 (Nikaido, 1989). E. coli K-12 produce two types, OMP F (outer membrane protein "F") and OMP C (outer membrane protein "C"). These two porins ("F" and

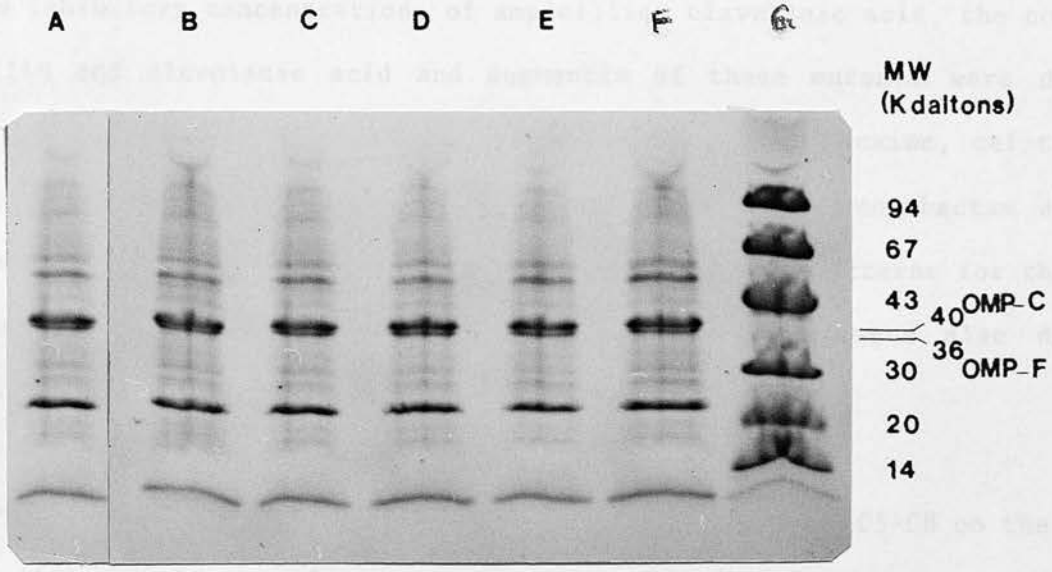
"C") are the main channels for the beta-lactam antibiotic passage into the cells and have the molecular weights of 36K and 40K respectively (Nikaido, 1989). The OMP "F" is a main channel for beta-lactams , especially cephalosporins.

Mutants "A" and "B" were examined for porin modification by extracting their OMP proteins (Materials and Methods) and analysing them by the Phast System on an SDS-PAGE gel (Materials and Methods). As it can be seen in fig 15, all the mutants "A" and "B" possessed unaltered OMP "F" and OMP"C" porins when compared to their control parent strain *E.coli* K-12 (R1). The expression of these two porins was found to be the same in comparison with their parent porins. It can be deduced from these results that as there were no alterations in the porin proteins of the mutants, there was no involvement of the outer membrane proteins sharing resistance mechanism to clavulanic acid and cefuroxime.

From the previous experiments it was confirmed that the hyperproduction of TEM-1 beta-lactamase was the main resistance mechanism among these mutants responsible for high MIC levels to ampicillin and resistance to augmentin, even though the resistance mechanisms for clavulanic acid alone and cefuroxime were not clarified

3.6.2B Selection of Clavulanic Acid Resistant Mutants "C" And "D" Directly from the *E.coli* K-12 Strain

The second part of these mutation studies was to investigate the possible resistance mechanisms responsible for clavulanic acid resistance in mutants "C"and "D" selected directly from the R1 plasmid-containing *E.coli* K-12 strain (fig 8). In the first stage, the mutants "C" were selected on 64 mg/l of clavulanic acid. At the second stage, some of the "C" mutants obtained from the first stage, including the original *E.coli*



This result was confirmed on two separate occasions.

Figure 15

SDS-PAGE (The Phast System) of Outer Membrane Proteins Extracted from Standard E. coli (R1) Strain, Mutants A and B1 - B4.

Track A - E. coli K12 (R1)
Track B - Mutant 'A' (LN182)
Track C - Mutant B1 (LN281)
Track D - Mutant B2 (LN282)
Track E - Mutant B3 (LN284)
Track F - Mutant B4 (LN285)
Track G - Molecular weight proteins

K-12 strain, were further exposed to a higher concentration of clavulanic acid (128 mg/1). The strains obtained here were designated "D" mutants (fig 8). Mutation rates are shown in table 39a.

3.6.2B1 Antibiotic Susceptibility Tests for the Mutants "C"

The minimum inhibitory concentrations of ampicillin, clavulanic acid, the combination of ampicillin and clavulanic acid and augmentin of these mutants were determined (table 45). In addition, each mutant was tested against cefuroxime, cefotaxime and ceftazidime, second and third generation cephalosporins. The monobactam aztreonam was also included in these susceptibility tests. Resistance patterns for the mutants against non-beta-lactam antibiotics at fixed concentrations were also determined (table 45).

The group "C" mutants were divided into two subgroups C1-C4 and C5-C8 on the basis of their ampicillin resistance levels (table 45). The subgroup C1-C4 had increased resistances to both ampicillin (MIC 1280 mg/1) and clavulanic acid (MIC 80 mg/1). these mutants had also acquired resistance to augmentin when compared with the original E. coli strain. The second subgroup (C5-C8 mutants) was the same as their parent strain apart from their elevated resistance level to clavulanic acid (MIC 80 mg/1) (table 45).

As with mutant "B", there were some "C" mutants that became resistant to the second generation cephalosporin cefuroxime (>4mg/1) (table 45). This was regardless of their (c1-C4 and C5-C8) differences in the MICs to ampicillin.

The important observations made from the sensitivity testing indicated that the acquisition of resistance to clavulanic acid (64 mg/1) could not

Table 45

Resistance Levels and Patterns of Mutants "C" (Selected on 64 mg/L of Clavulanic Acid)

Parent strain	Mutant no.	Minimum inhibitory concentrations (mg/L) Ap	Clav	Aug (2.5/0.5-160/32)	Resistance patterns	FIC indices
E. coli K12 (R1)	-	480	40	20 + 4	Ap Cm Sm Sp Sx	0.029
E. coli K12 (R1)	C (1*,2*,3*,4)	1280	80	40 + 8	Ap Cm Sm Sp Sx	0.0325
E. coli K12 (R1)	C (5,6*,7*,8)	480	80	20 + 4	Ap Cm Sm Sp Sx	0.029 - 0.05

* = Resistant to cefuroxime (> 4 mg/L)

FIC = Fractional inhibitory concentrations

FIC Indices - This is the sum of the fraction inhibitory concentrations of the individual drugs measured in the presence of the other at their point of maximum efficacy. If the combined effect of the drugs is additive, the FIC index would be 1.0. Any value of less than 0.7 indicates synergy.

enable the mutants "C" to become resistant to the combination of amp+clav, although the subgroup C1-C4 did show some increased MICs to augmentin (table 45). When the mutants were tested against all common antibiotics (table 45), they had exactly the same resistance profile as their parent strains.

3.6.2B2 Fractional Inhibitory Concentration Indices

The Fractional Inhibitory Concentration Indices (FIC) to ampicillin (1.25-2560 mg/l) and clavulanic acid (0.312-320 mg/l) for the mutants "C" suggested there was no antagonism between those two drugs as there was no significant change in the FIC Index values of the combination for the mutants in comparison with that of the original *E.coli* strain (table 45).

3.6.2B3 Resistance Levels and Patterns of Mutants "D"

This set of mutants selected on clavulanic acid at 128 mg/l were also divided into four subgroups: The first two subgroups, D1 and D2 were selected from the mutants C1 (ampicillin MIC 1280 mg/l) and C7 (ampicillin MIC 480 mg/l) respectively (fig 8 and Table 46) and the second two subgroups were, D3 and D4 mutants which were selected directly from the original *E.coli* K-12 (fig 8 and table 46).

The first subgroup mutants "D1" had no change in their resistance levels either to ampicillin or to augmentin when compared to their parent strain, mutant "C1"; however, one of the mutants in this group did became more resistant to cefuroxime (MIC >8 mg/l) than its parent mutant had been. These mutants had a 4-fold increased MIC to clavulanic acid (table 46).

Table 46

Resistance Levels and Patterns of Mutants "D" (Selected on 128 mg/L of Clavulanic Acid)

Parent strain	Mutant no.	Minimum inhibitory concentrations mg/L			Resistance Pattern	FIC indices
		Ap	Clav	Aug (2.5/0.5-160/32)		
E. coli K12 (R1)		480	40	20 + 4	Ap Cm Sm Sp Sx	0.029
Mutant C_1	D1 (a**,b)	1280	160	40 + 8	Ap Cm Sm Sp Sx	0.032
Mutant C_7	D2 (a-f)	480	160	20 + 4	Ap Cm Sm Sp Sx	0.051
E.coli K12 (R1)	D3 (a,b)***	2560	320	320 + 64	Ap Cm Sm Sp Sx	0.029
E. coli K12 (R1)	D4 (a)	2560	320	10 + 2	Ap Cm Sm Sp Sx	0.029

FIC = Fractional inhibitory concentrations

** = Resistant to cefuroxime (>8 mg/L)
*** = Resistant to both cefuroxime (>8 mg/L) and cefotaxime (>8 mg/L)

FIC Indices - This is the sum of the fraction inhibitory concentrations of the individual drugs measured in the presence of the other at their point of maximum efficacy. If the combined effect of the drugs is additive, the FIC index would be 1.0. Any value of less than 0.7 indicates synergy.

The subgroup "D2" did not show any changes in the resistance levels from their parent mutant "C7" (table 45) to the antibiotics tested (table 46), apart from the resistance to clavulanic acid (MIC 160 mg/l); however, they lost resistance to cefuroxime which was present in their parent strain, mutant "C7" (table 45).

The subgroup "D3" mutants, derived directly from *E.coli* K-12 strain, became significantly resistant to both ampicillin and clavulanic acid separately. The results are presented in table 46. Similarly, they became highly resistant to augmentin, reaching MIC of amoxycillin of 320 mg/l of in the presence of 64 mg/l of clavulanic acid. These were the mutants which possessed resistances to both cefuroxime (>8 mg/l) and cefotaxime (>8 mg/l) (table 46). The last subgroup "D4" mutant which was also directly obtained from *E.coli* K-12 strain was found to be different from the "D3" mutants. Except for possessing high levels of resistances to both ampicillin and clavulanic acid separately, no other special features were observed for mutants "D4" (table 46).

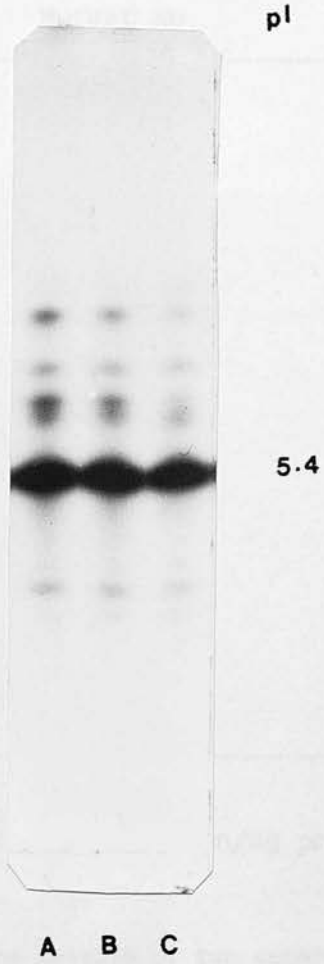
The common characteristics among these "D" mutants (D1-D4) were that they did not acquire resistance to the combination of ampicillin and clavulanic acid on exposure to clavulanic acid alone. Furthermore, they all retained the same resistance patterns to other common antibiotics (table 46) as their parent strains. In addition, the differences in the FIC indices of drug combination (amp+clav) for these mutants were negligible, as there was a slight variation between them and the FIC index of the original *E.coli* K-12 strain (table 46), confirming the presence of a synergetic effect produced by the combination of ampicillin and clavulanic acid against these mutants.

3.6.2B4 Possible Resistance Mechanisms amongst Clavulanic Acid Mutants "C" And "D"

Beta-lactamases produced in these mutants "C" and "D" were identified and compared to the normal TEM-1 of their parent strain *E.coli* to determine their part of contribution to acquired resistance to clavulanic acid. The results of analytical isoelectric focusing with a narrow range ampholine (a 1:1 mixture of pH 4-6 and pH 3.5-10) showed that TEM-1 enzymes from the mutants were very similar to the normal TEM-1 enzyme (fig 16).

Unlike the previous mutants, "A" and "B", the IEF results for the enzymes produced by these mutants did not suggest that they have hyperproduced TEM-1 beta-lactamase. The specific activities of beta-lactamases of the mutants "C" and "D" were measured and compared to the normal TEM-1 beta-lactamase. There were considerable differences between the specific activities of TEM-1 beta-lactamases amongst the mutants (table 47). The mutants "D1" and "D3" produced TEM-1 enzyme with a 6 and an 8-fold increased activities respectively. Conversely, there were not any noticeable differences between the specific activities of TEM-1 enzymes produced by the mutants "D2" and "D4"; and they were similar to that of the normal TEM-1 enzyme (table 47).

The results provided by the plasmid-DNA characterisation revealed that the plasmids harboured by the mutants had no plasmid rearrangement. The sizes and the number of plasmids isolated from the mutants were exactly the same as the original R1-plasmid of the *E.coli* strain (fig 17).



This experiment was performed on three separate occasions and the result shown is representative of that found on each occasion.

Figure 16

Isoelectric Focusing of TEM-1 Beta-lactamases from Mutants 'C' and 'D' Groups and their parent *E. coli* Strain.

Track A - Group 'C' mutants Track B - Group 'D' mutants
Track C - *E. coli* K12 (R1)

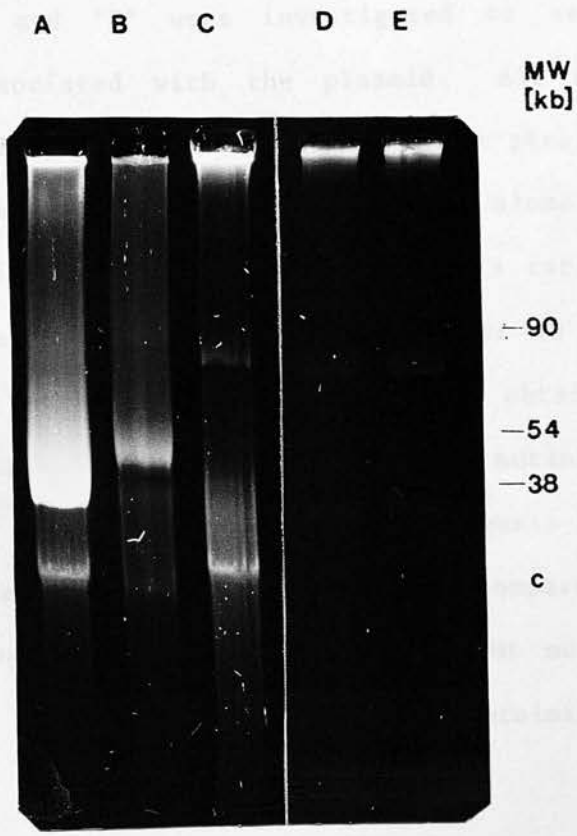
Table 47

Specific Activities of the Mutants 'C' and 'D'

Parent strain	Mutant No.	Specific activities*
E. coli K12 (R1)	-	17.3
E. coli K12 (R1)	C (1-4)	17.0 - 18.9
E. coli K12 (R1)	C (5-8)	15.0 - 17.0
Mutant C_1	D_1 (a,b)	113.0
Mutant C_7	D_2 (a-f)	18.0 - 20.0
E. coli K12 (R1)	D_3 (a,b)	158.9, 277.0
E. coli K12 (R1)	D_4 (a)	115.0

* μ moles nitrocephin hydrolysed/min/mg protein

This result represents the average of two separate determinations



This experiment was performed on two separate occasions and the result shown is representative of that found on each occasion.

Figure 17

Plasmid DNA Profile of Mutants 'C' and 'D' Groups with the Standard R1 Plasmid.

Track A - Plasmid R6K
Track B - Plasmid R1010
Track C - Plasmid R1
Track D - Mutants 'C' (LN249, 251-256)
Track E - Mutants 'D' (LN268-275,277-279)
C = chromosomal DNA

3.6.2B5 Transferability of the Clavulanic Acid Resistance from the Mutants "C" and "D"

The acquired resistances to clavulanic acid and other antibiotics (table 45 and 46) in the mutants "C" and "D" were investigated to see whether the resistance determinants were associated with the plasmid. All mutants "C" and "D" were subjected to conjugation with the standard recipient strain E. coli J62.2, to select the resistances for ampicillin and clavulanic acid alone. Overnight broth cultures of both donor and recipient strains were mixed in a ratio of 1:10 in a pre-warmed nutrient broth and incubated statically for another 18 hours as described in the Materials and Methods. As with the previous results obtained for the mutants "A" and "B", no other resistances were transferable from mutants "C" and "D" except the ampicillin resistance determinants. The transconjugants from these mutants selected on plates containing ampicillin were examined and compared to their parent strains. The transconjugants were different from their parent mutant strains but they were exactly the same as the original R1 plasmid containing E. coli strain in all respects.

The study of the mutants "C" and "D" revealed that the prime factor responsible for the acquired resistances to clavulanic acid, augmentin, cefuroxime and cefotaxime and also an increased resistance to ampicillin might have been diminished permeability of the drugs. This may have been in association with a high amount of beta-lactamase in some of the mutants obtained on exposure to clavulanic acid alone.

3.6.3 Factors Influencing the antibacterial activity of Beta-Lactams Plus Beta-lactamase Inhibitor in *E. coli* Strains Producing Extended-spectrum Beta-lactamases

Studies were performed to determine the relationship between beta-lactamase and antibacterial activity of the combination of ampicillin and clavulanic acid against TEM-3, TEM-5 and TEM-7 beta-lactamases produced in *E. coli* J53.

3.6.3A Effect of the Inoculum Size

Mutants of TEM-3, TEM-5 and TEM-7 enzymes which were resistant to ampicillin in combination with clavulanic acid (amp+clav) were selected at different antibiotic concentrations (12.5+2.5; 25+5 and 50+10 mg/1 of amp+clav). During the attempts to select the mutants, it was revealed that these strains producing extended-spectrum stable beta-lactamases were able to overcome the effect of the combination of amp+clav provided the inoculum size was high ($1x10^9$cfu).

The effect of the bacterial density of these strains in relation to their resistance to amp+clav was observed in two different ways. Bacterial cultures were diluted from $1x10^9$ cfu into a series of dilutions and each dilution was spread onto the plates containing increasing concentrations of amp+clav (table 48). These results suggested that the bacterial strains showed increased sensitivity as the inoculum size was reduced to amp+clav. No growth was seen at the higher concentrations of the drug combination (50+10 mg/1 of amp+clav) against the lowest dilution ($1x10^3$cfu) of the bacterial cultures (table 48); however, the MICs of a bacterial culture 10^5cfu (the normal concentration for MICs) showed growth but reduced in

Table 48

Viable Counts of TEM-3, TEM-5 and TEM-7 Beta-lactamases Producing Bacteria on Different Concentrations of Ampicillin + Clavulanic acid combination

	Bacterial densities (cfu/ml)											
Amp + clav[a] (mg/L)	10^3	10^5 (TEM-3)[b]	10^7	Neat	10^3	10^5 (TEM-5)[b]	10^7	Neat	10^3	10^5 (TEM-7)[b]	10^7	Neat
Control[c]	1045	TTC	TTC	ND	876	TTC	TTC	ND	2450	TTC	TTC	ND
12.5 + 2.5	20	1000	2052	TTC	10	45	500	TTC	28	1124	3050	TTC
25.0 + 5.0	10	94	808	TTC	-	4	38	495	18	125	1054	TTC
50.0 + 10.0	-	-	20	1700	-	-	-	45	-	-	100	8885

a = Ampicillin + Clavulanic acid (mg/L)

b = TEM-3, TEM-5 and TEM-7 beta-lactamases producing E. coli K12 (J53) strains.

TTC = Too many to count

comparison with a concentration of 10^9cfu of bacterial culture.

In a similar way, even at the highest inoculum ($1x10^9$cfu) of each bacterial culture, it was clear that the *E.coli* strains became sensitive as the concentration of amp+clav increased (12.5+2.5 < 25+5 < 50+10 mg/L) (table 48).

Some strains which seemed to be mutants grew at the highest concentration of the combined drug (50+10mg/l of amp+clav) after long exposure (60hrs) to the combination. It is possible, however, that the long exposure destroyed the beta-lactam antibiotics in the selection plates and that the strains that emerged were not resistant mutants. Despite this, two "mutants" were obtained from each E. coli strain producing TEM-3, TEM-5 and TEM-7 beta-lactamases. The presumed mutants were designated as 1M to 6M (mutants 1M-2M; 3M-4M; 5M-6M obtained from TEM-3, TEM-5 and TEM-7 producing E. coli strains respectively)(table 48).

3.6.3B Sensitivity Testing of Mutants "1M"-"6M"

They were maintained on the plates containing amp+clav as they seemed to be unstable mutants. These mutants were examined for their MIC levels to beta-lactams (ampicillin, clavulanic acid, amp+clav and augmentin) and also other non-beta-lactam antibiotics. The results revealed that none of the mutants "1M"-"6M" acquired a stable resistance to the antibiotic-inhibitor combination as they could not grow even at lowest concentration of ampicillin in the presence of clavulanic acid (<10+8mg/l) (table 49). On the other hand, all of them,including the corresponding parent strains,grew on plates containing augmentin (table 49).

Some other changes were noticed among these presumed mutants "1M-6M". The mutants 3M and 4M were the only ones which had

Table 49

Resistance Levels and Patterns of Mutants 1M - 6M

Parent strain	Mutant No.	MICs (mg/L)[a] Amp	A + C	Aug	Resistance patterns
E. coli K12[b] (J53)	-	>2560	-	20 + 4	Ap Ce Cm Km Sm Sp Tc Tp
E. coli K12[b] (J53)	1M	>2560	-	20 + 4	Ap Ce Cm Km Sm Sp Tc Tp
E. coli K12[b] (J53)	2M	>2560	-	20 + 4	Ap Ce Km Sm Sp Tc Tp
E. coli K12[c] (J53)	-	640	-	20 + 4	Ap Ce Cm Km Sm Sx Tc
E. coli K12[c] (J53)	3M	2560	-	20 + 4	Ap Ce Cm Km Sm Sx Tc
E. coli K12[c] (J53)	4M	2560	-	20 + 4	Ap Ce Km Sm Sx Tc
E. coli K12[d] (J53)	-	>2560	-	40 + 8	Ap Ce Gm Km Sx Tc
E. coli K12[d] (J53)	5M	1280	-	10 + 2	Ap Ce Gm Km Sx Tc
E. coli K12[d] (J53)	6M	1280	-	10 + 2	Ap Ce Gm Km Sx Tc

a = Minimum inhibitory concentrations
b = TEM-3 beta-lactamase producing E. coli K12 (J53) strain
c = TEM-5 beta-lactamase producing E. coli K12 (J53) strain
d = TEM-7 beta-lactamase producing E. coli K12 (J53) strain
A+C = Ampicillin + clavulanic acid (8 mg/L)

(4-fold) elevated MICs of ampicillin compared to their parent strain *E.coli*. the TEM-5 enzyme producing organism. A particularly interesting feature in the mutants 5M and 6M, obtained from TEM-7 enzyme producing *E.coli* strain, was a drop of its MIC levels both to ampicillin and augmentin (table 49).

The common feature among these mutants ("1M-6M") was that loss of resistance to chloramphenicol in some mutants and retention of other resistances compared to their parent strains (table 49).

3.6.3C Comparison between Resistances to Augmentin and Ampicillin/ Clavulanic acid among the Mutants and their Parent Strains

It was observed that the strains producing TEM-3, TEM-5 and TEM-7, the extended-spectrum beta-lactamases, were more resistant to augmentin than to ampicillin in association with clavulanic acid. Both parents and their mutants ("1M-6M") were able to grow in the presence of amoxycillin and clavulanic acid (MIC 20+8 mg/l for 1M-4M mutants and their parent strains and 40+8 mg/l for TEM-7 producing *E.coli* strain) (table 49). But the same strains with the same inoculum ($1x10^5$cfu) were not able to grow in the same concentration level of amp+clav. Despite their high levels of resistance to ampicillin and resistance to augmentin that they already possess, this did not enable them to acquire resistance to the combination amp+clav. However, the mutants 5M and 6M had 2-fold decrease in their resistance levels to both ampicillin and augmentin (table 49) after being exposed to ampicillin and clavulanic acid.

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3.6.4 Mutants Resistant to the Combination of Ampicillin and Clavulanic Acid from the R1010 Plasmid-containing Standard *E. coli* K12 Strain

The beta-lactamase encoded by the plasmid R1010 of the standard strain *E. coli* is SHV-1 enzyme, a broad-spectrum beta-lactamase, sensitive to inhibition by clavulanic acid. This enzyme is similar to TEM-1 beta-lactamase in its substrate profile. Thi clavulanic acid sensitive strain was exposed to a fixed combination of ampicillin (50 mg/l) and clavulanic acid (8 mg/l, a subinhibitory concentration). The resistan mutants were selected on the Oxoid DSTA plates with the incorporated antibiotics and purified on the same selective media. The calculation of mutational frequencies too place by counting the viable colonies on the purified plate and referring them to the total viable count of bacterial colonies on the control plates of MacConkey agar (see Table 50):

Table 50

Bacterial Strain	= *E. coli* K12 J53 (R1010)
Viable counts on control plates	= 1.98×10^{8}
Viable counts on antibiotic plates	= 1.57×10^{3}
Mutational frequency	= 7.9×10^{-5}

This result was confirmed on two separate occasions.

Of these mutants, six well grown and separated colonies were randomly selected and again purified. These mutants were designated as "S1-S6" and studied in detail.

3.6.4A Susceptibility Patterns of the mutants "S1-S6"

The selected mutant strains (S1-S6) concurrently with their parent strain E. coli K-12 J53 (R1010) were examined for their MICs of beta-lactam antibiotics (table 51). This sensitivity testing also included the second and third generation cephalosporins such as cefuroxime, cefotaxime and ceftazidime, and the monobactam aztreonam (table 51).

The results showed that all the mutants ("S1-S6") had raised MICs (>2560mg/1) of ampicillin (an 8-fold increase) (table 51). The MICs of ampicillin in the presence of clavulanic acid were not significant, even though these were mutants resistant to the drug combination. This can be shown by the fact that at a bacterial density of 10^5 there was only a 2-fold increase in resistance to the drug combination and even more elevated MICs were noticed with the increased bacterial inoculum (10^9) (table 51). On the other hand, all the mutants possessed a significant MIC (i.e. 80mg/1 of amoxycillin and 16mg/1 of clavulanic acid) to the drug augmentin compared to amp+clav combination (table 51) even at lower inoculum sizes (10^5cfu). In summary, the mutants acquired 2-fold increases in MIC for amp+clav and 4-fold increases in MIC for augmentin at a bacterial density of 10^5cfu in comparison with the standard parent strain.

In addition, the mutants became more resistant to the second and third generation cephalosporins and also the monobactam aztreonam (table 52). There was a 2-fold increased resistance to cefuroxime. The striking features were the resistance levels to ceftazidime which was about an 18-fold increase and a 4-fold increase in MIC levels of cefotaxime compared to the parent strain. The resistance to the drug monobactam (aztreonam) was also increased with these mutants (2-fold) (table 52) and one mutant

Table 51

Minimum Inhibitory Concentrations of Mutants S1 - S6

Parent strain	Mutant No.	MICs (mg/L) Amp[a] (10^5 cfu)[e]	A + C[b] (10^5 cfu)[e]	A + C[c] (10^9 cfu)[e]	Aug[d] (10^5 cfu)[e]
E. coli K12 (R1010)	-	320	10 + 8	10 + 8	20 + 4
E. coli K12 (R1010)	S1	>2560	20 + 8	80 + 8	80 + 16
E. coli K12 (R1010)	S2	>2560	20 + 8	80 + 8	80 + 16
E. coli K12 (R1010)	S3	>2560	20 + 8	80 + 8	80 + 16
E. coli K12 (R1010)	S4	>2560	40 + 8	80 + 8	80 + 16
E. coli K12 (R1010)	S5	>2560	40 + 8	80 + 8	80 + 16
E. coli K12 (R1010)	S6	>2560	20 + 8	80 + 8	80 + 16

a = Ampicillin

b = A + C, Ampicillin in the presence of 8mg/L of Clavulanic acid,

c = A + C, Ampicillin in the presence of 8 mg/L Clavulanic acid

d = Augmentin (Amoxycillin + Clavulanic acid; 2.5/0.5 - 160/32 mg/L)

e = bacterial density, cfu/ml.

Table 52

Resistance Levels and Resistance Patterns of Mutants S1 - S6

Parent Strain	Mutant No.	Resistance levels to cephalosporins (mg/L)				Resistance patterns
		Cxm[a]	Ctx[b]	Caz[c]	Az[d]	
E. coli K12 (R1010)	-	8	0.06	0.5	0.5	Ce Cm Sm Sx
E. coli K12 (R1010)	S1	>8	0.5	>8.0	1.0	Ce Cm Sm Sx
E. coli K12 (R1010)	S2	>8	0.5	>8.0	1.0	Ce Cm Sm Sx
E. coli K12 (R1010)	S3	>8	0.5	>8.0	1.0	Ce Cm Sm Sx
E. coli K12 (R1010)	S4	>8	0.5	>8.0	4.0	Ce Cm Sm Sx
E. coli K12 (R1010)	S5	>8	0.5	>8.0	1.0	Ce Cm Sm Sx
E. coli K12 (R1010)	S6	>8	0.5	>8.0	1.0	Ce Cm Sm Sx

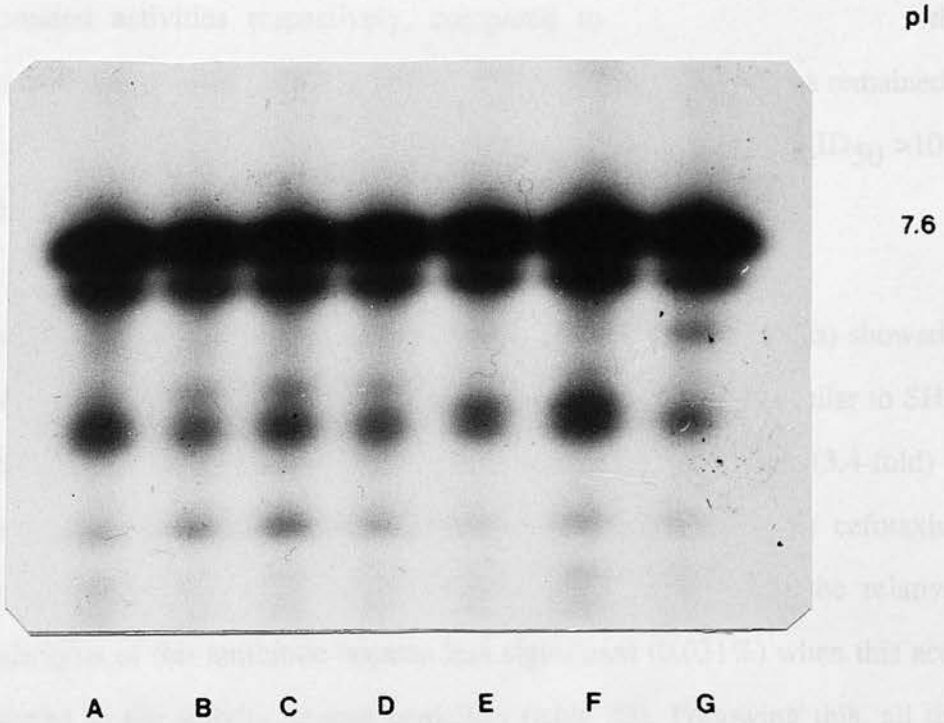
strain (S4) was shown to be significantly resistant to this drug (an 8 fold increase).

Both the mutants and standard *E.coli* strain had the same resistance patterns to other non-beta-lactam antibiotics (table 52).

It can be deduced from these results that the exposure to the combination of amp+clav diminished the bacterial sensitivity to the later generation cephalosporins whatever their resistance mechanisms might be; therefore, firstly, beta-lactamases produced by the mutants were investigated as a possible resistance mechanism.

3.6.4B Comparison of Beta-lactamases from Both the Mutants (S1-S6) and Original Parent Strain *E.coli* K12 (R1010)

Beta-lactamases produced by the mutants were identified by analytical isoelectric focusing. The comparison of the mutant enzymes and the standard SHV-1 enzyme was made on a polyacrylamide gel with a 1:1 mixture of 6-8 and 3.5-10.0 ampholines for a high resolution. The critical comparative studies revealed one of the main satellite bands disappeared in the mutant beta-lactamases which was prominently present in the standard SHV-1 enzyme (fig 18). Apart from this observation, mutant beta-lactamases were identical to their parent enzyme with respect to their band patterns. The effect of these mutant beta-lactamases, was tested against selected substrates to which the mutant strains became resistant.



This experiment was performed on four separate occasions and the result shown is representative of that found on each occasion.

Figure 18

SHV-1 Beta-lactamases Extracted from the Standard Strain E. coli K12 and from its Mutants S1 - S6

Tracks A-F - Mutants S1 - S6
Track G - E. coli K12 (R1010)

3.6.4c Biochemical Properties of the Beta-lactamases Produced by the Mutants "S1-S6"

The protein concentration and,subsequently, specific activities of the beta-lactamases of the cell-free extracts of the mutants were measured and compared to the standard SHV-1 enzyme (table 53). The results showed that there was an increase in the specific activities of the mutant enzymes with some variation between them. There was a 3-5 fold increased activity in 4 mutants compared with their parent strain. The other two mutants (S1 and S4) produced enzymes with a significant 17-fold and a 32-fold increased activities respectively, compared to the parent enzyme. Despite the increased specific activities, all the enzymes remained sensitive to clavulanic acid in the presence of nitrocephin as a substrate (ID_{50} >10^{-7}M) like their parent enzyme.

The substrate profiles (table 53) of these enzymes (S1-S6 mutants) showed that they had a rate of hydrolysis (penicillin 100%) against ampicillin similar to SHV-1 beta-lactamase; however, they had less effect on cephaloridine (3.4-fold) than the standard enzyme. They differed from SHV-1 by hydrolysing cefotaxime. Even though the hydrolysis of cefotaxime was clearly detectable, the relative rate of hydrolysis of this antibiotic became less significant (0.031%) when this activity was referred to the activity against penicillin (table 53). Following this, all the mutant strains ("S1-S6") were subjected to conjugation to examine the transferability of the resistances that they acquired.

3.6.4D Experiments on the Feasibility of the Transferable Resistances

Conjugation experiments were conducted employing the standard recipient strain *E.coli* J62.2; and an attempt was made to select for transconjugants on the DM plates

Table 53

Biochemical Properties of Mutants S1 - S6

Parent Strain	Mutant No.	Specific activities	Relative rates[a] of hydrolysis (%)			
			Pen	Amp	Cer	Ctx
E. coli K12 (R1010)	-	0.700	100	212	56	NH
E. coli K12 (R1010)	S1	12.19	ND	ND	ND	ND
E. coli K12 (R1010)	S2	2.2	ND	ND	ND	ND
E. coli K12 (R1010)	S3	3.32	ND	ND	ND	ND
E. coli K12 (R1010)	S4	22.7	100	143.9	16.6	0.031
E. coli K12 (R1010)	S5	2.24	ND	ND	ND	ND
E. coli K12 (R1010)	S6	3.6	ND	ND	ND	ND

a = Spectrophotometric assay method (expressed as a percentage of the value for benzyl penicillin)

Specific activities = μ moles hydrolysed/min/mg protein

ND = not done

NH = no hydrolysis

This result represents the average of two separate determinations

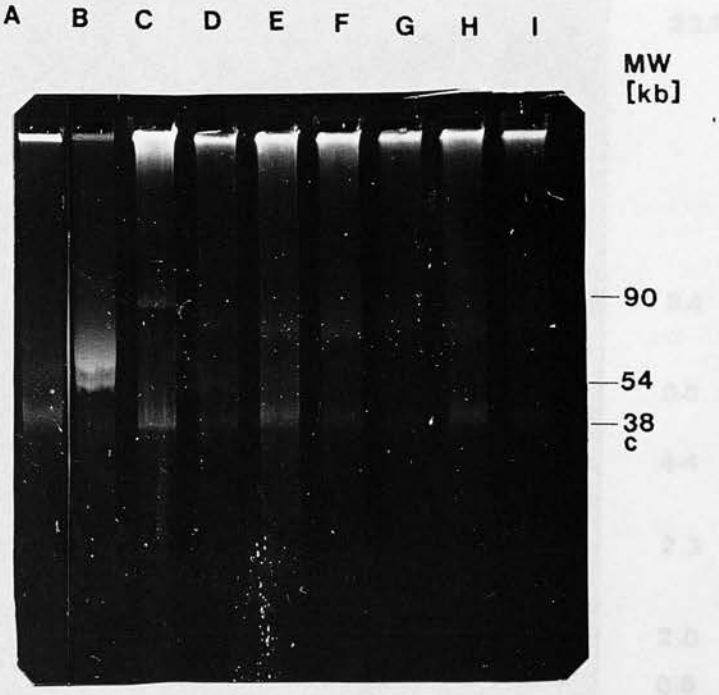
containing appropriate (ampicillin 10mg/l; cefuroxime, ceftazidime, cefotaxime and aztreonam 0.7mg/l) antibiotics. The results were negative showing no transferability even of ampicillin resistance.

Attempts to transfer these resistances were continued with the mobilising factor X^+. The method is described in the Materials and Methods section.
No resistances were mobilisable from these mutants; therefore, further investigation was carried out for characterisation of the plasmid-content of the mutants "S1-S6" which must be the genetic basis for the hyperproduction of the beta-lactamases.

3.6.4E Characterisation of Plasmid DNA

The extra-chromosomal DNA of the mutants ("S1-S6") was isolated and analysed by agarose gel (0.5%) electrophoresis according to the method described by Takahashi and Nagano (1984). The molecular mass of plasmids from the mutants were observed as slightly larger in their sizes with respect to the standard R1010 plasmid (fig 19). The uncertainty of the result suggest that a plasmid restriction digest might evaluate the plasmid size and if there had been any plasmid rearrangement. The plasmids were restricted by *Hind* III restriction enzyme (Materials and Methods). A single restricted fragment was detected in all the mutants and that included the standard plasmid R1010. All the restricted fragments migrated the same distance in the 0.7% agarose gel (fig 20). The sizes of these restricted fragments were calculated from the calibrated graph of known sizes of the lamda DNA which co-migrated in the gel with the sample DNA fragment.

The results might be due to a plasmid rearrangement in the form of "up mutations" of the promoter on the plasmids of the mutants, in



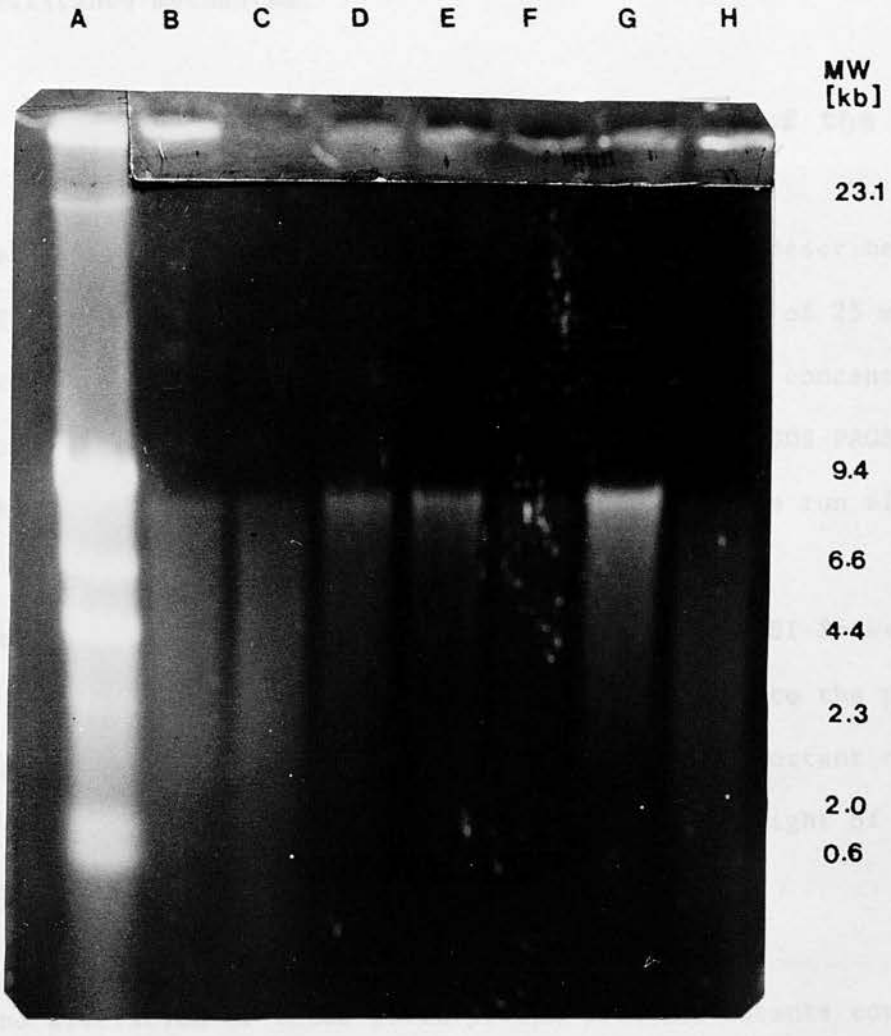
This experiment was performed on four separate occasions and the result shown is representative of that found on each occasion.

Figure 19

Plasmid DNA Profile of the Standard *E. coli* K12 (R1010) and of its Mutants S1 - S6.

Track A - Plasmid R6K
Track B - Plasmid R1010
Track C - Plasmid R1
Tracks D-I - Mutants S1 - S6 (LN306-311)

c = chromosomal DNA



This result was confirmed on two separate occasions.

Figure 20

Hind III Digestion of the Plasmids from the Standard E. coli K12 (R1010) and its Mutants S1 - S6.

Track A - λDNA
Tracks B-G - Mutants S1 - S6 (LN306-311)
Track H - E. coli K12 (R1010)

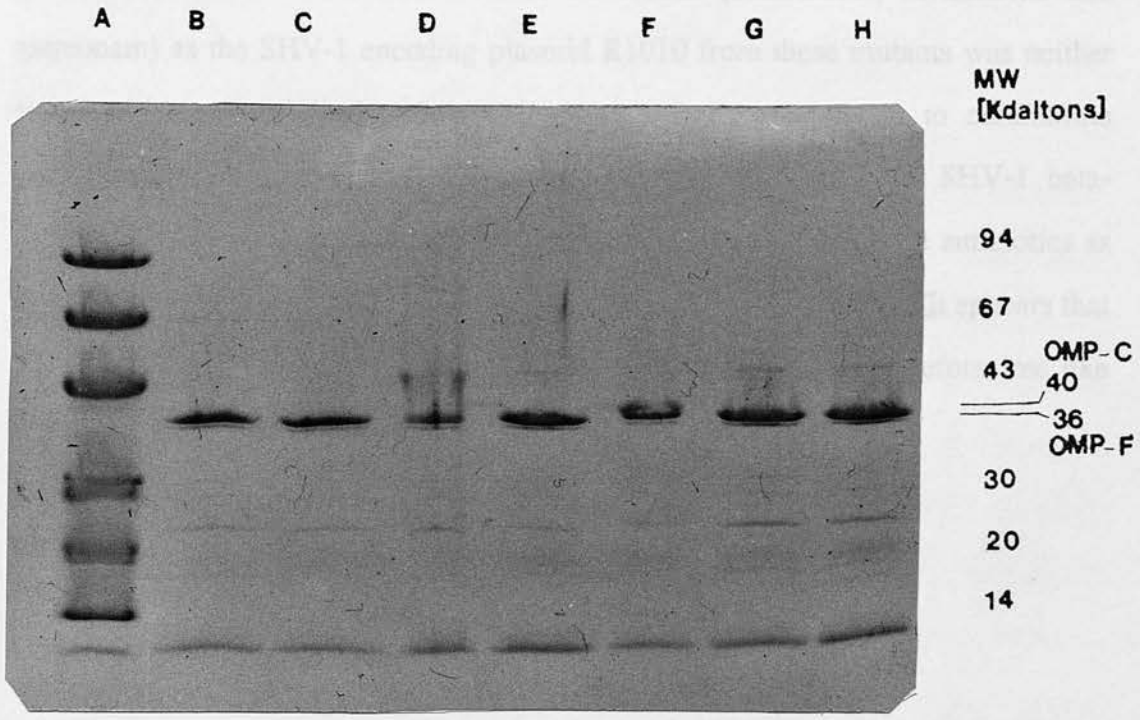
which case it was not possible to detect any changes in the plasmid. As it appeared that hyperproduction of beta-lactamases in the mutants was not sole resistance mechanism for their acquired resistances to the drug combination (amp+clav) and cephalosporins, the outer membrane proteins of the mutants were examined as a possible resistance mechanism.

3.6.4F Analysis of Outer Membrane Proteins of the Mutants "S1-S6"

The OMPs were extracted from 100 ml overnight cultures as described in Materials and Methods. The final OMP pellets were resuspended in 0.5 ml of 25 mM sodium phosphate buffer to increase the protein concentration. The protein concentrations were adjusted to 20-30 ng/μl. The Phast System Gradient gels (SDS-PAGE, 8-25) were used to focus the OMP bands. Molecular weight markers were also run simultaneously.

The profile of the outer membrane proteins of the mutants S1-S6 were no different from their parent strain E. coli K12 (R1010) with respect to the porins OMP "F" and OMP "C" (Fig. 21). These two porins are considered as important channels for beta-lactam antibiotics into the E. coli K12. The molecular weight of these porins are 36K (OMP "F") and 40K (OMP "C").

There was no alteration of these porin proteins in the mutants conferring resistances to the combination of ampicillin+clavulanic acid, the second (cefuroxime) and the third (cefotaxime and ceftazidime) generation cephalosporins, and also the monobactam aztreonam (Fig. 21).



This experiment was performed on two separate occasions and the result shown is representative of that found on each occasion.

Figure 21

SDS-PAGE (the Phast Sytem) of Outer Membrane Proteins Extracted from Standard E. coli (R1010) Strain and Mutants S1 - S6.

Track A - Molecular weight proteins
Track B - Mutant S1 (LN306)
Track C- Mutant S2 (LN307)
Track D - Mutant S3 (LN308)
Track E - Mutant S4 (LN309)
Track F - Mutant S5 (LN310)
Track G - Mutant S6 (LN311)
Track H - E. coli K12 (R1010)

The resistance to the drug combination (amp+clav), augmentin and high level ampicillin resistance must be the hyperproduction of SHV-1 beta-lactamase. Furthermore, this SHV-1 enzyme from the mutants was able to hydrolyse the third generation cephalosporin cefotaxime but not the other cephalosporins (cefuroxime or ceftazidime) and the monobactam aztreonam. It was difficult to determine the genetic location of resistances to these antibiotics (cefuroxime, ceftazidime and aztreonam) as the SHV-1 encoding plasmid R1010 from these mutants was neither transferable nor mobilisable. It could be argued that resistances to cefuroxime ceftazidime and aztreonam resulted from the hyperproduced SHV-1 beta-lactamase, but this hyperproduced enzyme could not break down these antibiotics as it had done with ampicillin +clavulanic acid and augmentin. It appears that mutated SHV-1 beta-lactamase is responsible for the hydrolysis of cefotaxime like SHV-2 plasmid-mediated beta-lactamase.

CHAPTER 4

DISCUSSION

4.1 Beta-lactam Resistance in South India

Bacterial evolution and the emergence of antibacterial drug resistance continue to interfere with the successful treatment of both community and nosocomial infections. Large amounts of antibiotics, especially tetracyclines, broad-spectrum penicillins/cephalosporins and sulphamethoxazole-trimethoprim, are being used throughout the world (Col and Conner, 1987). In most cases, the epidemiology is not studied and the spread of drug resistance not only goes unchecked but can also remain largely unnoticed. Almost all controlled surveys on beta-lactam resistance had been performed in Europe and North America (Medeiros, et al., 1980; Simpson, Harper and O'Callaghan, 1980; Medeiros, Gilleece and O'Brien, 1981; Roy et al., 1983, 1985; Gruneberg, 1984; Stobberingh, Houben and Van Boven, 1985; Simpson et al., 1986; Huovinen, Huovinen and Jacoby, 1988b; Reid et al., 1988b; Wiedemann, Kliebe and Kresken, 1989; Morfin-Otero et al., 1990) but not in developing countries. The first part of this thesis studied the incidence and type of beta-lactam resistance among enterobacterial pathogens isolated from significant bacteriuria, as well as *Salmonella* and *Shigella* isolates obtained from gastro-intestinal infections, over a three month period from the Christian Medical College Hospital, Vellore, South India (Young et al., 1986). In addition, biochemical, genetical and molecular biological aspects of the resistance mechanisms were investigated.

Ampicillin, amoxycillin and some cephalosporins had been in use for the

treatment of most infections (Urinary tract and gastro-intestinal) at the Christian Medical College Hospital in Vellore during the period over which the survey was conducted. These results revealed that there was a high incidence of ampicillin resistance (80.9%), and more than half of them were highly resistant to the drug (MIC >1280mg/l). The high proportion of the highly ampicillin resistant strains indicated the presence of plasmid-associated beta-lactamases among this resistant population. There have been different surveys on the epidemiology of plasmid-mediated beta-lactamases in Gram-negative bacteria isolated from different sources (faecal and urinary); but some surveys did not state the total number of strains obtained and source of the isolates (Roy et al., 1983, 1985; Simpson et al., 1986; Huovinen, Huovinen and Jacoby, 1988; Wiedemann, Kliebe and Kresken, 1989). The high incidence of ampicillin resistance of the present study had never been found before in similar surveys of urinary isolates of Enterobacteriaceae. Even in the recent Scottish multicentred survey (Reid et al., 1988b) where ampicillin resistance was believed to be unprecedented in the UK, the level of resistance was only 52.1%. In other surveys, *E.coli* had been the prevalent species (Gruneberg, 1984; Simpson et al., 1980, 1986) followed by *Klebsiella* spp (Simpson et al., 1980, 1986). The results of this survey were similar in that *E.coli* was the most common pathogen.

These Indian pathogens had hardly been exposed to cephalosporins (Collee and Amyes, 1986). Despite the lower usage of cephalosporins, most (65.1%) of the enterobacterial pathogens derived from urinary infections were resistant to cephaloridine; this included the *E.coli* strains (57.2%). This high proportion of resistance was even greater than in the earlier survey reports, i.e., 34% in London and 28.8% in Scotland (Gruneberg, 1984; Reid et al., 1988b). In this survey, only a very few isolates were highly resistant to cephaloridine (MIC >1280mg/l) which might indicate the lower cephalosporin usage. In the population of gastro-

intestinal pathogens (*Salmonella* and *Shigella* spp), only a few strains were resistant to cephaloridine.

The results of this thesis suggest that the high incidence of beta-lactam resistance among these Indian pathogens was derived from the general acquisition of the resistance genes by naturally sensitive bacteria (*E.coli*, *Salmonella*, *Shigella* spps) and was unbiased by the undue presence of intrinsically resistant species. As beta-lactam resistance has become prevalent among these sensitive bacteria (*E.coli*, *Salmonella* and *Shigella* spps), this has caused clinical resistance problems in India. In conclusion, the results for the incidences of ampicillin resistance in India were the highest in the world as previous surveys have shown variation in ampicillin resistance with the incidences range from 17-72% (Shales and Currie-McCumber,1989).

Resistance to later generation cephalosporins can also be a major problem in clinical situations and one of the main reasons for antibiotic research in this area. In most cases in this survey, only a small proportion of isolates (both urinary and gastro-intestinal) were shown to be resistant to either the second or third generation cephalosporins tested. A noticeable proportion of urinary pathogens (18%) and a remarkable percentage of *Salmonella* isolates (65.7%) were identified as cefuroxime resistant (MIC >8mg/l). No previous surveys have reported incidences reaching these levels. In addition, there were five *E.coli* isolates which were resistant to the whole series of cephalosporins tested; however, the resistances were not transferable and resistance mechanisms were assumed to be alterations in the membrane permeability (NB. TEM-1 was the only beta-lactamase produced by these bacteria). The same resistance mechanism was very likely to be responsible in the cefuroxime resistant urinary isolates. Usually, resistances to later generation

cephalosporins (3GC) are manifested by inducible chromosomal beta-lactamases (Quinn, DiVincenzo and Foster, 1987) or by slow hydrolysis of antibiotics mediated by beta-lactamase (Vu and Nikaido, 1985) or diminished penetration of these agents across the bacterial outer membrane (Bush et al., 1985; Nikaido, 1989). To this list may now be added active and stable plasmid-mediated beta-lactamases, which inactivate 3GC drugs (Philippon, Labia and Jacoby, 1989; Bush, 1989; Payne, Marriott and Amyes, 1989; Payne et al., 1989). These new enzymes now occur frequently (Philippon et al., 1989; Bush and Singer, 1989). The widespread cefuroxime resistance amongst *Salmonellae*, was found to be associated with R-plasmid mediated OXA-1 like enzyme and this is further discussed below.

The ampicillin susceptibility of the urinary pathogens, in the presence of the beta-lactamase inhibitor clavulanic acid had dropped to 41.5% (MIC of ampicillin >10-1280mg/l) and only 7 strains of *Klebsiella*, *Proteus* and *Enterobacter* spp. were highly resistant to this combination (MIC of ampicillin >1280mg/l). In contrast, a large proportion of salmonellae (62.7%) were resistant to this drug combination. On the other hand, very few isolates of shigellae were shown to resist ampicillin in the presence of clavulanic acid. The resistance to the combination of ampicillin and clavulanic acid can result (a) from changes in the cell wall, (b) from the production of new types of beta-lactamases and (c) most probably from the hyperproduction of the certain plasmid-mediated beta-lactamases such as TEM-1 or SHV-1 (Sanders et al., 1988; Martinez et al., 1988). Indeed, in the present study, three *E.coli* clinical isolates were found to produce the high quantities of R-plasmid-encoded TEM-1 beta-lactamases required to overcome the combination.

The most common mechanism by which clinically important bacteria become resistant to beta-lactam antibiotics is by acquisition of plasmid encoded beta-

lactamases. It is evident that R-plasmids evolve by a series of events that are initiated in many cases by the association of resistance determinants with transposable DNA sequences. Transfer of the resulting transposons to conjugative or nonconjugative mobilisable plasmids can lead to the spread of the determinants to other members of the species or closely, or even distantly, related species (Saunders, Hart and Saunders, 1986); and, thus, a worldwide spread of antibiotic resistance occurs. Transferable resistances in clinical isolates should be examined in epidemiological studies to evaluate the genetics of resistance determinants and their potential to disseminate. This is most comprehensively performed in parallel with biochemical investigations (e.g. beta-lactamase evaluation). Most previous studies in the literature only concentrated on biochemical analysis i.e. designation of plasmid-mediated beta-lactamases was made purely on the basis of biochemical results and no plasmid analysis was performed (Medeiros et al., 1980; Simpson, Harper and O'Callaghan, 1980; Medeiros, Gilleece and O'Brien, 1981; Roy et al., 1983, 1985; Simpson et al., 1986; Wiedemann, Kliebe and Kresken, 1989; Morfin-Otero et al., 1990). From the results in this thesis, it has been shown that many clinical isolates produced typical plasmid-mediated beta-lactamases, but the resistance genes encoding these enzymes were neither transferable or mobilisable. This result implies that determination of the genetic location of the beta-lactamases on the basis of biochemical evidence is not reliable. In a recent report, a new beta-lactamase (CMY-1), with a high pI 8.0 appeared to be chromosomally-determined. This was based on biochemical characteristics (isoelectric focusing and sensitivity testings) but conjugation experiments determined that it was a plasmid-mediated extended-spectrum beta-lactamase (Bauernfeind, Chong and Schweighart, 1989).

As it is possible to be misled by the employment of certain biochemical criteria, only a full characterisation of enzymes and the genetics of the gene will reveal the true nature of beta-lactam resistance.

A large number of ampicillin resistant clinical E. coli isolates (66.9%) possessed R-plasmids. This includes both autotransferable and mobilisable plasmids. The incidence of transferability was lower in other species of ampicillin resistant strains (Klebsiella spp, 51.4%; Shigella spp, 31%; and Salmonella spp, 15%). These data indicate only the self-transferable plasmids in those species. On most occasions transferability was found only in those strains which had high MICs of ampicillin (>1280 mg/l). This was true in all isolates except Shigella strains. Some transconjugants of shigellae showed higher MIC levels of ampicillin than their corresponding clinical isolates. This might result from better expression of resistance genes in the transconjugant strains than in their host strains. Certain plasmids sometimes are known to be expressed more efficiently in some host strains than others (Reid et al, 1988a).

On the other hand, resistance to cephaloridine was freely transferable from the strains of E. coli and some Klebsiella species despite the low MICs of the drug. Cephaloridine resistance was not transferable from the salmonellae. One interesting finding was that transferable resistance to ampicillin was often not accompanied by the transfer of resistance to cephaloridine (E. coli, Klebsiella, Salmonella and Shigella spp.) and vice versa (E. coli). This observation was made in the strains which produced the broad-spectrum TEM-1 and the penicillinase OXA-1 beta-lactamases and, amongst the Klebsiella spp., another broad-spectrum enzyme, SHV-1. Even though in some transconjugant strains TEM-1 was present in association with either OXA-1 or SHV-1, this combination did not enhance activity against cephaloridine in the intact cells.

These observations strongly suggest that the permeability of the clinical strains, in addition to the production of the beta-lactamases, might be a very important factor in the development of resistance. This can be explained as follows: in E. coli K12,

ampicillin permeates two to three fold more slowly than the cephaloridine which can be predicted from the differences in hydrophobicity. Penicillins have about a four times higher partition coefficient in octanol/water than the corresponding cephalosporins (Yoshimura and Nikaido, 1985). The relative rate of diffusion constant of cephaloridine is 167 whereas the equivalent for ampicillin is 46 (Yoshimura and Nikaido, 1985). These values indicate that ampicillin enters the periplasmic space of bacteria rather more slowly than cephaloridine. The TEM-1 enzymes have a much lower affinity for cephaloridine (16-20 fold) than for ampicill (Medeiros, Kent and O'Brien, 1974; Bush and Sykes, 1984); therefore, for TEM-1 producing strains, the differences in permeability to cephalosporins and penicillins in addition to differences in the TEM-1 affinity for the substrates might explain th differences in the susceptibilities to cephaloridine and ampicillin in the E. coli K12 J62.2 transconjugants. This also explains why, although in vitro the V_{max} value for the hydrolysis of cephaloridine are high, they are not rapid enough to inactivat sufficient beta-lactam and thus the amount of cephaloridine soon reaches a level which is lethal for E. coli K12.

In all individual bacterial spp. (E. coli, Klebsiella, Salmonella and Shigella) a wide variety of R-plasmid types were identified (minimum 8 types - maximum 38 types). This variation of plasmid type suggests that high-level beta-lactam resistance was not a consequence of the spread of a single or even a few plasmid types cross-infecting the whole bacterial population. Almost all the R-plasmids possessing beta lactam resistance genes also carried genes for other antibiotics; and, alarmingly, most of them conferred resistance to six or more drugs.

There are various factors interfering with plasmid transfer in vivo. The experimental models have revealed two major factors: 1) in general, there is a reduction of growth rate of

plasmid bearing bacteria in the mammals' digestive tract. This is a result of general dominance of antibiotic susceptible bacteria, 2) The efficiency of *in vivo* plasmid transfer does not differ from that occurring *in vitro* (Corpet,1986); however, very low rates of *in vivo* plasmid transfer are possible, These are related mainly to the population densities of *E.coli* in the gut (Corpet, 1986). These reports indicate the importance of the investigation of transferable resistances in the clinical isolates and to take account of the variation under different clinical conditions.

Plasmids in general vary in size up to greater than 400kb (Timmis et al., 1986). In the present study, there was considerable variation in the size of plasmids, ranging from as small as 11.5kb and as large as >200kb. Even though small (<16Md or <24kb) transferable plasmids are unlikely to occur (Wiedemann, 1981), there was a report of a plasmid (erythromycin, clindamycin and streptogramin resistant) as small as 10Md (15kb) found to be transmissible in *Bacteroides fragilis* (Jacoby, 1985). Some of these small plasmids could be transferable though it is not clear what the mechanism of transfer is.

In relation of ampicillin resistance to other common antibiotic resistances, the association of combined resistance genes of trimethoprim and sulphamethoxazole with the ampicillin resistance genes was widespread. This might reflect the high usage of co-trimoxazole in Vellore, South India, for the treatment of urinary infections (Young et al., 1986).

The distribution of beta-lactamases in the transconjugants of *E.coli*, *Klebsiella* spp, *Salmonella* spp and *Shigella* spp and the rest of the *E.coli* isolates from which resistance genes could not be transferred (either transfer or mobilisation) was studied by the isoelectric focusing (IEF) method. Isoelectric focusing has proved an

extremely useful tool for the detection of beta-lactamases in cell-free extracts, and has been used successfully in a number of previous surveys (Medeiros, 1984, 1989; Roy et al., 1983, 1985; Simpson et al., 1980, 1986; Reid et al, 1988b). This method has been one of the most sensitive for epidemiological purposes as it is able to produce very reproducible pIs and satellite banding patterns. This feature provides the capability to distinguish enzymes even if they differ by only a single amino acid residue. New enzymes can easily be differentiated from known beta-lactamases by IEF; and thus, only new beta-lactamases need be subjected to full biochemical characterisation.

In this study, the TEM-1 beta-lactamase was pre-eminent amongst the rest of the beta-lactamases identified in all individual species (*E.coli*, *Klebsiella* spp, *Salmonella* spp and *Shigella* spp). This is similar to previous surveys. The percentage of the TEM-1 enzyme was greater than 70% in all species. The distribution of the rest of the beta-lactamases types encountered in this study was not similar to the previous studies as the next commonest enzyme was the OXA-1 beta-lactamase. This was not so among *Klebsiella* isolates where SHV-1 enzyme is the second commonest. Indeed, the predominant enzyme in *Klebsiella* spp is usually the SHV-1 beta-lactamase (Roy et al., 1985). Overall, SHV-1 in *Klebsiellae* and OXA-1 in salmonellae and shigellae were the only other types of enzymes found besides TEM-1 enzyme. Amongst the *E.coli* population, a number of other previously documented enzymes; TEM-1, OXA-1, OXA-2, SHV-1, PSE-1 and PSE-2-were also found; but most of them were found on only a few occasions. In the previous literature, the largest number of different types of beta-lactamases were found in *Pseudomonas aeruginosa* (16) and *E.coli* (12), *Klebsiella* spp. (10), *Proteus mirabilis* (9), *Salmonella* spp. (6) and *Providencia* spp. (6) (Medeiros, 1989). These numbers have increased recently because of the

evolution of new broad-spectrum enzymes in the populations of *E.coli, Klebsiella* and *Salmonella* strains (Philippon, Labia and Jacoby, 1989; Bush, 1989).

In two transconjugant strains selected on a plate containing cephaloridine, no beta-lactamase was detected with the chromogenic cephalosporin nitrocephin. This might be because of either their poor or no activity against the substrate nitrocephin as it has been observed for two plasmid-mediated beta-lactamases. One of them, an enzyme (pI 8.0), produced by *Haemophilus influenzae*, was found to be non-reactive with nitrocephin (Rubin et al., 1981). The other enzyme (pI 5.7-5.9) from *Enterobacter cloacae* and *Klebsiella pneumoniae* gave a slow nitrocephin reaction (Corkill, Hart and Shears, 1989). These findings raise the possibility of beta-lactam treatment failure because of infections caused by apparently beta-lactamase negative clinical strains. Indeed, this did happen with the *H. influenzae* strain which produced the beta-lactamase non-reactive with nitrocephin (Rubin et al., 1981).

It was found in this study that 34.2% of the *E.coli* clinical isolates which neither transferred nor mobilised their resistance genes produced the TEM-1 beta-lactamase. This indicates that resistance genes encoding TEM-1 enzyme have probably been located on the chromosome. It has been noted that the migration of certain plasmid-associated antibiotic resistance genes into the bacterial chromosome has occurred, most notably, with trimethoprim resistance (Amyes, Doherty and Young, 1986). Uniquely, one *E.coli* strain produced both PSE-1 and PSE-2 beta-lactamases; and, although both have been found in members of the Enterobacteriaceae before (Medeiros, Hedges and Jacoby, 1982; Roy et al., 1983; Livermore, Maskell and Williams, 1984), they have not been found together in the same strain. The interesting feature of the strain producing these two PSEs was that it remained sensitive to a combination of clavulanic acid and ampicillin even though biochemical studies

have shown these enzymes were not sensitive to inhibition by clavulanic acid (Reid, A.J., Ph.D. Thesis). Another unusual E. coli isolate produced the PSE-1 enzyme in conjunction with the TEM-1 enzyme.

In the E. coli population, two other unusual plasmid-mediated enzymes and a further two novel beta-lactamases were identified. The two unusual enzymes were 1) hyper-produced TEM-1 beta-lactamase (TEM-1^{++}) which conferred resistance to the combination of ampicillin and clavulanic acid (MIC 40+8mg/l of Amp and Clav). This hyperproduced enzyme was encoded by plasmids of greater than 100kb. Hyperproduction is often a consequence of multiple copies of the plasmid or a more active resistance gene. Multiple copies almost invariably occur with small plasmids (<24kb) (Timmis et al., 1986). Hyperproduction by large plasmids often results from tandem repeats of the gene (gene amplification). In this case, it is likely that tandem repetitions of the ampicillin resistance genes or up mutations in the promoter region of the gene are responsible for hyperproduction (Korfmann, Kliebe and Wiedemann, 1986). The biochemical properties of the TEM-1^{++} enzyme were identical to those of the normal TEM-1 beta-lactamase and, therefore, the resistance to ampicillin/clavulanic acid most probably derives from the high production of the enzyme.

The second unusual beta-lactamase was TEM-1 (Caz^1) which could hydrolyse ceftazidime at a much higher rate than the normal TEM-1 enzyme could. This enzyme had a similar pI and banding pattern on IEF to TEM-1 enzyme; however, TEM-1 (Caz^1) focused with marginally higher pI on IEF with narrow range ampholines than the standard TEM-1. The TEM derived plasmid mediated extended-spectrum beta-lactamases currently being isolated in Europe and the USA

TEM-5, TEM-7, TEM-9, TEM-E1 and TEM-E2, have very similar pIs which lie between pI5.4 (TEM-1) and pI5.6 (TEM-2) (Sirot, Labia and Thabaut, 1987; Gutmann et al., 1988; Spencer et al., 1987; Payne, Marriott and Amyes, 1989; Payne et al., 1989). This feature has made it confusing to identify these enzymes unless they run side-by-side on a narrow range ampholines IEF gel; therefore, it is very easy to miss this type of enzyme on IEF gels with broad range ampholines under quick screening procedures.

Finally, the further two novel beta-lactamases identified among the *E.coli* transconjugants were CARB-6 and SAR-2.

CARB-6 is a carbenicillin hydrolysing enzyme of high pI. Although this enzyme had a broad-spectrum of activity, it had shown more activity towards carbenicillin. The plasmid encoding this enzyme was not stable in the strain and required antibiotic pressure to maintain it. Some previous studies indicated instability of plasmids may be observed after transfer to another host such as a laboratory strain of *E.coli* (Richmond and Sykes, 1972; Timmis, et al., 1986). This instability might result from certain circumstances such as inefficient expression of plasmid genes and host systems where the DNA polymerases are not suitable. Most plasmids are well adapted to the hosts in which they are found (Bennett and Linton, 1986). The original *E.coli* clinical strain which produced CARB-6 also harboured resistance genes for TEM-1 production. These TEM-1 resistance genes could not transfer or be mobilised into a recipient, indicating their presence might be either on a different plasmid or the chromosome. The expression of this CARB-6 was very poor on an IEF gel compared with TEM-1 beta-lactamase from the original clinical isolate. Comparing the biochemical properties, it does not appear that the production of these two enzymes in the same strain has any additional advantages over other

isolates.

The other novel enzyme was SAR-2 (Nandivada and Amyes, 1989). SAR-2 beta-lactamase had a higher pI (at 8.3) than any previously characterised plasmid encoded beta-lactamases including CEP-1 (Bobrowski et al., 1976) and CEP-2 (Levesque et al., 1982). The classification of beta-lactamases is largely dependent on their substrate and inhibitor profile (Richmond and Sykes, 1973; Sykes and Matthew, 1976); therefore, this enzyme was classified as a broad-spectrum beta-lactamase as it could hydrolyse a series of antibiotics such as benzyl penicillin, ampicillin, carbenicillin, oxacillin, methicillin, cephaloridine and cefotaxime. The rates of hydrolysis revealed that it had more affinity for penicillins and it was different from other broad-spectrum beta-lactamases with higher rates of hydrolysis against methicillin and oxacillin. The main difference between the SAR-2 and the rest of the broad-spectrum enzymes was its inhibition profile. It was not sensitive to inhibition either clavulanic acid or p-chloromercuribenzoic acid; however its activity was restricted by the inhibitor cloxacillin.

Amongst salmonellae populations, the spread of an OXA-1 like enzyme was responsible for a high incidence of cefuroxime resistance (65.7%) even though these strains were susceptible to cephaloridine. Of these strains, only four clinical isolates could transfer the resistance genes encoding this OXA-1 like enzyme. In susceptibility patterns to ampicillin, oxacillin, cephaloridine and cefuroxime, there were no significant differences between the strains producing the standard OXA-1 and the new OXA-1 like enzymes. These two enzymes showed noticeable differences biochemically, i.e., band pattern on IEF and substrate profile pattern. These beta-lactamases co-focused at pI7.4, the main differences were two satellite bands which were absent in the OXA-1 like enzyme but were clearly visible

with the standard OXA-1 enzyme.

When the rates of hydrolysis of various substrates by both the OXA-1 like and OXA-1 enzymes were analysed under the same experimental conditions, there were some dissimilarities, especially for cefuroxime hydrolysis. When the rates were related to the hydrolysis of penicillin G (100%), OXA-1 like and OXA-1 hydrolysed cephaloridine (49.6% and 46.9% respectively), cefotaxime (18.4% and 12.4%) and ampicillin (755% and 573%) to similar extents. Significantly, the OXA-1 like enzyme had a considerably higher activity (8-fold) against cefuroxime than OXA-1 (12.5% and 1.5% respectively). The OXA-1 could hydrolyse cefoxitin (1.9%) whereas OXA-1 like enzyme could not. The OXA-1 like enzyme had higher activity (>2-fold) than OXA-1 against cephalothin (13.0% and 5.8% respectively), cephamandole (39.3% and 15.7%) and nitrocephin (654% and 250%). On the other hand, the OXA-1 like enzyme was less effective than the OXA-1 enzyme against substrates such as cephradine (0.33% and 3.2%) and carbenicillin (107% and 260%).

With respect to inhibitor profile, both these enzymes share common properties. They were resistant to clavulanic acid, cloxacillin, p-chloromercuribenzoic acid, sodium chloride, cefoxitin and cefotetan. It was previously reported that a characteristic feature of all the members of OXA family of enzymes was the sensitivity to inhibition by sodium-chloride and this was the main difference between these enzymes and rest of the plasmid mediated beta-lactamases (Medeiros, 1989). This result what was not obtained in this study. The occurrence of the OXA-1 enzyme is not an unusual event in species of *Salmonellae*. This enzyme was previously isolated from different epidemic strains (*S. typhimurium* from Iran, *S. typhi* from Algeria, *S. typhimurium* from UK and *S. wien* from Paris) of

ampicillin resistant salmonellae (Medeiros, 1984). The OXA-1 was also identified in ampicillin resistant E. coli clinical isolates from different countries: Thailand, Brazil, S. Africa and UK; and, in P. mirabilis and Salmonella St Paul from the USA associated with the TEM-1 beta-lactamase (Medeiros, 1984) similar to the findings of this study. A high incidence of OXA-1 like enzymes in Salmonella isolates was unknown before this present study. A parallel could be drawn between the prevalence of TEM-1 enzyme among the ampicillin resistant salmonellae (81%) (Medeiros, 1984) from the USA and the results obtained in the Indian Salmonella strains (85.7%).

The necessity for the increased activity of OXA-1 like enzymes for cefuroxime was unclear as cephalosporins were used little at the time these strains were collected in Vellore, S. India in 1984. It has recently been shown that higher usage of penicillins and aminoglycosides could lead to cefuroxime resistance. This has happened among the isolates obtained from urinary infections and also from intensive care patients in the Edinburgh Royal Infirmary (John Hood, personal communication).

Even though cefuroxime resistance in the Salmonella strains was accompanied by the widespread distribution of novel OXA-1 like enzymes, there must be other resistance mechanisms playing a subsidiary role. The most probable additional resistance mechanism could be a membrane permeability barrier. Theoretically, the outer membrane proteins of Salmonella strains are similar to those of E. coli. Both OMP "F" and OMP "C" porin proteins are important for influx of beta-lactam antibiotics; and OMP "F", a larger channel provides a good passage for cephalosporins (Medeiros et al, 1987). Zwitterionic compounds (cephaloridine) diffuse more rapidly than monoanionic compounds (cefuroxime) (Nikaido, 1989); therefore, the cephaloridine

quickly enters the bacterial periplasm in large amounts and so that the quantity of bacterial enzyme might be insufficient to destroy the drug in the intact cells. This would explain bacterial sensitivities to cephalosporins (e.g. cephaloridine and cefuroxime) in permeability mutants (Medeiros et al., 1987; Nikaido, 1989). In *Salmonella* isolates there must be an involvement of the outer membrane in addition to the OXA-1 like enzyme. This would explain why so many transconjugants of these isolates had low MICs of cefuroxime.

This problem with cefuroxime resistance is now particularly poignant following the release of cefuroxime axetil. Cefuroxime axetil (an oral compound) has been proven to be efficient in treating upper and lower respiratory tract infections, soft tissue infections and sexually transmitted infections (Emmerson, 1988). This drug's efficacy has been found to be comparable with that of beta-lactam combinations (augmentin) (Emmerson, 1988) in terms of beta-lactamase stability and wide-spectrum activity. It is recommended as a valuable therapy for the treatment of urinary tract infections, particularly those resulting from beta-lactamase producing bacteria (Leigh et al., 1989). In order to keep the efficacy of this drug, the other common penicillins and cephalosporins which are still in use for common and uncomplicated infections must not be misused; otherwise, this could lead to cross-resistance problems by selective pressure as has happened in S.India and in the Edinburgh Royal Infirmary (John Hood, personal communication).

The results of the present study on both urinary and gastro-intestinal pathogens clearly indicates that the incidence of antimicrobial resistance was considerably higher in Vellore, South India than in the UK. The widespread resistance is probably a reflection of either intensive use of antibiotics or their uncontrolled availability. The comparison was made between the antibiotic usage given to inpatients in the

Christian Medical College Hospital, in Vellore in 1984, and antibiotic distribution to similar patients in the Royal Infirmary, Edinburgh, at the same time. The hospital sizes are similar (about 1000 beds). The official consumption in the Vellore hospital of ampicillin/amoxycillin was 2.8 kg/month and of cephalosporins 0.6 kg/month. The sum of this was approximately half the equivalent consumption of the sum of these two groups of drugs in the Edinburgh Royal Infirmary where the usage of ampicillin/amoxycillin was 2.2 kg/month and of cephalosporins was 4.3 kg/month (Collee and Amyes, 1986). Despite the higher usage of beta-lactams in Edinburgh, the incidence of beta-lactam resistance was just over half that found in Vellore, South India (Reid et al, 1988b). The recorded consumption of beta-lactam antibiotics does not explain the high incidence of beta-lactam resistance at the Christian Medical College, Vellore. The possible explanation might be given considering other factors: ampicillin/amoxycillin are poorly absorbed by the gut, and residual antibiotic provides a selective environment for the emergence of resistant strains from commensal gut bacteria (Knudsen, Rolinson and Stevens, 1961; Garrod, Lambert and O'Grady, 1981); and ampicillin and amoxycillin remain in widespread clinical use outside the hospital.

In addition to conducting surveys on clinically associated pathogens, studies on commensal gut flora should be performed on normal healthy volunteers as they represent a "gene pool" from which future infections involving beta-lactamase producing isolates might arise. The first survey on ampicillin resistant E. coli (17%) in the community was conducted in the UK in 1966 (Smith and Halls, 1966), followed by another study in 1969 among children in the general community (Moorehouse, 1969). It was found that ampicillin-resistant E. coli in the children was high (62%), as children, in general, are less hygienic than their elders, and this was taken as a reason for this high proportion of resistant E. coli isolates. The first survey on ampicillin

resistant *E.coli* from nosocomial infections in UK was held in 1969 (Datta, 1969). Recently, surveys on ampicillin resistant enterobacterial strains from healthy volunteers have been carried out in the UK, South India and Mexico (Simpson et al., 1986; Tait et al ., 1990 ; Morfin-Otera et al., 1990). In addition to these studies, the assessment of qualitative and quantitative factors of the principal resistance mechanisms, especially beta-lactamase types responsible for resistance in clinical populations must be combined with the establishment of the range of activities (e.g. spectrum of resistance) associated with each. This information is valuable in assessing the relative merits of new antibiotics (Simpson et al., 1986).

4.2 Combinations of Ampicillin and Clavulanic Acid

The second part of this thesis involved the investigations on the efficacy of the antibiotic combination ampicillin + clavulanic acid as this combination and later generation cephalosporins are amongst the main current strategies to overcome widespread bacterial beta-lactam resistance (Turck, 1988). . The broad-spectrum activities of the later generation cephalosporins (3GC) and the monobactams are being hampered by extended-spectrum plasmid mediated beta-lactamases derived from the common TEM and SHV enzymes (table 54). Within the past seven years a group of multiresistant Gram-negative bacteria have been identified with resistance patterns that include aminoglycosides and extended spectrum beta-lactam antibiotics. There have recently been several reports on the genetics (Philippon, Labia and Jacoby, 1989), biochemical characteristics (Bush and Singer, 1989) and epidemiology of these extended spectrum beta-lactamases (Philippon et al., 1989).

All extended-spectrum beta-lactamases have been identified among nosocomial

Table 54

Country (*species) in which the enzyme was first found	β-lactamase	pI	Reference
France[1,2]	TEM-3 (CTX-1)	6.3	Sirot et al (1987)
France[3]	TEM-4	5.9	Paul et al (1987)
France[1]	TEM-5	5.55	Petit et al (1988)
F.R. Germany[3]	TEM-6	5.9	Bauernfeind and Horl (1987)
France[4]	TEM-7	5.41	Gutmann et al (1988)
England[1]	TEM-9 (RHH-1)	5.5	Spencer et al (1987)
U.S.A[1]	TEM-10	5.57	Quinn et al (1989)
F.R. Germany[6,2]	SHV-2	7.6	Knothe et al (1983)
France[1]	SHV-3	7.0	Jarlier et al (1988)
France[1]	CAZ-2	5.9	Chanal et al (1988)
France[1]	CAZ-3	5.2	Labia et al (1988b)
France[1]	SHV-4 (CAZ-5)	7.75	Bure et al (1988)
Chile[1]	SHV-5 (CAZ-4)	8.2	Gutmann et al (1989)
France[1]	CAZ-6	6.5	Channal et al (1989)
France[1,3]	CAZ-7	6.3	Channal et al (1989)

contd/-

Country (*species) in which the enzyme was first found	β-lactamase	pI	Reference
Belgium[1]	CAZ lo	6.5	Vuye et al (1989)
Belgium[1]	CAZ hi	6.5	Vuye et al (1989)
USA[1]	PMG 25	5.55	Jacoby et al (1988)
—	CMY-1[1]	8.0	Bauernfeind et al (1989)
France[7]	MJ-1	5.53	Deschaseaux et al (1988)
France[5]	MJ-2	5.55	Deschaseux et al (1988)
Belgium[1]	FUR	7.5	Vuye et al (1989)
Japan[3]	FEC-1	–	Matsumato et al (1988)
UK[8]	UN	5.7 (5.9)	Corkill et al (1989)
Belgium[3]	TEM-E1	5.4	Payne, Marriott and Amyes (1989)
UK[1]	TEM-E2	5.3	Payne, Marriott and Amyes (1990)
England[3]	TEM-E3	5.55	Payne et al (1989)

*, 1, *Klebsiella pneumoniae*; 2, *Serratia marcescens*; 3, *Escherichia coli*; 4, *Citrobacter freundii*; 5, *Citrobacter amalonaticus*; 6, *Klebsiella azaenae*; 7, *Klebsiella oxytoca*; 8, *Enterobacter cloacae*. UN, unnamed

isolates of Enterobacterial strains (urinary, blood, wound, sputum cultures), mostly from intensive care units (Philippon et al., 1989). The broad-spectrum beta-lactamases TEM and SHV are efficient in controlling the activities of a wide variety of new potent beta-lactams because they are able to produce mutations capable of dealing with these drugs and this has resulted in therapeutic failures. Thus, this part of the thesis was directed towards the potential of the TEM and SHV enzymes to produce resistance to ampicillin and clavulanic acid.

The TEM-1 and SHV-1 beta-lactamases producing E. coli K12 (R1) and E. coli K12 (R1010) standard laboratory strains were chosen for challenge with the drug combination (ampicillin and clavulanic acid). Simultaneously, mutation experiments were also carried out with E. coli K12 (J53) producing the extended-spectrum beta-lactamases TEM-3, TEM-5 and TEM-7 to evaluate the efficacy of these drug combinations against these enzymes. In the clinical situation, beta-lactam combination therapy has not yet been compromised by bacterial resistances despite some minor resistance problems. These experiments were carried out to see what type of mutations occurred which could confer resistance to various combinations of the beta-lactam (ampicillin) and beta-lactamase inhibitor (clavulanic acid).

Challenge of the original E. coli strain producing the original TEM-1 enzyme with the combination (amp+clav, 100+20mg/l respectively) resulted in mutant "A" in which there was a rearrangement of plasmid R1 to give small, multicopy non-transferable plasmids. In this mutant, there were copies of the original R1 plasmid as well. Wiedemann (1981) showed that under selective presure (e.g. antibiotic stress), several natural manipulations could occur in plasmids such as insertion of IS elements, insertion of transposons, general recombination, mutations, formation of multicopy plasmids, amplification, and perhaps deletion. Therefore he had postulated that one or two big

plasmids fall apart into a coaggregated status of small plasmids, which can under certain circumstances, reaggregate to a big molecule. It is this type of rearrangement that has produced the genetic background in the present experiment which resulted in mutant "A" which contained two small additional plasmids. This rearrangement increased the level of enzyme produced resulting in 12-fold elevation in specific activities. Despite the hyperactivity of the enzyme, the beta-lactamase itself possessed similar biochemical properties to the normal TEM-1 enzyme. It also had similar ID_{50} values of clavulanic acid with nitrocephin as a substrate. However, despite the apparently identical nature of the enzyme, the hyperproduction of the enzyme resulted in the acquisition of resistance to the combination (MIC 160+8mg/1, amp+clav) and elevated MIC of ampicillin (mic 2560 mg/1).

The genetic basis for the development of resistance in mutant "A" was found to be much the same as that in resistant clinical strains. There have been recent reports of beta-lactam/clavulanic acid resistant bacteria of Enterobacteriaceae in England (Williams et al., 1988), Spain (Martinez et al., 1987, 1988; Rodriguez-Creixems et al., 1988) and USA (Medeiros et al., 1988; Sanders et al., 1988; Cooksey et al., 1990). In all these cases resistance to the combination had been based on overproduction of TEM-1 enzyme. In addition to TEM-1 enzyme, SHV-1 and class IV beta-lactamases were also reported to be hyperproduced in the clinical strains isolated from the patients at the University of Texas M.D. Anderson Cancer Hospital (Sanders et al., 1988). In one exceptional circumstance resistance resulted from the rapid hydrolysis by PSE-1 with low level enzyme production in Klebsiella pneumoniae strain at the same Cancer Hospital.

Later the genetic basis of beta-lactamase hyperproduction was found in ten

resistant E. coli clinical strains in Spain (Martinez et al., 1989). They found fro transformation experiments that very small plasmids with sizes ranging from 2.5 t 7.0kb with 10 copies per chromosome were responsible for hyperactivity of the TEM- beta-lactamase. They also observed a conjugative plasmid in the same cell with size of 55kb with one to two copies per chromosome. All these small plasmids wer found to be mainly non-conjugative, even though some could be mobilised (Martinez e al., 1989). No positive association between the large and small plasmids was shown.

Further challenge of mutant "A" by clavulanic acid alone resulted in the loss of th R1 plasmid (mutants "B"). The small multi-copy plasmids were retained but they wer unable, of course, to transfer ampicillin resistance alone even with the aid o mobilising factor X^+. Smaller plasmids (>24kb) usually cannot auto-transfer but ar often capable of mobilisation by unknown mechanisms. Homology between the mobilised and the mobilising plasmid is necessary in order to obtain transfer (Olsen, 1978) Mutant "B" had further increased ampicillin resistance but the loss of the R1 plasmi had led to the loss of the other antibiotic resistance determinants associated wit this plasmid, namely chloramphenicol, streptomycin, spectinomycin an sulphamethoxazole. This suggests that small plasmids carried resistance genes onl to ampicillin or beta-lactams. Similar observations had been made in a previou study that plasmid R1767 S. typhimurium had been split into and gave several plasmid under antibiotic pressure. Some plasmids with a molecular weight as low as 5M (7.5kb) coded for resistance to only one drug while others carried quite a lot o resistance genes (Wiedemann, 1981).

Mutants "B" were almost exactly the same as their parent strain with respect t

sensitivity to inhibition by clavulanic acid alone regardless of their further elevated resistance to ampicillin. This can be explained because both mutants "A" and "B" produced elevated levels of enzyme (increased number of molecules) which can easily overcome the inhibition by clavulanic acid. Additional interpretation may come from the fact that clavulanic acid, even though Michaelis-Menton kinetics suggest it to be a competitive inhibitor, functions 75% as a competitive and 25% as a suicidal inhibitor (D.M. Livermore, personal communication). The inhibitor is disadvantaged when challenged with an overproduced enzyme as the number of clavulanic acid molecules are insufficient to bind all the enzyme molecules. Sanders et al in studies (1988) with PSE-1 enzyme and 100μM of nitrocephin as a substrate determined that clavulanic acid is irreversible once saturing concentrations of the inhibitor are achieved. This suggests that any mechanism (such as overproduction) that prevents saturation of beta-lactamases by clavulanic acid is likely to be an efficient mechanism to overcome the combination.

The kinetic parameters (V_{max} and K_m values for substrate and K_i values for inhibitor profiles) should be determined in the evaluation of new antibiotics against beta-lactamases (Bush and Sykes, 1986). The TEM-1 enzyme from mutant "A" did have a slightly lower K_m for nitrocephin and K_i values of clavulanic acid than the normal TEM-1 enzyme though these differences are probably not significant. In a recent report on clinical isolates of E. coli (amp+sulbactam resistant), hyperproduced TEM-1 beta-lactamase has been found to be the resistance mechanism; and this enzyme appears to hydrolyse nitrocephin more efficiently (V_{max}, 1.000 for hyperactive TEM-1 and 0.725 for normal TEM-1) but with decreased affinity for the substrate (2-fold increase in Km value) and to be less susceptible to sulbactam inhibition

(Cooksey et al., 1990). Differences in susceptibilities of hyperproduced TEM-1 enzymes to different inhibitors such as clavulanic acid and sulbactam might indicate differences in the efficiencies of the inhibitors. From these results it can be deduced that clavulanic acid is more stable to the hyperproduced beta-lactamases. Indeed, it has been postulated before that clavulanic acid is a more efficient inhibitor than sulbactam (Bush, 1988b).

The increased level of enzyme production must be the main resistance mechanism in the mutants "A" and "B" as there were no alterations in the outer membranes of these mutants when compared with their parent strain E. coli K12. Both the OMP F and OMP C, the channels for beta-lactamas, were produced in the mutants to the same level as in the parent E. coli strain. As reported earlier, it is possible to see transferable plasmids affecting the permeability of the outer membrane (Iyer et al., 1978; Rossouw and Rowbury, 1984).

For the mutant "A", as only the large plasmid (R1) was transferable into the transconjugants, the recipient strains were identical to the original parent strain E. coli. This confirms the involvement of the small plasmids of the mutant "A", in bacterial resistance to the combination (amp+clav) and elevated resistance to ampicillin because these features were not transferable. In conclusion, the small multi-copy plasmids (<10kb (mutants "A" and "B"), 38kb ("B" mutants) and about 48kb (mutant "A")) were responsible for hyperproduction of the enzyme. Unlike their laboratory counterparts, the multi-copy small plasmids (2.5kb to 10.0kb) found in clinical isolates of E. coli which encoded the greater amount of TEM-1 beta-lactamase were either mobilisable or transformable (Martinez et al., 1989); and, this

augurs badly for the future.

Like non-transferable small plasmids in the mutant "A", the small plasmids of mutant "B" were also neither self-transferable nor mobilisable; they could be transformed into a sensitive strain E. coli MC 1022 (streptomycin resistant). The transformants that were obtained were highly resistant to ampicillin (MIC >2560mg/1) and augmentin (>80 + 16mg/1, amoxycillin + clavulanic acid). Although these transformants acquired the correct resistances, it was difficult to visualise the transformed plasmids. It is possible the plasmids might have been integrated into the host bacterial chromosome. In the conjugation process, a plasmid with a host range for conjugation that exceeds its range for replication is a potential "suicide" vector. It is able to introduce resistance genes which can only survive if they can integrate into the host chromosome or another plasmid (Jacoby, 1985). It is unlikely to be with the plasmids as this transformation was performed between two similar E. coli strains.

Another explanation might be that TEM-1 encoding resistance genes are on transposons. The small plasmids or transposons which possessed only multicopy ampicillin resistance genes might have been unstable and have difficulty in existing as individual plasmid forms. During the procedure of transformation, these DNA units might have been incorporated into the chromosome. On the other hand, the technique used might simply not have detected these plasmids. In a recent report, non-transferable small plasmids (2.5kb to 10.0kb), responsible for resistance to agumentin in the clinical strains were identified following transformation. The plasmids in the transformants were clearly detectable (Martinez et al., 1989). In conclusion, the resistance mechanisms resulted from small multicopy plasmids (>10kb (mutants "A" and "B"), 38kb (mutants "B") and 48kb (mutant "A"))

producing a very active TEM-1 enzyme. In the Spanish study, the genetic basis for hyperproduction of the enzymes in clinical strains of E. coli was shown to result from the emergence of small, multicopy plasmids (Martinez et al., 1989). In other clinical studies, hyperproduction of the enzyme has been shown to be a resistance mechanism but, in these cases, the genetic basis was not established (Williams et al., 1988; Rodriguez-Creixems et al., 1988; Sanders et al., 1988; Cooksey et al., 1990).

Challenge of the original TEM-1 producing strain with a lower concentration of clavulanic acid (64mg/1) alone produced "C" mutants (MIC 80mg/1). Mutant "C" had an elevated MIC of ampicillin (>1280mg/1) and combined drug (amoxycillin+clavulanic acid, MIC 40+8mg/1) resistances. In these mutants neither the plasmid nor the level of enzyme produced was altered. Regardless of the resistance levels to ampicillin, some of the "C" mutants had become resistant to cefuroxime (MIC >4mg/1); but none of these resistance genes, except for ampicillin, were transferable. The ampicillin-resistant transconjugants were found to be no different from the standard TEM-1 producing strain. This finding indicates that the resistance mechanisms of these mutants are unlikely to be beta-lactamase derived; and they probably result from alterations of the outer membrane permeability of clavulanic acid. It is unlikely to come from alterations of penicillin binding proteins which are potent mechanisms of resistance in Gram-positive organisms which have no membrane barriers; but this is unusual in these types of Gram-negative organisms (Spratt, 1989).

Challenge of some of the mutants "C" as well as the original E. coli K12 strain with higher clavulanic acid concentrations (128 mg/1) had the effect of increasing the resistances to beta-lactam drugs in the resultant mutant "D"; however, it did not affect plasmid integrity. Most of the "D" mutants produced elevated levels of TEM-1 beta-lactamase. Of these "D" mutants, only those directly derived from the strain

E. coli K12 had very high MICs of ampicillin, clavulanic acid and augmentin (in one mutant) and two of them also conferred resistance to cefuroxime and cefotaxime (>8mg/1).

The results suggest that exposure to clavulanic acid alone brings about changes in the bacterial cells yielding resistant mutants. It had been reported that the elimination rate of clavulanic acid from patients with different degrees of renal insufficiency varied and took longer time than in the patients with normal renal function (Muench, Luethy, and Siegnthaler, 1981). When two drugs are administered together, it is possible that some parts of the body may only be exposed to one of the drugs at either higher or lower concentrations. It was because of this reason that attempts were made to select the resistant mutants to clavulanic acid at concentrations above the MIC. It had been postulated that this would demonstrate the effect that the pharmacokinetics of this inhibitor might have on the development of bacterial resistance. Most pharmacokinetic studies of this inhibitor, in conjunction with the beta-lactam antibiotic, have shown that it can penetrate through all tissues, eventually achieving the required concentrations as a combination in each part of the body (Cox, Meewis and Horton, 1989; Meier, Adam and Heilmann, 1989; Weismeier et al., 1989). Amoxycillin, ampicillin and clavulanic acid do have different penetration rates and there are times during therapy when high concentrations of clavulanic acid are achieved in the absence of the penicillin. It has been suggested that employment of antibiotics that display their additive or synergic activity for a broad range of concentrations would counter balance the possible variations in drug level that can take place in vivo due to differences in pharmacokinetics, distribution and metabolism (Grassi, 1981).

As with the previous mutants ("A", "B" and "C"), in mutant "D" only the ampicillin

resistance gene was transferable. As there were no additional acquired small multicopy plasmids, the hyperproduction in some mutants ("D1", "D2" and "D3") is probably the result of mutation in the promoter region of beta-lactamase *bla* gene and not from plasmid rearrangement to small plasmids.

Mutants ("S1-S6") selected from SHV-1 beta-lactamase producing and R1010 plasmid harbouring *E.coli* strain by exposure to the combination (50 + 8 mg/l, amp + clav) also exhibited similar results to that of the *E.coli* K12 (R1 plasmid) mutants. The main resistance mechanism among these mutants ("S1-S6") had been the enzyme hyperproduction (SHV-1 beta-lactamase). The important and interesting feature of these mutants was the acquisition of resistances to cefuroxime, cefotaxime, ceftazidime and aztreonam in addition to their significantly increased resistance to ampicillin and moderately acquired resistance to the drug combination (amp+clav).

The mutants ("S1-S6") had shown higher MICs of the combination with increased bacterial density. The increased MICs of the drug combinations associated with increased inoculum size is possible only with those strains producing beta-lactamases, especially, in larger amounts of the enzyme (Martinez, et al., 1988). These mutants were found to be more resistant to the combination of amoxycillin and clavulanic acid than the combination of ampicillin and clavulanic acid at the same inoculum size.

Although the genetic basis for these resistances was hyperproduced, plasmid-mediated beta-lactamase SHV-1, the plasmid encoding the enzyme could not transfer resistance genes even to ampicillin resistance. As it was difficult to establish the genetic basis for the resistances of the mutants, biochemical characterisation of the enzyme produced by these mutants ("S1-S6") was carried out. The results revealed that the SHV-1 enzyme from the mutant could hydrolyse

cefotaxime, but not cefuroxime, ceftazidime and aztreonam to which the intact bacterial cells were resistant. From a few mutational steps only, the SHV-1 beta-lactamase has been converted into the new enzyme SHV-2 and the strain producing this enzyme became cefotaxime resistant (Korfmann, Kliebe and Wiedemann, 1986). This concept may be applicable to the mutated SHV-1 enzyme of the present study. Although the enzyme is not identical to SHV-2 it certainly had become capable of hydrolysing cefotaxime. Analytical isoelectric focusing clearly revealed that the SHV-1 enzyme from all the mutants differed from the standard SHV-1 by loss of one satellite band. This might imply some structural change in the SHV-1 which would enable it to hydrolyse cefotaxime.

The enzyme could hydrolyse ampicillin and cephaloridine at rates similar to that of the original SHV-1 enzyme. The inhibitor profile with clavulanic acid showed its unchanged sensitivity to inhibition by this inhibitor in comparison with the standard SHV-1 enzyme (ID_{50} $>10^{-7}$), despite its high specific activity and the resistance to the drug combination. Sanders et al., (1988) found in her study hyperproduction of SHV-1 enzyme with different specific activities in *Klebsiella pneumoniae* was responsible for resistance to ticarcillin-potassium clavulanate. One *Klebsiella* strain produced SHV-1 in association with TEM-1 beta-lactamase. In all strains there was a correlation between the amount of enzyme produced and the resistance level to the combination even though major differences in permeability might have masked the true relationship between quantity of enzyme produced and resistance (Sanders et al., 1988). Some studies showed a lack of correlation between the specific activity of the beta-lactamase present in the cell and the beta-lactam resistance level (Richmond and Curtis, 1974; Medeiros, 1984; Reid - Ph. D thesis). This has been contrary to the situation of resistance to beta-lactam combination. These clinical isolates remained sensitive to inhibition by

clavulanic acid (ID_{50} 0.022μM) which is lower than the value obtained for the mutants "S1-S6" of this study (ID_{50} 0.1μM).

As the biochemical properties of the enzyme from the mutants ("S1-S6") showed no activity against some cephalosporins, the outer membrane proteins (OMPs) of the mutants were extracted and analysed by SDS-PAGE electrophoresis concurrently with the OMPs of the standard *E.coli.* The pattern and the expression of the beta-lactam porins OMP "F" and OMP "C" of the mutants (S1-S6) showed no differences from the porin proteins of the standard *E.coli.* On the basis of the results it could be determined that hyperproduction of the enzyme must have been the main resistance mechanism even for resistances to cefuroxime, ceftazidime and aztreonam, even though there might be other resistance mechanisms such as permeability through lipopolysaccharide layer (LPS) (Nikaido, 1989). However, the LPS barrier has been recognised to be a suitable passage for penicillins because they can pass through easily. . This barrier can not be considered as a potential resistance mechanism as it can not be a channel for cephalosporins' influx (Yamaguchi, Hiruma and Sawai, 1982). Attention should perhaps be focused on the hyperproduced beta-lactamase which might be able to trap the cephalosporins, even though they have an inability to destroy them. The large amounts of the enzyme in the periplasm of the bacterial cells could trap low numbers of antibiotic molecules and confer resistance to them. This kind of resistance mechanism could be possible for the antibiotics such as cephalosporins. Similar findings have been found elsewhere in *Pseudomonas aeruginosa* and *Enterobacter cloacae* which became resistant to cephalosporins because of the trapping mechanism (Then and Angern, 1982). In another report latamoxef-resistance in beta-lactamase derepressed *Pseudomonas aeruginosa* was seen by "covalent-trapping" (Livermore, 1987c).

The plasmid analysis of these mutants "S1-S6" showed that even though the mutants apparently had slightly larger plasmids than the original plasmid R1010, the digestion of these plasmids with *Hind* III restriction enzyme revealed that there was no difference between the patterns of the restricted plasmid fragments of the mutants and the standard *E.coli* strain. This suggests there might be "up" mutations in the promoter region of the beta-lactamase gene giving rise to high amounts of enzyme production. In general, under continuing antibiotic stress, a plasmid could extend its molecular weight by selectively multiplying those DNA segments responsible for that particular antibiotic resistance. This would form duplications within the molecule resulting in can increased ability to withstand higher concentrations of the drugs it was exposed to. This was the case with plasmid pBP11 (encoding OXA-2 enzyme) when exposed to ampicillin (Wiedemann, 1981). Another plasmid pBP10 (encoding TEM-1 enzyme) showed an overproduction of the whole plasmid molecule under antibiotic exposure (multicopy plasmid with no change in the plasmid size) (Wiedemann, 1981)

The outcome of these mutation experiments revealed that exposure to the drug combination and also to clavulanic acid alone could select mutants but only at a low frequency rate. These mutants conferred resistance not only to the drug combination but also to later generation cephalosporins. None of these third generation cephalosporin resistance determinants was transferable. Although there have been reports of clinically isolated strains which are resistant to the drug combination by hyperproduction or reduced membrane permeability, the reports did not identify concurrent conferment of resistance to later generation cephalosporins (Williams et al., 1988; Martinez et al., 1987, 1989; Rodriguez-Creixems et al., 1988; Medeiros et al., 1988; Sanders et al., 1988; Cooksey et al., 1990). It appears this is likely to arise and may become epidemic during beta-lactam combination therapy if

care is not taken during dosage (i.e. correct concentrations and correct combination of beta-lactam and beta-lactamase inhibitor to be included in a combined drug) and compliance.

It has been postulated that strains with beta-lactamase determinants in small multicopy plasmids or strains with hyperproduced TEM-1 enzyme might have existed long before the introduction of amoxycillin in clinical therapeutics (Sanders et al., 1988; Martinez et al., 1989). This must be true, as there were three E. coli isolates that produce hyperactive TEM-1 beta-lactamases among the Indian urinary pathogens of this study in the absence of exposure to the beta-lactam combinations in clinical therapy. These drug inhibitor combinations still prove to be effective against clinical isolates producing very stable extended-spectrum beta-lactamases of the TEM and SHV types. All these beta-lactamases are inhibited well by clavulanic acid with ID_{50} values ranging from 4.3 to 12nM compared with 130nM for TEM-2. Inhibition by sulbactam was also found to be better for the extended broad-spectrum beta-lactamases than for the TEM-2 beta-lactamase with ID_{50} values of 12-940nM for the extended broad-spectrum enzymes compared to 1600nM for the TEM-2 beta-lactamase (Bush and Singer, 1989).

When E. coli strains producing TEM-3 (hydrolyses cefotaxime preferentially), TEM-5 (hydrolyses ceftazidime preferentially) and TEM-7 (more resistant to ceftazidime) enzymes were challenged with different concentrations of ampicillin and clavulanic acid combinations, they could overcome the combined drugs, only the bacterial density was high. MICs for these enzyme-producing strains of the combination were progressively reduced as the concentrations of both the beta-lactam and the inhibitor were increased.

To treat the infections with the pathogens producing expanded-spectrum beta-

lactamases, it may be necessary to use higher concentrations of the antibiotic combination. It is impossible to find a fixed ratio that can be valid for all bacterial species to achieve the adequate dosage for the treatment of severe infections (Muench, Luethy and Siegenthaler, 1981). However, it is important that the components are present in the most appropriate ratio to develop their antibacterial activity.

Six well-grown colonies of strains producing TEM-3, TEM-5 or TEM-7 beta-lactamases (2 colonies from each type of beta-lactamase producing strain) were chosen from the plate containing higher concentrations of ampicillin and clavulanic acid. Studies carried out on these strains ("1M-6M") to determine if there were any mutations leading to modified resistance patterns or hyperproduced beta-lactamases revealed that they had not significantly differed from their parent strains. On exposure to the combination, mutants 3M and 4M (from TEM-5 producing strain) had highly increased MICs of ampicillin and 4M mutant lost its resistance to chloramphenicol. Mutants 5M and 6M (from the strain producing TEM-7) surprisingly dropped their resistance level to ampicillin in comparison with their parent strain. Mutants 1M and 2M (from the strain producing TEM-3 beta-lactamase) were similar to their parent strain; but the mutant 2M lost its resistance to chloramphenicol. Apart from these observations, none of these strains acquired resistance to the drug combinations such as ampicillin/clavulanic acid or elevated resistances to amoxycillin in combination with clavulanic acid.

From the results, it can be deduced that combined beta-lactam antibiotics would be useful against the extended-spectrum beta-lactamases. Not only the concentrations of the antibiotics but also the type of the beta-lactam to be included in a combination might be of considerable importance, depending on the type of bacteria to be

treated. Beta-lactams such as ampicillin, amoxycillin and early cephalosporins (Livermore, 1987a) are often strong inducers of chromosomal beta-lactamases at levels below their MICs; and they are labile to the class 1 beta-lactamases that they induce. The achievement of the required concentrations of the beta-lactam and beta-lactamase inhibitor at the site of infection is vital, especially in the treatment of bacteria capable of producing inducible beta-lactamases. The inducible enzymes can lead to an antagonism between beta-lactam (i.e. ticarcillin) and beta-lactamase inhibitor (clavulanic acid) as has happened in Pseudomonas clinical strains (Livermore, 1987b). In one study, induction of chromosomal beta-lactamases by ticarcillin in combination with clavulanic acid did not result in any decrease in ticarcillin susceptibility in four clinical strains (Stobberingh, 1988). The other tests conducted on non-inducible beta-lactamase producing bacteria with different combinations of beta-lactams and beta-lactamase inhibitors demonstrated no antagonism except between cefotaxime and clavulanic acid (Kitiz et al., 2989).

On the basis of the literature available and the data obtained from this thesis, it can be concluded that beta-lactamases play an important role in beta-lactam resistance and the TEM-1 beta-lactamase is the most prevalent in Gram-negative bacteria worldwide. Strains producing the TEM-1 beta-lactamase are likely to be dominant even over the bacterial strains harbouring genes for extended-spectrum beta-lactamase production. Both TEM-1 and SHV-1 beta-lactamases are responsible for current beta-lactam resistance problems, as they have been shown to be highly efficient in their ability to mutate. Structural changes in their enzymes overcome newer beta-lactam antibiotics. The prime cause of the evolution of new potent beta-lactamases is the antibiotic cefotaxime which has been widely used (Casellas and Goldberg, 1989; Philippon, Labia and Jacoby, 1989) in the treatment of complicated and hospital

acquired urinary tract infections because of its wide spectrum (Naber, 1989) antibacterial activity. It has been postulated that the extensive use of other newer antibiotics also contributes to the emergence of the new beta-lactamases (Philippon et al., 1989). The continued success of combination therapy in the clinical situation is only threatened by the same enzymes (TEM-1 and SHV-1) if these are hyperproduced. Hyperproduction of these enzymes does not change their sensitivity to clavulanic acid and they retain their activity against beta-lactam antibiotics. The inhibitory effect of clavulanic acid for all kinds of plasmid-mediated beta-lactamases shows that combination therapy could be well chosen for different types of infections. Indeed, the extended-spectrum enzymes remain sensitive to inhibitors such as clavulanic acid, sulbactam and tazobactam (Philippon, Labia and Jacoby, 1989). Along with increased affinity for cephalosporin substrates goes enhanced affinity for these beta-lactamase inhibitors so that combinations of an inhibitor with a hydrolysable beta-lactam should prove effective against infections by bacteria with new enzyme production (Philippon, Labia and Jacoby, 1989).

Recently, it has been documented that the potentiation effect of clavulanic acid or sulbactam, in combination with third-generation cephalosporins against 46 Klebsiella pnaeumoniae strains from Argentina producing expanded-spectrum enzymes has been shown to be positive synergy (Casellas and Goldberg, 1989).

Although generalisations can be made about the appropriate use of agents on the basis of published susceptibility patterns, significant regional, demographic, and institutional variables exist that require each hospital to establish its own antibiotic data base and antibiogram (Parry, 1989). It is necessary to monitor

carefully the development of resistance to new beta-lactams. Even though advances in the chemistry of beta-lactams should offer solutions to these real problems, it seems likely that enzymes will be found wherever antibiotics are used which will favour their appearance, either by promoting the emergence of novel beta-lactamase or the interplay between the various mechanisms of resistance which can be reflected in the emergence of resistance to a new antibiotic.

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