

**MULTI DRUG RESISTANCE IN GRAM-NEGATIVE BACTERIAL
PATHOGENS**

**THESIS SUBMITTED
UNIVERSITY OF MUMBAI**

**FOR THE DEGREE OF
MASTER OF SCIENCE
IN
MICROBIOLOGY**

**SUBMITTED BY
ANINE ISAAC**

**UNDER THE GUIDANCE OF
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**HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
MUMBAI**

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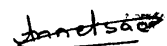
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(ANNE ISAAC)
CANDIDATE

Forwarded through the Research Guide


Dr. S. V. GADRE
RESEARCH GUIDE

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INTRODUCTION

When first discovered, antibiotics were touted as a miracle cure and they literally were. Infections that were fatal before the turn of the century were tamed to mere inconveniences during this century. But we've come almost full circle. The misuse, over prescription, and abuse of antibiotics has allowed resistant strains of bacteria to develop and once again threaten health and life.

The development and spread of antibiotic resistance is usually attributed to the overuse or the misuse of antibiotics. Some of the other factors that contribute to the development of resistance are the use of antibiotics for agricultural purposes and as food supplements for poultry and cattle. In India, over prescribing of antibiotics is rampant. Therapy with antibiotic combinations is usually started even before the culture specimen is sent to the laboratory. Patients also have easy access to antibiotics over the counter. Wrong dosage and a duration for treatment that is too short also contributes to the emergence of drug resistance (Senghal 1999). There are important clinical ramifications to the problem of resistance. Resistance of pathogenic bacteria to antibiotics has had a severe effect on the treatment of infectious diseases. Many drugs, such as tetracycline which was called a wonder drug had great potential

for effective control when it was first introduced, only to have bacteria adapt to them and greatly reduce their applicability.

Plasmid mediated resistance accounts for most high level resistance found in bacteria and has resulted in the rapid spread and accumulation of the most efficient resistance mechanisms. Many outbreaks of plasmid mediated resistance causing nosocomial infections has been reported and single or closely related plasmids have been shown to be responsible. As it seems that there is no way either to control the dissemination of resistance genes or to restrict the injudicious use of antibiotics sufficiently to diminish the spread of resistance, the only alternatives left are to search out new antibiotics to take the place of old ones or to study the resistances themselves, with the hope of learning enough to control them.

AIMS AND OBJECTIVES

More than 250 antibacterial agents are in use through out the world today. With such an abundance at the prescriber's disposal, it might be imagined that the bacterial infection has become a minor problem. However, nothing would be further from the truth. Bacteria seem to posses a limitless ingenuity in avoiding the effects of antimicrobial agents. Bacteria can assemble resistance genes for unrelated classes of antibiotics on plasmids that can be easily transmitted between bacterial species and sometimes between genera.

Infections caused by gram negative bacteria such as E.coli, Klebsiella, Proteus, Shigella and Pseudomonas have become an increasing cause of concern as these organisms have acquired multiple resistance to antibiotics. This resistance has frequently been associated with conjugative plasmid DNA. The problem of transferable drug resistance in gram-negative pathogenic bacteria is of great importance because of its clinical and epidemiological implications. Hence, it is not wise to be complacent about the problem of resistance.

The global problem of resistance must be countered by the vigorous control of infection, effective regulation, distribution and use of the drugs and studying the genetic mechanisms involved in antimicrobial drug resistance. A better understanding of R

plasmids and their role in resistance will lead to more appropriate regimens of antibiotic therapy.

The present study was conducted with the following aims and objectives in mind.

1. To determine the Multi Drug Resistance (MDR) patterns of the clinical isolates of gram negative bacterial pathogens collected from 1997 to 1998.
2. To determine the role played by the plasmid in conferring drug resistance by the following experiments:
 - a) Curing the resistant isolates of their R- plasmids, if any, and demonstrating the consequent loss of resistance to several antibiotics.
 - b) Transferring the resistance determinants via conjugation to a suitable recipient.
 - c) Isolation of the R- plasmid and its physical demonstration by Gel Electrophoresis.

LITERATURE SURVEY

It is a truism that the development of safer effective antimicrobial drugs has revolutionized medicine. Unfortunately it is also true that microorganisms are nothing if not versatile, and the brilliance of the chemotherapeutic achievement has been somewhat dimmed by the emergence of microbial strains presenting a formidable array of defenses against growth inhibiting drugs

1.1 Historical Perspective:

Resistance to antimicrobial agents is not a new phenomenon in bacteria. Abraham and Chain (cited by J. Davies 1994) recorded the first resistance mechanism in 1940. They identified a bacterial enzyme that catalyzed the hydrolysis of the β -lactam ring of the antibiotic Penicillin and eliminated its antibacterial activity. Sulfonamides were used in the treatment of bacillary dysentery in Japan in the 1940s. However, its effectiveness had a duration of only five years and by 1945, Sulfonamide resistant *Shigella* appeared rapidly (Mitsuhashi 1976). Subsequently, newer antibiotics such as Streptomycin (Sm), Chloramphenicol (Cm) and Tetracycline were employed for the treatment of Sulfonamide (Su) resistant *Shigella*. However *Shigella* strains resistant to these antibiotics also soon appeared

(Watanabe 1963, Mitsuhashi 1976). In 1959, Akiba and Ochiai demonstrated independently, that multiple drug resistance might be carried by an infective agent analogous to the F factor of *E.coli*. Their experiments proved that multiple drug resistance could easily be transferred *en bloc* between *Shigella* and *Escherichia coli* by mixed cultivation (Akiba 1960, cited by Watanabe 1963). Watanabe and Fukasawa further proved that these resistance genes are located in various combinations on transmissible plasmids now called **resistance (R) plasmids**. Mitsuhashi et al. (1960) also found that F factor is not necessary for the transfer of drug resistance among the substrains of *E.coli* strain K12. Harada et al (Harada 1960) found that multiple drug resistance could be transferred to many other bacteria, including most genera of Enterobacteriaceae, by mixed culture. The rapid spread of R factors is what accounts for the rise in the number of multi resistant gram negative bacteria through out the world.

Let me first give you an outline of the genetic background to the problem of drug resistance, and then describe the general types of biochemical mechanisms that give rise to resistance.

1.2 The Genetics of Drug Resistance:

Antibiotic resistance is classified into two broad types:

Intrinsic/ Innate Resistance:

Intrinsic resistance suggests that the inherent properties of the bacterium are responsible for preventing antibiotic action. It is the natural/innate resistance possessed by a bacterial species and is usually specified by chromosomal genes (Courvalin 1996). For example, most gram-negative organisms are resistant to vancomycin and methicillin; enterococci are intrinsically resistant to cephalosporins and in the *Acinetobacter* genus, structural genes for 6-aminoglycoside acetyl transferases have been detected in every species studied so far.

Acquired Resistance :

Acquired resistance may be Indigenous i.e.; resistance may arise as a consequence of mutations to chromosomal genes because of changes in the DNA sequence for eg, chromosomal mutation to streptomycin, rifampin, and fluoroquinolone resistance. Though mutations have traditionally been thought to occur in genes encoded on the chromosome, they may also occur in plasmid or transposon encoded genes. Beside mutations in structural genes, mutations may also occur in regulatory genes for eg, mutations in the regulatory region of DNA can cause resistance to third generation cephalosporins, due to high level

constitutive production of chromosomal cephalosporinases (cited by Murray 1991).

Acquired resistance may also be Exogenous where the bacteria can obtain foreign DNA by 3 mechanisms: transduction, transformation and conjugation. The role of transduction in nature may be related to closely related species, by the high degree of specificity of the adsorption step in bacteriophage invasion. Similarly, transformation may be confined to intragenetic transfer (Mazodier 1991) and is thought to play a minor role in the spread of antibiotic resistance in nature. The spread of resistance by conjugation in gram negative bacteria is recognized as an important threat to the treatment of diseases.

Conjugation and R factors:

Conjugation is the transfer of DNA from one bacterial cell to another by a mechanism that requires cell to cell contact. The phenomenon of conjugation in gram-negative bacteria was discovered some years before its importance in drug resistance was appreciated. The realization that drug resistance could be transferred during cell conjugation, came about in 1959, when it was found by Japanese investigators that multiple drug resistance can be easily transferred between *Shigella* and *E.coli* by mixed cultivation (Akiba 1960, Watanabe 1963).

Two types of genetic elements are transferable by conjugation: plasmids and transposons. Plasmids appear to be the most

common means of disseminating resistance genes in nature. However even they have their limitations as most plasmids have narrow host ranges and may not even transfer to other organisms within the same family.

R plasmids consist of two distinct but frequently linked entities:

- a) The resistance transfer factor or RTF
- b) The resistance determinant (r-det).

The RTF of R plasmids carries genes that determine autonomous replication and conjugal transfer. The r-det portion of the R plasmid is a highly variable component encoding anywhere from one to eight different resistance genes (Mosher 1992). Depending on the linkage of the resistance genes and the RTF, the R plasmids are classified as plasmid cointegrates and plasmid coaggregates. Watanbe and coworkers (Watanbe 1961) proposed the cointegrate model. They proposed that R plasmids are composed of two linked units: The RTF segment and the unit of drug resistance genes (r-det) (cited by Helinski 1973). An alternative to the cointegrate model, the coaggregate model was proposed by Anderson and coworkers (Anderson 1965). According to their model, the RTF and the r-det are independent replicons. Without the r-det, the RTF's although communicable, have no drug resistance to transfer and without the RTFs, the determinants are transmitted in a lineal fashion only in the host bacteria (Anderson 1968).

R plasmids can confer resistance to more than one or sometimes as many as seven antibiotics. How have plasmids accumulated such a diversity of resistance genes? The r-det portion in the R plasmid is variable and variability is due to the location of nearly all r-det antibiotic genes on transposons (Mosher 1992). Genes that are capable of translocating intact from one location on a plasmid to another plasmid or to a chromosome are called transposons. Hedges and Jacob discovered the first transposon in 1974. They discovered a transposable element Tn1, which encoded for a TEM- β lactamase, determining resistance to several β lactams.

Most transposons can be divided into two broad groups called composite transposons (class 1) and complex transposons (class 2). Composite transposons can transpose only after a round of DNA replication, as there is no replication of the transposons during the replication process. On the other hand, complex transposons, contain a transposase, which sets up a cointegrate intermediate between the transposon containing replicon and the target replicon, and a resolvase which separates the cointegrate into the original two replicons with the copy of the transposon in each (Schmitt 1986, Amyes 1998).

Transposons are less restrictive than plasmids in their host range. A special type of transposon, conjugative transposons, can transpose from the chromosome of one strain to the chromosome

of the another. They are DNA segments, which are normally inserted into the bacterial genome. To transfer, they first excise themselves to form a nonreplicating circular intermediate. This is then transferred by conjugation to a recipient where it integrates into the recipient's genome. Conjugative transposons were first discovered in gram-positive cocci and Bacteriodes species, are now found in a variety of enteric bacteria. E.g., the tetM gene originally found in enterococci is now the most widespread gene known. This gene is known to exist on a conjugative transposon like Tn916 (Poyart-Salmeron 1995). Conjugative transposons of Gram positive cocci represent an efficient mode of transfer of antibiotic resistance genes between phylogenetically distinct bacterial genera.

The ultimate rule of organization for migrant genetic information is provided by integrons. In this system, isolated gene "cassettes" can be integrated by site specific recombination in a genetic unit that, in addition to the recombination system, also provides a promoter for the expression of the incoming DNA (Hall 1995). The integron has been shown to be responsible for the dramatic success of the Tn21 like transposon. The Tn21 like transposon, appears to be one transposon that has a number of different resistance genes that code for resistance to various β -lactams, Trimethoprim, Aminoglycosides, Chloramphenicol etc. This is explained by the fact that this transposon contains a site for the specific insertion of the resistance gene cassettes, along

with a promoter, a terminator and genes that code for an integrase that facilitates the integration of these cassettes into the "hot spots" (Murray 1991). Thus one or more resistance genes can be incorporated into the same transposon, creating new and multi resistant transposons.

Conferring the role of the plasmid in multi drug resistance:

The chromosomal genes code for all the essential functions of a cell. Plasmids code for genes that give the cell a survival or growth advantage under particular environmental conditions. Although plasmids are stably inherited in any growing population of bacteria, sometimes there is a tendency for the clones in the culture to lose their plasmid due to an error in the process of plasmid replication. These plasmidless segregants thus lose the set of plasmid determined characters. Thus the instability of a bacterial property may indicate if genes present on a plasmid coded for it or not. Spontaneous loss of the plasmid is infrequent. However, bacteria can be treated with "curing" agents to enhance the natural frequency of plasmid loss. These agents however are individually effective only against some plasmids and their likely response is unpredictable. In those instances where a curing experiment has resulted in a significant increase in the number of bacteria lacking the property under study, plasmid involvement is

indicated. However, confirmation of this will require supportive evidence.

Curing experiments using DNA intercalating dyes was first performed by Hirota and co-workers in 1960. Their work showed that the F^+ cells of E.coli can easily be rendered F^- by treatment with acridine orange and acriflavine (Hirota 1960, Hirota and Iijima 1957). Hirota further found that the F factor in the integrated state (Hfr) couldn't be eliminated by acridines. In 1961, Watanabe and Fukasawa reported that the resistance factor could be eliminated from E.coli and Shigella, by treating the resistant cells with acridine dyes albeit at a very low frequency. Watanabe's speculation that the low elimination frequency was a consequence of an integration of R factors into the bacterial chromosome has not been confirmed as yet.

In this study we used curing agents like acridine orange and ethidium bromide. They are both planar heterocyclic molecules with dimensions roughly the same as those of a purine pyrimidine pair. In aqueous solution, acridine orange/ ethidium bromide, get inserted or intercalated between the levels of base pairs into the double helix. The spaces for the insertions are created through the local untwisting of DNA by an estimated 12° of rotation per one intercalated molecule that causes a separation between normally adjacent base pairs of approximately $3.5\text{\AA}$. Intercalation into supercoiled DNA produces characteristic conformational transitions in this type of DNA. Increasing numbers of individual intercalations causes progressive unwinding of superhelices by

local untwisting and gradual decrease in the number of right handed super coils. At a defined equivalence point, superhelical DNA becomes unstrained circular DNA. Further intercalation produces left-handed unnatural supercoils.

Besides curing an organism of its plasmid, the ability of a property to be transferred from one bacterium to another provides good presumptive evidence of plasmid involvement. Attempts to demonstrate such transfer can therefore be used as evidence to support the fact that the property is plasmid encoded, if the plasmid involvement has already been indicated by curing experiments or DNA examination. In the present study conjugational transfer of the resistance genes was used as a presumptive test for determining the role of the plasmid in conferring drug resistance.

With the development of methods for isolating plasmid DNA the presence of a plasmid can be determined directly. Modern small-scale preparative procedures and the use of agarose gel electrophoresis allow plasmids to be detected in very small culture volumes.

1.3 Mechanisms of Drug Resistance

β -lactam Antibiotics

The β -lactams belong to a family of antibiotics, which are characterized by a β -lactam ring. Penicillins, cephalosporins, clavams (or oxapenams), cephamycins and carbapenems are members of this family. The classic Penicillins are penams, characterized by a fused heterocyclic structure composed of a β -lactam ring and a 5 membered sulphur containing thiazolidine ring. In the cephalosporins the fused dihydrothiazine ring has an extra carbon with an unsaturated bond between C-3 and C-4, giving a cephem structure.

Mode of action:

β -lactams block the final crosslinking stage of the pathway in the biosynthesis of peptidoglycan which occurs in the cell wall. Here the linear glycan strand via the peptide chains attach the mature peptidoglycan in the cell wall. Crosslinking is catalyzed by a group of enzymes called the transpeptidase. The β -lactams effectively inhibit the transpeptidase by acting as an alternate substrate. **They mimic the D-alanyl-D-alanine residues and react with the transpeptidase.** All β -lactams act in a similar manner (Lambert 1998).

Mechanism of Resistance:

There are three major mechanisms by which clinical isolates of gram negative bacteria resist β -lactam antibiotics. These include,

- a) production of periplasmic β -lactamases
- b) altered outer membrane permeability
- c) and the diminished affinity of the Penicillin Binding Proteins (PBPs), the direct targets of β -lactam antibiotics.

Of these three mechanisms, production of β -lactamases is by far the most frequently encountered in clinical isolates (Sanders 1987).

a) Production of β -lactamases:

β -lactamases are the major mechanisms of resistance to penicillins, cephalosporins and other β -lactam antibiotics. Several schemes have been proposed to classify β -lactamases. One scheme by K. Bush, G.A Jacoby and A.A Medeiros (Bush 1995) allocates the enzymes into 4 groups according to their substrate and inhibitor profiles. Another classification initiated by Ambler divides the enzymes into four structural classes (A-D) on the basis of the amino acid similarities (Geroge 1994).

Most of the resistance to β -lactams among gram negative bacteria is due to plasmid or transposon encoded β -lactamases such as TEM-1, TEM-2, SHV-1, PSEs, OXAs etc. The TEM enzyme was the first R- plasmid specified β -lactamase to be recognized in gram negative bacteria (Datta 1965). Since it was

first found in the plasmid from *E. coli* some thirty years ago, TEM-1 has become one of the most prevalent resistant genes in the world (George 1994). The reason for this may be that the TEM-1 β -lactamase genes are located on the highly successful class 2 transposon Tn3, which has promoted its spread. Both TEM-2 and SHV-1 β -lactamase genes have also been found on efficient transposons. β -lactamase encoding genes are also common in multi drug resistant transposons of the Tn 21 type (Levesque 1988). Mutations in TEM-1, TEM-2 and SHV-1 can generate enzymes that are active against the third generation cephalosporins. They are known as extended spectrum β -lactamases (ESBLs). These enzymes were first found in the 1980s among strains of *E.coli* and *Klebsiella* that were found to be resistant to the third generation cephalosporins. Most ESBLs confer moderate or high level resistance to ceftazidime and low level resistance to cefotaxime. Among Enterobacteriaceae these ESBLs are most predominantly seen among *Klebsiella pneumoniae* (Nordmann 1998, Amyes 1992, and Murray 1991).

Most enterobacterial species express low level of chromosomally encoded beta-lactamases, which may contribute to intrinsic resistance. They may carry a chromosomal ampC beta-lactamase gene. In *Escherichia coli* and *Shigella*, expression from ampC is constitutively expressed and the beta-lactamase is synthesized at low levels. Mutations leading to increased beta-lactamase synthesis occur rather infrequently, making resistance to modern cephalosporins a rare event in these

species. In other Enterobacteria and Pseudomonas, ampC beta-lactamase synthesis is induced by beta-lactams (Normark S 1986).

b) Alteration/ Modification of target site:

In a few species of gram negative bacteria, resistance has emerged by the development of altered penicillin binding proteins (PBPs), with decreased affinity for β -lactam antibiotics. This mechanism of resistance is found in naturally transformable organisms like *Streptococcus pneumoniae*, *Neisseria gonorrhoeae* etc. In non-transformable organisms, a novel low-affinity PBP has been acquired, rather than there being a decrease in the affinities of the normal PBPs (Christopher 1994).

c) Reduction in the permeability of the cell:

Some of the gram-negative organisms show a decreased expression of the Omp F protein, the channel through which the cephalosporins cross the outer cell wall of gram negative organisms, coupled with increased expression of porin C, a channel through which the cephalosporins do not easily pass. This results in a decreased amount of cephalosporins and penicillins accumulating in the cell.

Quinolones

The quinolones comprise of a relatively large and rapidly expanding group of synthetic compounds based on the 4-quinolone nucleus. The first of the quinolones was Naladixic acid, a 1,8- naphthyridine, which was introduced for clinical use in 1962. This was used exclusively for urinary tract infections. Later, compounds such as Ciprofloxacin and Ofloxacin were used as they have a much broader clinical utility. The quinolones exhibit relatively potent activity against a wide range of Enterobacteriaceae, but have little or no activity against the non-fermenting aerobic gram negative bacilli such as Acinetobacter and Pseudomonas species (Brown and Reeves).

Mode of action:

The principal target of the 4-quinolones is the enzyme DNA gyrase, the only bacterial enzyme capable of introducing negative supercoils into DNA (Sutcliffe 1989, Gellert 1976, cited in Amyes 1992). DNA supercoiling compacts the chromosome and is involved in the regulation of gene transcription (Fisher 1984). The enzyme is a tetramer consisting of two A and two B subunits. The A subunit cuts both strands of DNA simultaneously at intervals four bases apart and holds the strands apart but covalently bound to the enzymes. The B subunit, containing ATP for energy introduces negative supercoils into the DNA and the A strand then reseals the two strands (Amyes1992). The 4-

quinolones appear to bind to the A subunit and prevent the resealing of the DNA strands as a result, the chromosome the chromosome in treated cells becomes fragmented (Gellert 1977).

Mechanism of resistance:

Bacterial resistance to an antimicrobial agent is usually mediated either via a plasmid-encoded factor or a chromosomal mutation. Plasmid mediated resistance to the 4 quinolones has not yet been identified (Lewin C.S 1990). Two instances of plasmid mediated naladixic acid resistance has been reported in *Shigella dysenteriae* strains isolated in Kashmir (Panhotra 1985) and Bangladesh (Munshi 1987). Whether the resistance was actually conferred by the plasmid has been questioned as neither of the reports proved conclusively that naladixic acid resistance was transferable. Furthermore, the possibility that the plasmid was a mutator plasmid rather than a plasmid carrying a gene for resistance to naladixic acid cannot be eliminated (Crumplin 1987).

The only mechanisms by which bacteria are able to develop resistance to quinolones are through chromosomal mutations. This occurs by one of two mechanisms: Alterations in the target enzyme or by a mutation causing a reduction in the permeability of the cell for the drug (Lewin 1990, John 1989, and Hooper1987).

a)Alterations in DNA gyrase:

This may occur by changes in either of the two subunits, i.e., gyrA or gyrB. In the Enterobacteriaceae, resistance to the fluoro-quinolones has been found to be associated with alterations in gyrA (Linda 1998). Mutations in the gyrA gene which codes for the A subunit of DNA gyrase, confers high level resistance to all

4-quinolones (cited by Lewin1990). Such mutations have been identified in *E.coli* (cfx, gyrA, nalA, nfxA) (Nakamura1989, Hooper1986, Aoyama 1987,Chapman 1989), *Pseudomonas aeruginosa* (nalA, cfxA) (Hirai 1987, Robillard 1988) and *Citrobacter freundii* (Aoyam 1988). The quinolone resistant mutants of *E.coli* strain KL 16 were studied and the mutations in the GyrA gene were found at nucleotides 248, 3 and 119. These mutations are situated in an area near Tyr-122, which is a site in DNA gyrase, that is covalently bound to DNA when the enzyme breaks the phosphodiester bonds of DNA (cited by Lewin). Changes in the B subunit were observed by Yamagishi et al (Yamagishi 1990), who reported that nalC was a gyrB change at position 447, and that nalD conferred a shift at position 426. These two regions of the gyrB subunit are thought to be the ATP binding sites.

b) Reduction in the permeability of the cell :

The second mechanism of resistance involves mutations that affect the permeability of the quinolones, thereby leading to reduced uptake of not just the quinolones but also structurally

unrelated drugs such as β -lactams, tetracycline and chloramphenicol into the bacterial cell. The most frequent mutation associated with porin change is linked to OmpF a protein coded for, by the ompF gene. This may result in the diminished production of Omp F, alterations to the composition and structure of Omps and the synthesis of novel Omps. In E.coli, resistance may be mediated not only by an alteration in the ompF gene itself, but also by mutations in regulatory genes like nfxB, norB, cfxB and marA, that control the expression of ompF at a post transcriptional level. nfxB, cfxB or norB do not map in the ompF region, but have been shown to decrease ompF expression and hence cause reduced intracellular drug accumulation (Wolfson 1989).

c) Efflux:

The increased efflux of quinolones is another mechanism associated with quinolone resistance. However, the overall level of resistance due to efflux is a lot lesser than that seen with alterations in DNA gyrase.

Aminoglycosides

Aminoglycosides (Streptomycin, kanamycin, tobramycin, amikacin) are compounds that are characterized by the presence of an aminocyclitol ring linked to aminosugars in their structure. They have a broad antimicrobial spectrum of activity. They are active against aerobic and facultative aerobic Gram-negative bacilli and some Gram-positive organisms.

Mode of action:

The aminoglycosides are cidal making them unique among the antibacterials that act by inhibiting protein synthesis. They act by tightly binding to a structural component of the 30S ribosomal subunit to inhibit protein synthesis. Some aminoglycosides cause an alteration in the codon:anticodon matching. The resulting misreading of the mRNA allows an incorrect charged tRNA to bind to the "acceptor" (A) site on the ribosome. The protein produced has an incorrect primary amino acid sequence making it a defective protein that fails to function correctly. Other effects on protein synthesis have been noted, as well. The drugs are most effective on growing organisms (Internet site 1).

Mechanism of resistance to aminoglycosides:

There are three mechanisms by which resistance to aminoglycosides may occur in bacteria - Ribosome alteration, decreased permeability, and inactivation of the drugs by aminoglycoside modifying enzymes. The latter mechanism is of most clinical importance since the genes encoding aminoglycoside-modifying enzymes can be disseminated by plasmids or transposons.

a) Enzymatic modification:

Aminoglycoside modifying enzymes (AME) may modify the aminoglycoside in several ways: Aminoglycoside acetyltransferase (AAC) brings about the N-acetylation of aminoglycosides, Aminoglycoside phosphoryl transferase (APH) brings about the O-phosphorylation of aminoglycosides and Aminoglycoside adenyltransferase (AAD) modifies certain hydroxyl groups in the antibiotic molecule by adenylating them.

3'-O-phosphotransferase is very commonly found in *Pseudomonas*, *Klebsiella*, *E. coli*, and *Staphylococcus aureus*. Kanamycin is inactivated by being phosphorylated, but Gentamicin and Amikacin are resistant to this enzyme. *Pseudomonas* organisms occasionally have a plasmid coding for a 3'-N-acetyltransferase that acetylates and inactivates Gentamicin, but not Amikacin (Internet Site 2). Most genes coding for AMEs are plasmid specified and many are known to be carried on transposons.

b)Ribosome alteration :

High level resistance to streptomycin can result from single step mutations in chromosomal genes encoding ribosomal proteins: rpsL in E.coli (or strA), rpsD (or ramA or sud2), rpsE (eps or spc or spcA). These mutations in genes coding for ribosomal proteins prevent the antibiotics from binding to them (Internet Site 3).

Gentamicin resistance could be due to a mutation in rplF affecting ribosomal protein L6, which alters drug binding to the ribosome, in combination with an unc mutation which impairs transport across the membrane(Foster 1983).

Tetracycline

Tetracyclines are antibiotics, which inhibit the bacterial growth by stopping protein synthesis. They have been widely used for the past forty years as therapeutic agents in human and veterinary medicine. The classical tetracyclines were derived from Streptomyces species, but the newer derivatives are semisynthetic. Its clinical usefulness has been declining because of the appearance of an increasing number of tetracycline-resistant isolates of clinically important bacteria.

Mode of action:

Tetracyclines bind reversibly to the 30S subunits of bacterial (and eukaryotic) ribosomes at a site involving the 3' end of the 16S rRNA, a number of associated ribosomal proteins and magnesium ions. Hence they interfere with binding of charged-tRNA to the "Acceptor" site and block protein synthesis. They are "bacteriostatic" rather than cidal.

Mechanism of resistance:

Three different specific mechanisms of tetracycline resistance have been identified so far: tetracycline efflux, ribosome protection and tetracycline modification.

a)Tetracycline efflux:

An export protein from the major facilitator superfamily (MFS) achieves tetracycline efflux. The export protein catalyzes the exchange of tetracycline-divalent-metal-cation complex for a proton. In Gram-negative bacteria, the efflux proteins are linked to repressor proteins, which in the absence of tetracycline block transcription of the repressor and structural efflux genes (Roberts 1994, Speer BS 1992).

b) Ribosomal Protection:

Tet M, O, S, B and Q determinants encode for proteins that share amino acid sequence homology, to the elongation factor EF-Tu and EF-G. Marilyn Roberts in her reports suggests that these proteins that resemble the elongation factors, might function as Tetracycline resistant EF's or might block the binding of Tetracycline to the ribosome (Roberts 1994).

c) Enzymatic Alteration of Tetracycline:

The only example of enzymatic mediated resistance to Tetracycline is the TetX determinant that has been cloned from anaerobic Bacteroides. Efflux resistance genes are generally found on plasmids, whereas genes involved in ribosome protection have been found on both plasmids and self-transmissible chromosomal elements (conjugative transposons). In gram negative organisms, the Tet determinants are usually associated with transposons inserted into diverse groups of conjugative plasmids. The TetB determinant has the widest host range among gram negative species. The TetE determinant are associated with large non conjugative plasmids. The ribosomal protection TetM and TetQ determinants are often associated with conjugative chromosomal elements and are freely transmissible between gram positive and gram negative organisms (Roberts 1994, Pseer 1992)

Chloramphenicol

Chloramphenicol causes aplastic anemia and other blood dyscrasias in the patients treated. Originally isolated from *Streptomyces venezuelae*, chloramphenicol is now made synthetically. It has a broad spectrum of activity, which covers both gram positive and gram negative bacteria.

Mode of action:

Chloramphenicol binds reversibly to the 50S ribosomal subunit in the region of the A site involving the 23S rRNA. It binds to the peptidyl transferase enzyme to inhibit the transfer of the growing polypeptide to the next amino acid occupying the "Acceptor" site. The bacterial ribosome (and eukaryotic mitochondrial ribosome) is more sensitive, but protein synthesis is also decreased in patients receiving the drug as the concentration rises.

Mechanism of Resistance:

a) Enzymatic Alteration of the drug:

Resistance is due to the deactivation of the drug by acetylation of the hydroxyl group, amidase hydrolysis of the amide group, or reduction of the nitro group. Plasmid or transposon encoded Chloramphenicol acetyl transferases (CAT) are responsible for resistance by inactivating the antibiotic by acetylation of the hydroxyl group. CATs are found in many gram

negative bacteria and they are expressed constitutively. Chloramphenicol resistance results from the formation of 3-acetoxy-Cm, followed by 1,3-diacetoxy-Cm. The acetoxy derivatives of Chloramphenicol are inactive as they cannot bind to the ribosomes (Foster1983). The most widespread CAT is the type 1 specified by the Tn9 transposon

b)Nonenzymatic resistance:

The mar locus in E.coli regulates resistance to Chloramphenicol that does not involve acetyltransferase. Resistant E.coli cells show an active efflux of the antibiotic from the cells. Though the gene coding for the efflux system cannot be at the mar locus, it may be positively influenced by it (McMurry 1994).

Trimethoprim and Sulfonamides

Trimethoprim (TMP) and Sulfonamides (SUL) are synthetic antibacterial agents. Since 1968, TMP has been used in combination with SULs because the combination was synergistic in vitro (cited in Huovinen 1995). TMP is active against the dihydrofolate reductase, which acts later in the same metabolic pathway as dihydropteroate synthetase, the enzyme that is inhibited by sulfonamides. Hence, TMP and Sulfonamides were always administered together. However, soon after the clinical

introduction of the TMP-sulphamethoxazole combination (co-trimoxazole) resistance to TMP emerged (Amyes 1989).

Acquired resistance to Trimethoprim (TMP) may be chromosomally encoded or by antibiotic resistance plasmids.

a) Chromosomal TMP resistance:

Dihydrofolate reductase (DHFR) is an essential enzyme in all living cells. TMP is a competitive inhibitor of this enzyme. Chromosomal resistance could be of the following types (Huovinen 1995):

1. Chromosomal location of transposon Tn7
2. Low level resistance to TMP was found to be the result of a mutational loss in bacteria of their ability to methylate deoxyuridilic acid to thymidilic acid, making them dependent on an external supply of thymine.
3. Mutational changes in the gene for DHFR.

b) Plasmid borne TMP resistance :

The plasmid mediated TMP resistance mechanism involves the production of an additional dihydrofolate reductase (DHFR), which unlike the chromosomal enzyme was resistant to the action of TMP (Amyes 1989). An alternative type of resistance where mutations result in the overproduction of the normal chromosomally encoded DHFR has also been reported (Amyes 1992). Mutants of this type are rare in clinical isolates.

a) Chromosomal SUL resistance:

SULs are the structural analogs of the enzyme dihydropteroate synthetase, which catalyses the formation of hydropteroic acid in bacterial cells. Chromosomal mutations in the *dhps* genes have isolated.

b) Plasmid borne SUL resistance:

Clinically occurring SUL resistance in bacteria is usually plasmid borne and is due to the presence of alternative drug resistant variants of DHPS.

MATERIALS AND METHODS

2.1 Collection of the Clinical Isolates:

80 clinical isolates were collected from the year 1997 to 1998 from the J.J Group of Hospitals, the Kasturba Hospital for Infectious Diseases and the Bacteriology Department of Haffkine Institute.

61 of these isolates, were used for the present study.

2.2 Identification of the Clinical Isolates:

All the clinical isolates were subjected to identification on the basis of their morphological, cultural and biochemical characteristics (Cowan 1974, Stokes 1987). The following tests were employed for the identification of the clinical isolates.

- ❖ Gram staining to determine the gram character and the morphology of the isolates.
- ❖ The motility test
- ❖ Isolation on MacConkey's agar, Nutrient agar, and Cetrimide agar to determine the cultural characteristics of the organisms and also to confirm their purity.
- ❖ Biochemical tests to check for the fermentation of sugars such as sucrose, glucose, lactose and mannitol. Tests were also performed to check for the production of indole from tryptophan. Besides these, the Methyl Red, Vogues Proskauer and the Simmon's Citrate tests were also performed. The

Phenylpyruvic acid test was performed, to differentiate *Proteus* from the other gram-negative isolates.

2.3 Determination of Drug Resistance Patterns:

The antibiograms of each of the 61 isolates was examined by the disk diffusion technique of Bauer and associates (Bauer 1966) against the following 12 antibiotics: Ampicillin (Am), Chloramphenicol (Cm), Gentamicin (Gm), Kanamycin (Km), Amikacin (Ak), Streptomycin(Sm), Ceftazidime (Cz), Cefotaxime (Cf), Naladixic acid (Na), Ciprofloxacin (Cp), Tetracycline (Tc) and Trimethoprim+ Sulphamethoxazole (T+S).

Media and Antibiotic discs:

- ❖ Mueller Hinton's broth sterilized at 121°C (15-lbs. pressure) for 15 minutes was used for the preliminary growth of the cultures.
- ❖ Mueller Hinton's agar sterilized at 121°C (15-lbs. pressure) for 15 minutes was used for the antibiotic sensitivity test.
- ❖ The antibiotic discs containing appropriate concentrations of the required antibiotics were prepared in the department. For details on the preparation of the antibiotic discs, please refer to the Appendix (A4).

Protocol:

1. The colonies to be tested were picked up with a wire loop from the original culture plate and inoculated into a test tube

- containing 5ml of M.H broth. This was then incubated at 37°C for 24 hours.
2. 0.1 ml of this overnight culture was inoculated in fresh M H broth and incubated at 37°C for about 3-4 hours.
 3. M.H agar is poured into sterile petri plates so as to obtain a layer of about 4mm thickness (20ml in an 8.5-cm dish) on solidification. The plates are then dried for 20-30 minutes before inoculation, so as to eliminate surface moisture.
 4. Sterile cotton swabs are soaked in the M.H inoculum tubes, and surplus suspension is removed from the swab, by being rotated against the side of the tube, before the plates are seeded.
 5. These cotton swabs are then used to surface spread on M H agar plates in order to get a confluent growth.
 6. After the inoculum has dried, each drug-containing disc is placed on the agar surface with flamed forceps.
 7. Standard strain *E. coli* ATCC 10148, which is sensitive to all the drugs, was employed with each set of experiments so as to check on the activity of the discs and to check the reproducibility of the test.
 8. Following overnight incubation the zone diameters (including the 6mm disc) was measured on the underside of the plate.
 9. The zone diameters are recorded and interpreted according to the standard charts provided by NCCLS (NCCLS 1988).

2.4 Maintenance of Cultures:

After confirming the purity of the isolates and determining their antibiotic resistance patterns, the clinical isolates were all maintained on Nutrient agar slants containing 100µgms of Ampicillin. The slants were stored at 4°C and were subcultured regularly.

2.5 Conferring the Role of the Plasmid

The role of the plasmid in conferring drug resistance can be determined by the following experiments:

- i) Curing the resistant isolates of its R plasmid and demonstrating the consequent loss of resistance to the antibiotics that it was originally resistant to.
- ii) The conjugal transfer of the resistance determinants to a suitable recipient like E.coli K12.
- iii) Plasmid DNA isolation and its physical demonstration by agarose gel electrophoresis. By this, the presence of the plasmid can be determined directly.

2.5.1 Curing of the plasmid:

Elimination of antibiotic resistance at a high frequency is required in order to assert the extra chromosomal location of the drug resistance determinants. Curing cells of their antibiotic resistance markers, using intercalating dyes such as Acridine orange and Ethidium bromide (Watanabe 1961, Bouanchaud 1969) have been successful in the past although at a very low frequency.

Watanabe suggested that the low frequency of elimination might be due to the chromosomal integration of the resistance factors. According to their reports there was an increase in the frequencies of elimination, when the cells were exposed to U.V, prior to exposure to acridine orange. They suggested that this may be due to the conversion of the integrated resistance factor to the autonomous state. On the basis of this hypothesis, resistant cells that could not be cured by treatment with acridine orange or ethidium bromide, were then subjected to U.V treatment prior to exposure to ethidium bromide.

Bacterial strains used:

Resistant Isolates	MDR Pattern
<i>Klebsiella pneumoniae</i> (K 15)	Resistant to all 12 antibiotics
<i>Shigella dysenteriae</i> (J 21)	Am-Sm Tc-Cm-T+S
<i>Escherichia coli</i> (J 27)	Am-Ak-Km-Sm-Na-Tc-Cm
<i>Citrobacter freundii</i> (J 36)	Am-Km-Sm-Tc
<i>Salmonella typhi</i> (J1)	Am-Sm-Tc-Cm-T+S
<i>Salmonella typhi</i> (J2)	Am-Sm-Tc-Cm-T+S

Salmonella typhimurium containing a curable Ampicillin plasmid was used as the control .

Media and Reagents used:

Luria Bertani broth sterilized at 121°C (15-lbs. pressure) for 15 minutes was used for the preliminary growth of the cultures

- ♦ Luria Bertani agar sterilized at 121°C (15-lbs. pressure) for 15 minutes, was used to determine the viable count of the culture treated with the mutagen.
- ♦ Ethidium Bromide (EtBr) stock solution : EtBr was dissolved in distilled water at 5mg/ml and autoclaved.
- ♦ Acridine orange stock solution: Acridine orange was dissolved in distilled water at 10mg/ml and autoclaved.

Protocol:

Treatment with Acridine Orange/ Ethidium Bromide:

1. 0.1 ml of an overnight resistant culture was inoculated into 4.9 ml of fresh L.B broth and incubated at 37°C for 3-4 hours.
2. 0.1 ml of this culture was then inoculated into tubes of L.B broth (pH 7.6) containing various concentrations of Ethidium Bromide/Acridine Orange. The optimal concentration depends on the bacterial strain used. The inoculated media was then incubated in the dark at 37°C for 24 hours.
3. The next day, the tubes were serially diluted in 0.85% NaCl and assayed for viable cells by plating 0.1 ml on L.B agar plates.
4. From these plates, 35 colonies were picked up and patched on plates containing the requisite amounts of the appropriate antibiotics.

Treatment with UV and Ethidium Bromide:

1. 0.1ml of an overnight resistant culture was inoculated into 4.9 ml of fresh L.B broth and incubated at 37°C for 3-4 hours.
2. 5 ml of this culture was transferred to a sterile petri dish and subjected to UV radiation.
3. The U.V irradiated cultures were then incubated at 37°C for one hour in the dark.
4. This treated culture is then exposed to different concentrations of Ethidium bromide according to the above-mentioned protocol.

2.5.2 Conjugal Transferability of Drug Resistance:

The drug resistant isolates were examined for their ability to transfer their drug resistant markers to the recipient strain (*E.coli* K12), using the broth mating method of conjugation (Willets 1988). In essence, a conjugation experiment is carried out by mixing appropriate quantities of broth cultures of the Donor and Recipient strains, under appropriate conditions and selecting plasmid containing transconjugants of the recipient strain. Transfer of resistance determinants has been reported to be temperature sensitive. Hence conjugation was carried out at both 28°C and at 37°C.

Bacterial strains:

Rachna Thapliyal (Thapliyal 1995) reported in 1995 that ciprofloxacin resistant isolates failed to transfer their resistant markers to the recipient strain. This is probably because resistance to Quinolones in some cases may be the result of changes in the structure of the Omp proteins present in the LPS layer of the bacterial cells (Lewin 1990, Wolfson 1989). Such changes in the cell surface of bacteria may cause ineffective cell to cell contact, and hence they may not be able to conjugate. Hence in our study, only Ciprofloxacin sensitive or intermediately resistant isolates were chosen as donors in the conjugation experiments.

Donor strains	MDR Pattern
<i>Klebsiella pneumoniae</i> (K15)	Resistant to all 12 antibiotics
<i>Shigella dysenteriae</i> (J 21)	Am-Sm Tc-Cm-T+S
<i>Escherichia coli</i> (J 27)	Am-Ak-Km-Sm-Na-Tc-Cm
<i>Citrobacter freundii</i> (J 36)	Am-Km-Sm-Tc
<i>Salmonella typhi</i> (J1)	Am-Sm-Tc-Cm-T+S
<i>Salmonella typhi</i> (J2)	Am-Sm-Tc-Cm-T+S

Recipient strain used: *E.coli* K12 (F⁻ Lac⁺ Nal^r)

Media used:

- ◆ Nutrient broth sterilized at 121°C (15-lbs. pressure) for 15 minutes was used for the preliminary growth of the cultures.
- ◆ MacConkeys agar sterilized at 121°C (15-lbs. pressure) for 15 minutes was used to determine both the Donor count and the Transconjugant count. It was also used along with the requisite amount of antibiotics to confirm the antibiotic resistance markers present in the transconjugants.

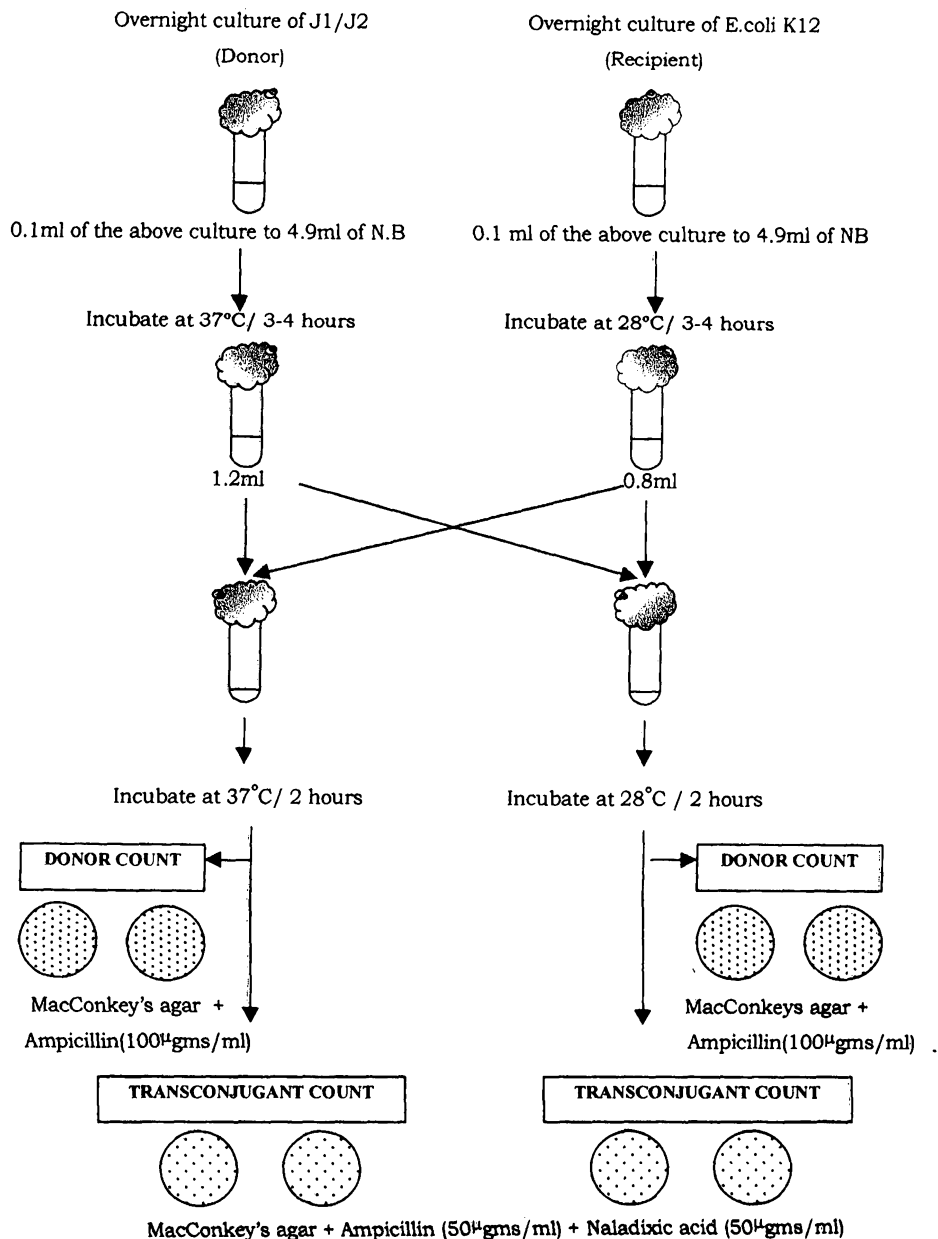
Conjugational conditions:

- ◆ The Donors and the Recipient were checked for the above-mentioned antibiotic markers, by plating them on MacConkey's agar containing the appropriate antibiotics.
- ◆ Separate 4.9-ml tubes of Nutrient broth were inoculated with overnight cultures of Donor and Recipient cells (*E.coli* K12). This is then incubated at 37°C for 3-4 hours i.e., till the cultures grow to mid log phase.
- ◆ 1.2 ml of the mid log phase culture of the Donor and 0.8 ml of the Recipient are mixed together and incubated at 37°C and 28°C for two hours/18 hours.
- ◆ Donor count: At the end of the two hour mating period, samples from the mating mixture were serially diluted using 0.85% NaCl solution and viable counts of the donors were made on MacConkeys agar supplemented with the appropriate amounts of the antibiotic that will help select donors. If there are no

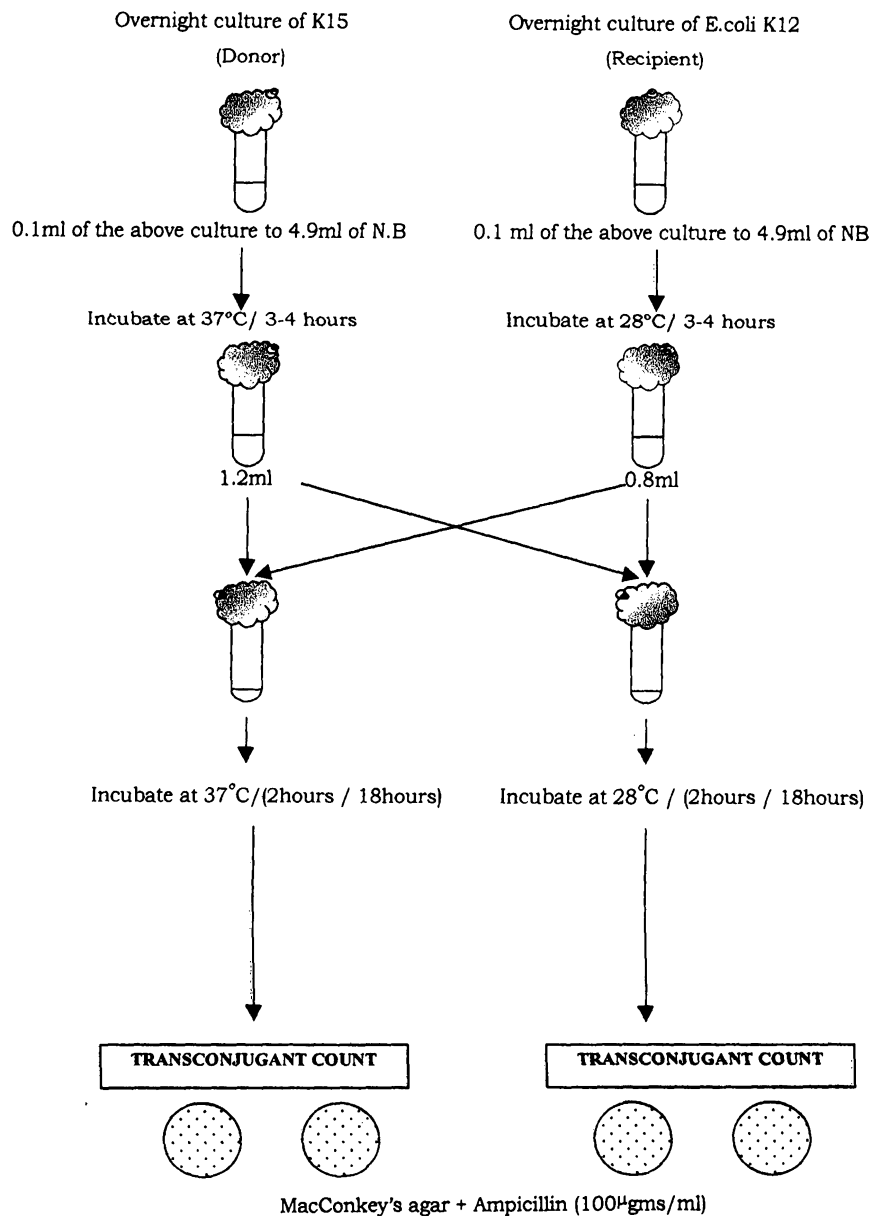
antibiotic markers to select only the donor cells from the conjugation mixture, they will have to be differentiated from the transconjugants on the basis of their morphological characteristics.

- ♦ *Transconjugant count:* At the end of the two hour incubation, samples from the mating mixture were serially diluted using 0.85% NaCl solution and viable counts of the transconjugants were made on MacConkeys agar supplemented with the required amounts of the antibiotics that used for the selection of the transconjugants.
- ♦ ***The transfer frequency is calculated as the number of viable cells of the drug resistant transconjugant to the number of viable cells of the donor strain after incubation of the mixture***

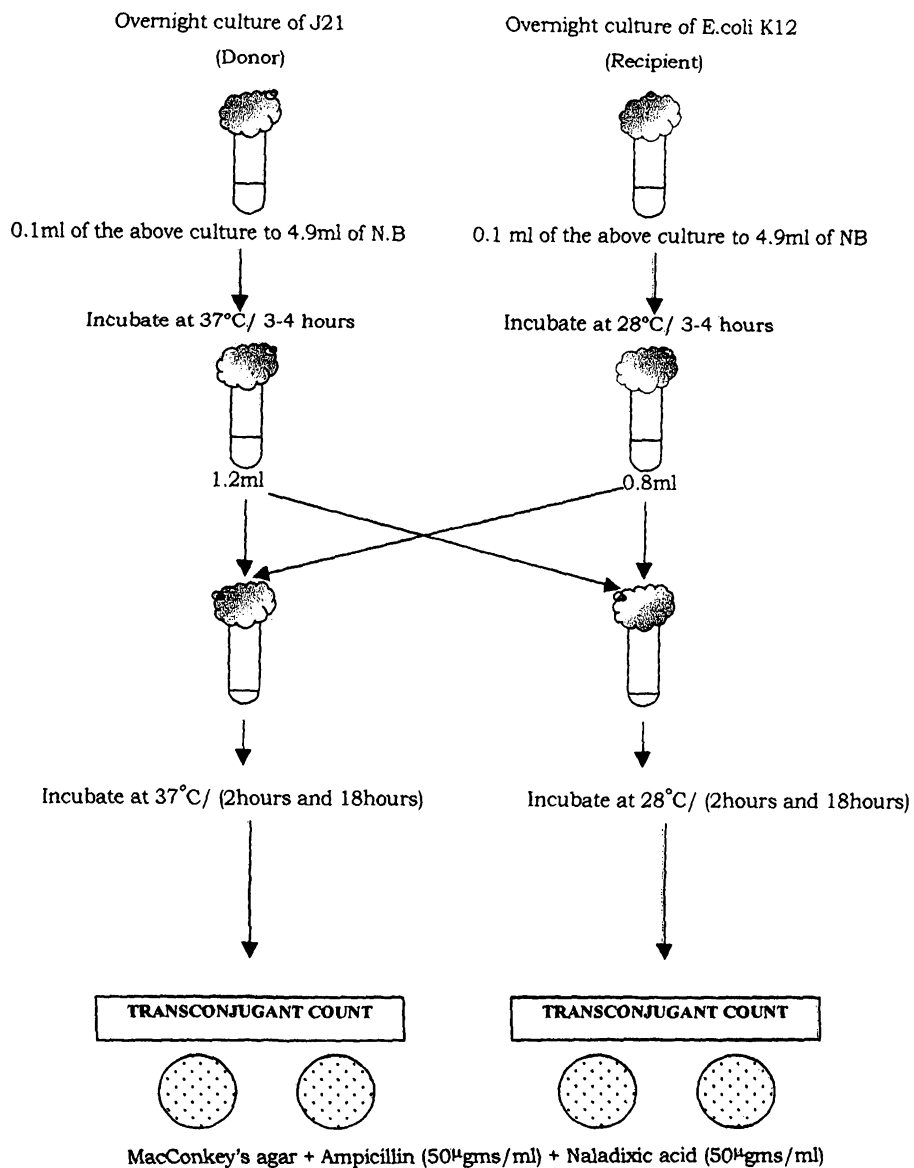
CONJUGATION OF J1/J2 WITH E.coli K12



CONJUGATION OF K15 WITH E.coli K12



CONJUGATION OF J21 WITH E.coli K12



2.5.3 Isolation of the plasmid:

To determine if a given bacterium harbors a plasmid, it is necessary to isolate the plasmid DNA and demonstrate its presence by gel electrophoresis.

Bacterial strains used:

Clinical Isolates:

Resistant Strains	MDR Pattern
<i>Klebsiella pneumoniae</i> (K15)	Resistant to all 12 antibiotics
<i>Shigella dysenteriae</i> (J 21)	Am-Sm Tc-Cm-T+S
<i>Escherichia coli</i> (J 27)	Am-Ak-Km-Sm-Na-Tc-Cm
<i>Citrobacter freundii</i> (J 36)	Am-Km-Sm-Tc
<i>Salmonella typhi</i> (J1)	Am-Sm-Tc-Cm-T+S
<i>Salmonella typhi</i> (J2)	Am-Sm-Tc-Cm-T+S

Transconjugants:

TJ1(Transconjugant of J1 and *E.coli* K12)

TJ2(Transconjugant of J2 and *E.coli* K12)

TJ21(Transconjugant of J21 and *E.coli* K12)

TK15 (Transconjugant of K15 and *E.coli* K12).

Cured strains:

J1 cured of all its resistance markers and J2 cured of all its resistance markers.

Controls: PBR and *E.coli* K12

The Alkaline Lysis method for isolation of plasmid DNA :

The Alkaline lysis method of plasmid extraction described by Brinboim and Doly(1979) was followed with slight modifications.

Reagents used:

SOLUTION 1	SOLUTION 2	SOLUTION 3	T.E BUFFER
Glucose 50mM	SDS 2%	Na Acetate 3M	Tris Acetate 10mM
Tris.Cl 25mM	NaOH 5N		EDTA 1mM
EDTA 10mM			PH 8.0
Lysozyme (2mg/ml)			

Protocol:

1. Harvesting of Cells:

The organism is inoculated in 5 ml of Luria Bertani broth containing 100µgms/ml of the Ampicillin stock solution. It is then incubated at 37°C for 24 hours with vigorous agitation. The cells are spun down at 12,000 rpm for 15 minutes in a Sorvall SS 34 rotor.

2. Lysis of the cells:

- ◆ Suspend the bacterial pellet in 100µl of chilled Solution1 containing 2mg /ml of lysozyme.

The EDTA in the Solution 1 chelates divalent cations, disrupting the outer membrane. This allows the lysozyme to attack the peptidoglycan layer. It inactivates cation dependent

nucleases and also prevents the disruption of phosphodiester bonds. Lysozyme hydrolyses the β 1'4 glycosidic bonds of the polysaccharide backbone of the peptidoglycan layer, hence causing the destruction of the cell membrane and the release of DNA and other cellular components.

- ◆ 200 μ l of Solution 2 is added to the preparation. Close the tube and mix gently by inverting 5 times. Keep on ice for two minutes.

Solution 2 contains an anionic detergent Sodium Dodecyl Sulphate (SDS) and NaOH. SDS disrupts the cytoplasmic membrane and denatures much of the cell protein. It also favors the dissociation of protein-DNA complexes. The NaOH raises the pH, which denatures double stranded DNA.

- ◆ 150 μ l of ice cold Solution 3 which consists of 3M Sodium Acetate at a pH of 5.2 is added to the above preparation. The tube was closed and vortexed gently. The tube containing the preparation was then kept on ice for 5 minutes and then centrifuged at 12,000 rpm for 5 minutes at 4°C in a Sorvall SS 34 rotor.

Sodium Acetate at a pH of 5.2 is added to neutralize the pH of the preparation, allowing the single stranded DNA to reanneal. The rate of reassosiation is proportional to the length of DNA. Since the plasmid is small and intertwined, it reanneals much faster than chromosomal DNA. When the solution is centrifuged, the reannealed plasmid DNA remains in the

solution, but large aggregates of chromosomal DNA, RNA and protein are pelleted out.

3. Removal of proteins :

- ◆ 400µl of the supernatant was transferred to a fresh tube. Equal volume of Chloroform: Isoamylalcohol (24:1) was added to the supernatant. The tube was then centrifuged at 13,000 rpm for 2 minutes at 4°C in a Sorvall SS 34 rotor.

Before the plasmid is precipitated, the solution must be deproteinised. This is brought about with Chloroform: Isoamylalcohol. Chloroform brings about the denaturation of proteins and isoamylalcohol reduces foaming and stabilizes the interface between the aqueous phase and the organic phases where the protein collects.

4. Precipitation of the DNA:

- ◆ The upper aqueous phase was transferred to a fresh tube and two volumes of ice cold ethanol were added. This was kept at room temperature to precipitate the plasmid DNA. Centrifuge the tube at 4°C for 30 minutes in a Sorvall SS 34 rotor.

DNA is precipitated using ethanol. Ethanol is used because DNA becomes insoluble due to its ionic nature if the aqueous medium is made less polar by addition of an organic solvent.

- ◆ Discard the supernatant and air-dry the pellet for 10 minutes.

The pellet is then re-suspended in 50µl of Tris-EDTA buffer.

The Boiling Method for isolation of plasmid DNA:

The Alkaline Lysis method of plasmid isolation is time consuming as it involves treating the cells with lysozyme prior to lysing them with a detergent under highly alkaline conditions. Using lysozyme in an ionic buffer is unsuitable in some cases, as lysis depends on the stage of cell growth, and on the temperature and length of incubation. This may not result in reproducible results in some cases. These time consuming steps are avoided in the Boiling method (Kado 1981). This method is ideal for isolating small molecular weight plasmid DNA from cells.

Reagents used:

LYSIS SOLUTION	"E" BUFFER	T.E BUFFER
3% SDS	Tris Acetate 40mM	Tris Acetate 1 M
Tris 50mM	EDTA 2mM	EDTA 0.5 M
2N NaOH	Glacial acetic acid	PH 8.0
pH 12.6	pH 7.9	

Protocol:

1. The cells were grown in 10 ml of nutrient broth under shaken conditions. The culture was then centrifuged at 10,000 rpm for 15 minutes at 4°C in a Sorvall SS 34 rotor.
2. The cell pellet was suspended in 3ml of E Buffer.
3. The cells were then lysed using 6 ml of the Lysis solution.

4. The solution was then heated at 50°C to 65°C for 20 minutes in a water bath.
5. The temperature treatment was used to eliminate the “lathery” effect of the chromosomal DNA that is observed in agarose gel electrophoresis. The temperature treatment is also used to eliminate RNA and chromosomal DNA altogether.
6. Two volumes of unbuffered phenol:chloroform solution (1:1 vol/vol) were added to the above preparation.
7. The lysate is extracted using unbuffered phenol:chloroform. This mixture was designed to minimize the formation of brown oxidation pigments that occurs when phenol is used alone. Using the unbuffered mixture also helps in lowering the pH of the lysate to 9.3. It is also conveniently separated into the hydrophobic and aqueous phases and facilitates the denaturation and extraction of proteins.
8. The solution is centrifuged at 6000 rpm for 15 minutes in a Sorvall SS34 rotor.
9. Avoiding the precipitate at the interphase, the aqueous phase was transferred into another tube.
10. Two volumes of ice cold ethanol were added to this preparation. This was kept at room temperature to precipitate the plasmid DNA and then centrifuged at 4°C for 30 minutes.
11. The supernatant was discarded and the pellet was air-dried for 10 minutes.

12. The pellet was then resuspended in 50 μ l of Tris- EDTA buffer.

2.5.4 Agarose Gel Electrophoresis

The presence of the plasmid in the preparation can be detected by visualizing the DNA on an agarose gel. The technique was used from Maniatis et al (1982).

Reagents used:

T.B.E BUFFER (10X)	Loading Buffer	Ethidium bromide
Tris base 890mM	Bromophenol blue 0.25%	1mg/ml of EtBr stock solution
Boric Acid 890mM	40% Sucrose	
EDTA 20mM		

Protocol:

1. Preparation of 0.7% Agarose gel:

0.14 grams of Agarose were added to 18ml of distilled water. To this 2 ml of 10x Tris Borate EDTA buffer and Ethidium Bromide at a concentration of 0.5 μ gms/ml were added. This was then digested completely in a water bath. The solution was cooled and poured into a cast and set to form a gel.

2. 250 ml of the Electrophoresis buffer (1X TBE buffer), was added to the electrophorsis tank.

3. 20 μl of the plasmid preparation was mixed with 5 μl of the loading dye on a sterile aluminum foil. This was then loaded into the well using a micropipette.
4. Electrophoresis is carried out at a constant voltage of 80 volts for 2 hours, till the tracking dye reaches the end of the gel.
5. The gel is then viewed on a U.V transilluminator (260nm)

RESULTS

3.1 Identification of the Clinical Isolates:

A total of 80 clinical isolates of gram negative bacterial pathogens were collected from J.J Group of Hospitals, the Kasturba Hospital for Infectious Diseases and the Bacteriology Department of Haffkine Institute from 1997-1998. 61 of these isolates were used in the present study.

These isolates were identified on the basis of their morphological, cultural and biochemical characteristics.

Of the 61 isolates 11 isolates belonged to the genus *Klebsiella*. They showed characteristic large, pink, mucoid, dome shaped colonies on MacConkey's agar and were non-motile.

17 of the 61 isolates belonged to the genus *Escherichia* and showed small, bright pink, translucent, butyrous colonies with an entire margin on MacConkeys agar.

14 of the 61 isolates belonged to the genus *Salmonella* and showed small, colorless, translucent colonies on MacConkeys agar. These were actively motile.

10 isolates belonged to the genus *Pseudomonas* and showed characteristic pyocin producing green colored colonies on Cetrimide agar.

6 isolates belonged to the genus *Proteus* and showed swarming, on both MacConkeys and Nutrient agar.

Among the 61 isolates there was one that belonged to the genus *Shigella*. These organisms were non-motile.

There was one isolate that belonged to the genus *Citrobacter freundii* and one that belonged to the genus *Providencia*.

All the 61 isolates were subjected to further identification based on their biochemical characteristics (Table 1-A and 1-B). The number of isolates belonging to each species is given in Table 2. Since serotyping was not done for *Salmonella*, they were not further identified upto the individual serotypes.



TABLE 1-A
STANDARD BIOCHEMICAL CHART

	<i>Escherichia coli</i>	<i>Proteus mirabilis</i>	<i>Proteus vulgaris</i>	<i>Klebsiella pneumoniae</i>	<i>Klebsiella Oxytoca</i>	<i>Pseudomonas aeruginosa</i>
Glucose	AG	AG	AG	AG	AG	(v)
Sucrose	AG	-	+	AG	AG	-
Lactose	AG	-	-	AG	AG	-
Mannitol	AG	-	-	AG	AG	-
Indole	+	-	+	-	+	-
M.R	+	+	+	-	-	-
V.P	-	-	-	+	+	-
Citrate	-	-	-	+	+	+
TSI						
Slant	Acidic	Alkaline	Alkaline	Acidic	Acidic	Alkaline
Butt	Acidic	Acidic	Acidic	Acidic	Acidic	No change
Gas	+	+	+	+	+	-
H ₂ S	-	+	+	-	-	-
Oxidase	-	-	-	-	-	+
PPA	-	+	+	-	-	-
Motility	+	+	+	-	-	+

Key:- AG:- Acid and gas production

A :- Only Acid production

+

- :- Negative reaction

(v) :- Variable results

MR :-Methyl Red

VP :- Vogues Proskauer

PPA:- Phenylpyruvic acid test

TABLE 1-B
STANDARD BIOCHEMICAL CHART

	<i>Salmonella typhi</i>	<i>Salmonella paratyphi</i>	<i>Providencia rettgeri</i>	<i>Shigella dysenteriae</i>	<i>Citrobacter freundii</i>
Glucose	A	AG	A	A	AG
Sucrose	-	-	-	-	(v)
Lactose	-	-	-	-	(v)
Mannitol	+	AG	+	-	+
Indole	-	-	+	+	-
M.R	+	+	+	+	+
V.P	-	-	-	-	-
Citrate	-	-	+	-	+
TSI					
Slant	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline
Butt	Acidic	Acidic	Acidic	Acidic	Acidic
Gas	-	+	-	-	+
H ₂ S	+	(v)	-	-	(v)
Oxidase	-	-	-	-	-
PPA	-	-	+	-	-
Motility	+	+	+	-	+

Key :- AG:- Acid and gas production

A :- Only Acid production

+

- :- Negative reaction

(v) :- Variable results

MR :-Methyl Red

VP :- Vogues Proskauer

PPA:- Phenylpyruvic acid test

TABLE 2

BACTERIAL SPECIES	NO: OF ISOLATES
<i>Escherichia coli</i>	17
<i>Klebsiella pneumoniae</i>	8
<i>Klebsiella oxytoca</i>	3
<i>Salmonella typhi</i>	13
<i>Salmonella paratyphi</i>	1
<i>Pseudomonas aeruginosa</i>	10
<i>Proteus mirabilis</i>	3
<i>Proteus vulgaris</i>	3
<i>Shigella dysenteriae</i>	1
<i>Citrobacter freundii</i>	1
<i>Providencia retgerii</i>	1

3.2 Determination of the Antibiotic Resistance patterns:

Antibiograms of the 61 isolates collected were determined by the Kirby Bauer disk sensitivity method against 12 antibiotics:

Ampicillin (Am), Chloramphenicol (Cm), Gentamicin (Gm), Kanamycin (Km), Amikacin (Ak), Streptomycin(Sm), Ceftazidime (Cz), Cefotaxime (Cf), Naladixic acid (Na), Ciprofloxacin (Cp), Tetracycline (Tc) and Trimethoprim+ Sulphamethoxazole (T+S).

Tables 4-A, 4-B, and 4-C give the zone diameters obtained for each of the 61 isolates collected. The zone diameters are interpreted according to Table 3 and the organisms are classified as resistant, intermediately resistant or sensitive to the antibiotics used.

Graph 1 gives a clear picture of the percentage of resistant isolates obtained for each of the antibiotics used.

- ❖ All 61 of the isolates i.e., 100% of the isolates were resistant to Ampicillin, Tetracycline and Streptomycin.
- ❖ 80% of the isolates were resistant to Aminoglycoside Gentamicin, 83 % were resistant to Kanamycin and 73% of the isolates were resistant to Amikacin.
- ❖ 70.5% resistance was seen to Ciprofloxacin while 92% of the isolates were resistant to Naladixic acid.
- ❖ Resistance to Chloramphenicol was 93%

- ❖ 93.5% of the isolates were resistant to the combination of Trimethoprim and Sulphamethoxazole.
- ❖ 69% of the isolates were resistant to ceftazidime, while only 52.5% (incorrectly reported in the synopsis as 67%) of the isolates were resistant to cefotaxime.

A detailed analysis of the resistance patterns indicates that 20 (32.8%) of the 61 isolates were resistant to all the 12 antibiotics used and 21 (34.4%) of the 61 isolates were resistant to 11 to the antibiotics used.

TABLE 3**ZONE SIZES AND THEIR INTERPRETATIONS FOR THE
ANTIBIOTICS USED**

ANTIBIOTIC	DISK POTENCY	INHIBITION ZONE DIAMETER (In mm)		
		RESISTANT	INTERMEDIATE	SENSITIVE
Ampicillin	10µgms	20 or less	21-28	29 or more
Cefotaxime	30µgms	14 or less	15-22	23 or more
Ceftazidime	µgms	14 or less	15-17	18 or more
Amikacin	30µgms	14 or less	15-16	17 or more
Gentamicin	10µgms	12 or less	13-14	15 or more
Kanamycin	30µgms	13 or less	14-17	18 or more
Streptomycin	10µgms	11 or less	12-14	15 or more
Naladixic Acid	30µgms	13 or less	14-18	19 or more
Ciprofloxacin	5µgms	15 or less	16-20	21 or more
Tetracycline	30µgms	14 or less	15-18	19 or more
Chloramphenicol	30µgms	12 or less	13-17	18 or more
T+S	1.25:23.75 µgms	10 or less	11-17	16 or more

TABLE 4 - A

ISOLATE	ZONE DIAMETER (In mm).											
	AMP	CFX	CFZ	AMK	GEN	KAN	STR	NAL	CIP	TET	CHL	T+S
K2	-	20	19	17	11	9	-	-	-	10	-	5
K3	-	18	15	15	12	15	-	-	19	-	-	-
K4	-	17	14	14	13	14	-	-	20	-	-	-
K5	-	24	15	9	-	15	-	-	-	-	-	-
K6	-	20	13	14	12	13	-	-	19	-	-	-
K7	-	28	18	18	16	21	-	-	17	-	-	-
K9	-	26	15	8	-	-	-	-	-	-	-	-
K10	-	11	-	10	7	-	-	-	8	-	-	-
K11	-	10	-	-	8	10	-	-	16	-	-	-
K13	-	28	18	18	17	23	-	-	17	-	-	-
K15	-	18	-	-	-	-	-	-	15	-	-	-
K16	-	17	18	-	-	-	-	-	14	-	-	-
K17	-	9	14	9	6	-	-	-	17	-	-	-
K18	-	-	5	-	-	-	-	-	10	-	-	-
K23	-	11	-	12	10	-	-	-	13	-	-	-
K25	-	21	15	10	-	-	-	-	-	-	-	-
K27	-	11	9	9	-	-	-	-	-	-	10	-
K28	-	19	16	14	-	-	-	-	-	-	-	-
K29	-	12	-	5	-	-	-	-	-	-	-	-
K30	-	10	-	-	-	-	-	-	-	-	5	-

Key:- Ampicillin (AMP), Chloramphenicol (CHL), Gentamicin (GEN), Kanamycin (KAN), Amikacin (AMK), Streptomycin(STR), Ceftazidime (CFZ), Cefotaxime (CFX), Naladixic acid (NAL), Ciprofloxacin (CIP), Tetracycline (TET) and Trimethoprim+ Sulphamethoxazole (T+S).

TABLE 4 – B

ISOLATE	ZONE DIAMETER (In mm).											
	AMP	CFX	CFZ	AMK	GEN	KAN	STR	NAL	CIP	TET	CHL	T+S
K31	-	8	-	-	-	-	-	-	-	-	-	-
K36	-	26	16	13	-	-	-	-	-	-	-	-
K38	-	24	15	15	13	-	8	-	11	-	-	-
K39	-	21	15	15	-	-	-	-	-	-	-	-
K42	-	13	11	11	-	-	-	-	-	-	-	-
J1	-	25	19	22	21	20	-	19	25	-	-	-
J2	-	23	19	19	18	22	-	20	28	-	-	-
J3	-	16	16	11	-	-	-	-	9	-	8	-
J4	-	9	-	-	-	-	-	-	-	-	10	-
J5	-	32	22	20	21	20	-	16	26	-	-	-
J6	-	24	12	18	-	-	-	16	14	-	-	-
J7	-	32	22	20	21	20	-	15	28	-	-	-
J8	-	23	-	19	15	-	-	-	21	-	30	-
J9	-	-	12	-	-	-	-	-	-	-	-	-
J10	-	24	18	21	-	18	-	16	21	-	-	-
J11	-	25	19	22	-	17	-	16	27	-	-	-
J15	11	17	18	30	13	-	-	-	26	14	25	-
J16	10	18	15	18	-	-	-	-	-	-	-	-
J17	-	18	18	23	22	6	-	-	24	-	-	-
J18	-	25	15	14	-	12	-	-	-	-	-	-
J19	-	25	-	8	-	-	-	-	12	-	-	-

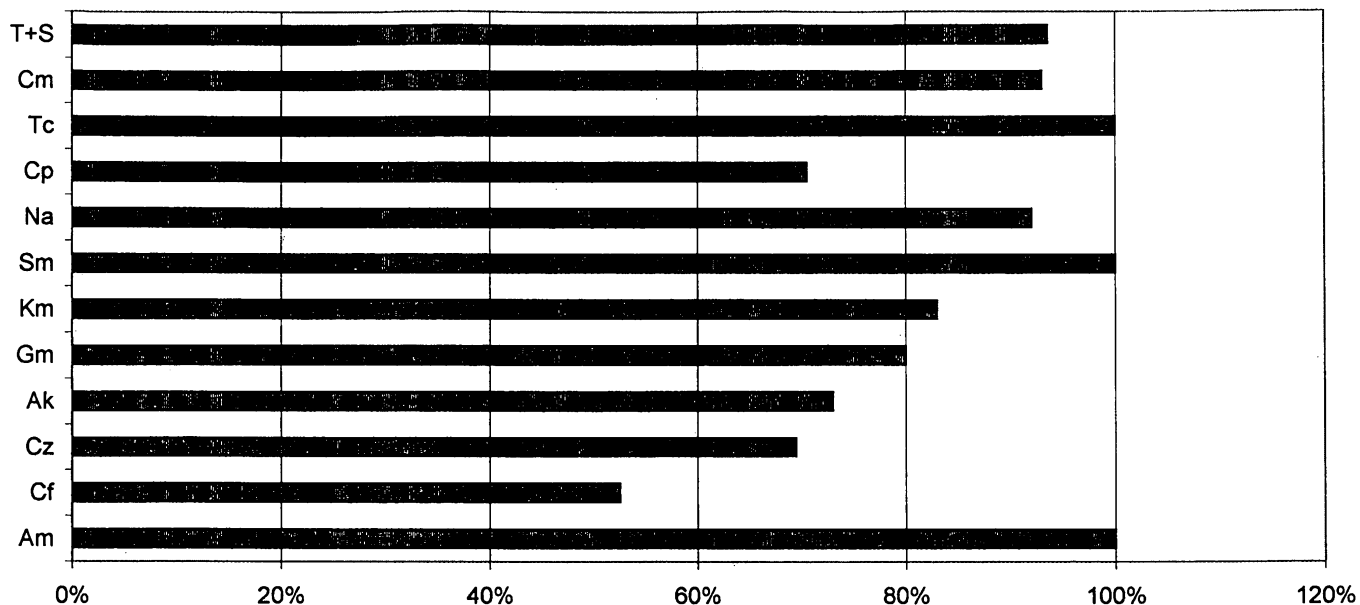
Key:- Ampicillin (AMP), Chloramphenicol (CHL), Gentamicin (GEN), Kanamycin (KAN), Amikacin (AMK), Streptomycin(STR), Ceftazidime (CFZ), Cefotaxime (CFX), Naladixic acid (NAL), Ciprofloxacin (CIP), Tetracycline (TET) and Trimethoprim+ Sulphamethoxazole (T+S).

TABLE 4 – C

ISOLATE	ZONE DIAMETER (In mm).											
	AMP	CFX	CFZ	AMK	GEN	KAN	STR	NAL	CIP	TET	CHL	T+S
J21	-	27	24	15	20	19	-	21	25	-	8	-
J23	-	24	15	11	-	-	-	-	11	-	12	-
J24	-	17	19	14	11	-	-	-	21	9	-	-
J25	-	25	-	14	-	-	-	-	-	-	-	-
J26	-	18	19	17	-	-	-	-	16	15	-	-
J27	-	30	20	12	18	-	-	-	21	-	-	17
J28	-	-	12	9	-	-	-	-	-	6	6	-
J30	-	17	12	8	-	-	-	-	-	10	7	-
J31	-	16	9	14	-	-	-	-	-	-	-	-
J32	-	22	6	12	-	-	-	-	-	13	-	-
J33	-	24	13	-	-	-	-	-	-	-	-	-
J34	-	25	9	-	-	-	-	-	-	-	-	7
J35	-	22	20	-	-	-	-	-	-	-	7	-
J36	-	26	20	18	16	-	-	20	25	13	20	16
J37	-	21	-	15	-	-	-	-	-	-	13	-
J38	-	10	11	12	18	14	-	14	-	-	18	16
J39	-	22	12	15	-	-	-	-	-	-	-	-
J40	-	23	-	12	-	-	-	-	9	-	-	-
J41	-	16	-	14	-	-	-	-	-	-	-	17
J42	-	13	12	11	-	-	-	-	-	-	-	-
<i>E.coli</i> 10148	19	28	24	21	20	18	16	20	30	17	26	26

Key:- Ampicillin (AMP), Chloramphenicol (CHL), Gentamicin (GEN), Kanamycin (KAN), Amikacin (AMK), Streptomycin(STR), Ceftazidime (CFZ), Cefotaxime (CFX), Naladixic acid (NAL), Ciprofloxacin (CIP), Tetracycline (TET) and Trimethoprim+ Sulphamethoxazole (T+S).

Percentage of resistant organisms to individual antibiotics (Graph 1)



Percentage of resistant organisms

Ampicillin(Am), Chloramphenicol(Cm), Gentamicin(Gm), Kanamycin(Km),
Amikacin(Ak), Streptomycin(Sm), Cefazidime(Cz), Cefotaxime(Cf), Naladixic acid(Na),
Ciprofloxacin(Cp), Tetracycline(Tc) and Trimethoprim+ Sulphamethoxazole(T+S).

3.3 Curing of the Resistance Determinants

Attempts were made to cure the MDR clinical isolates K15, J21, J27, J36, J1 and J2 of their resistant determinants using intercalating dyes such as Ethidium Bromide and Acridine Orange. Resistant strains were also exposed to U.V prior to treatment with Ethidium bromide. A control strain *Salmonella typhimurium* TA100 carrying a curable Amp^r was also subjected to the above mentioned mutagens.

The following results were obtained:

- ❖ K15, J21, J36 and J27 could not be cured of any of their resistance markers on treatment with Acridine Orange, Ethidium Bromide, or by exposing the resistant culture to U.V before treating it with Ethidium bromide.
- ❖ On exposing J1 and J2 to varying concentrations of Ethidium bromide, two types of strains were obtained. One that had lost resistance to only Tetracycline but had retained resistance to Streptomycin, Ampicillin, and Chloramphenicol. The other type of strain lost all its resistance markers and was simultaneously sensitive to all the antibiotics that it was initially resistant to i.e., before treatment with Ethidium Bromide.

Table 5 (A) and 5(B) give a detailed account of the percentage of drug susceptible colonies obtained on treatment of J1 and J2 with Ethidium bromide.

- ❖ The control strain *Salmonella typhimurium* TA100 lost its Amp^r marker at a frequency of 75 %.

TREATMENT OF J1 AND J2 WITH ETHIDIUM BROMIDE

Table 5 - A

Isolate	Conc. EtBr (µgms/ml)	No: of colonies patched	Survivors on L.B +Am	Survivors on L.B +Tc	Survivors on L.B +Cm	Survivors on L.B
J1	50	35	35	32	35	35
	100	35	32	25	32	35
Controls						
J1(+ve)	0	1	1	1	1	1
E.coli 10148 (-ve)	0	1	0	0	0	1
J2	50	35	35	30	35	35
	100	35	34	32	34	35
Controls						
J2 (+ve)	0	1	1	1	1	1
E.coli 10148 (-ve)	0	1	0	0	0	1

PERCENTAGE OF DRUG SUSCEPTIBLE COLONIES

Table 5 - B

ISOLATE	EtBr CONC. (µgms/ml)	PERCENTAGE OF DRUG SUSCEPTIBLE COLONIES	
		Am-Tc-Cm Sensitive	Tc Sensitive Am-Cm Resistant
J1	50	-	8.6%
	100	8.6%	20%
J2	50	-	14.3%
	100	2.8%	5.7%

3.4 Conjugal transfer of drug resistance determinants :

Conjugation experiments were performed using the MDR clinical isolates K15, J21, J27, J36, J1 and J2 as donors and *E.coli* K12 as the Recipient. The conjugations were performed for 2 hours and for 18 hours at 28°C and at 37 °C.

The following results were obtained:

- ❖ K15 transferred its resistance determinants to the Recipient *E.coli* K12 after 18 hours of incubation. All the resistance determinants were transferred except for resistance to Gentamicin and Tetracycline. Transconjugants were not seen after 2 hours of incubation.
- ❖ J21 transferred its resistance markers *en bloc* to the Recipient *E.coli* K12 after 18 hours of incubation. Transconjugants were not seen after 2 hours of incubation.
- ❖ J1 transferred all its resistance determinants *en bloc* to the Recipient *E.coli* K12 at 28°C after 2 hours of incubation at a frequency of 4.97×10^{-1} . No transconjugants were seen when the conjugation mixture was incubated at 37°C.
- ❖ J2 transferred all its resistance determinants *en bloc* to the Recipient *E.coli* K12 at 28°C after 2 hours of incubation at a frequency of 4.74×10^{-1} .
- ❖ J27 and J36 did not transfer any of their resistance markers to *E.coli* K12.

TABLE 6

CONJUGATION FREQUENCIES

Donor X Recipient	No: of viable Donor Cells	No: of viable Transconjugant Cells	Transfer frequency
J1 x E.coliK12	6.80×10^3	3.38×10^3	4.97×10^{-1}
J2 x E.coli K12	5.90×10^3	2.8×10^3	4.74×10^{-1}

3.5 Plasmid Isolation and Demonstration by Gel Electrophoresis:

The following organisms were used for plasmid analysis:

Six Clinical isolates: (K15, J21, J27, J36, J1 and J2)

Four transconjugants:

TJ1 (Transconjugant of J1 and *E.coli* K12)

TJ2 (Transconjugant of J2 and *E.coli* K12)

TJ21 (Transconjugant of J21 and *E.coli* K12)

TK15 (Transconjugant of K15 and *E.coli* K12)

Cured strains: J1 and J2 cured of all their resistance markers. **Controls:** *E.coli* pBR 322 and *E.coli* K12

The plasmid DNA was isolated from all the above mentioned organisms, subjected to agarose gel electrophoresis, and visualized on a U.V transilluminator.

The following results were obtained:

- ❖ K15 showed the presence of a high molecular weight plasmid. The plasmid banded just below the chromosome.
- ❖ J21 showed the presence of two small molecular weight plasmids.
- ❖ J27 and J36 did not show any plasmids bands on gel electrophoresis
- ❖ J1 and J2 both showed similar plasmid profiles. They both showed the presence of a large molecular weight plasmid

that banded just below the chromosome and two small molecular weight plasmid bands.

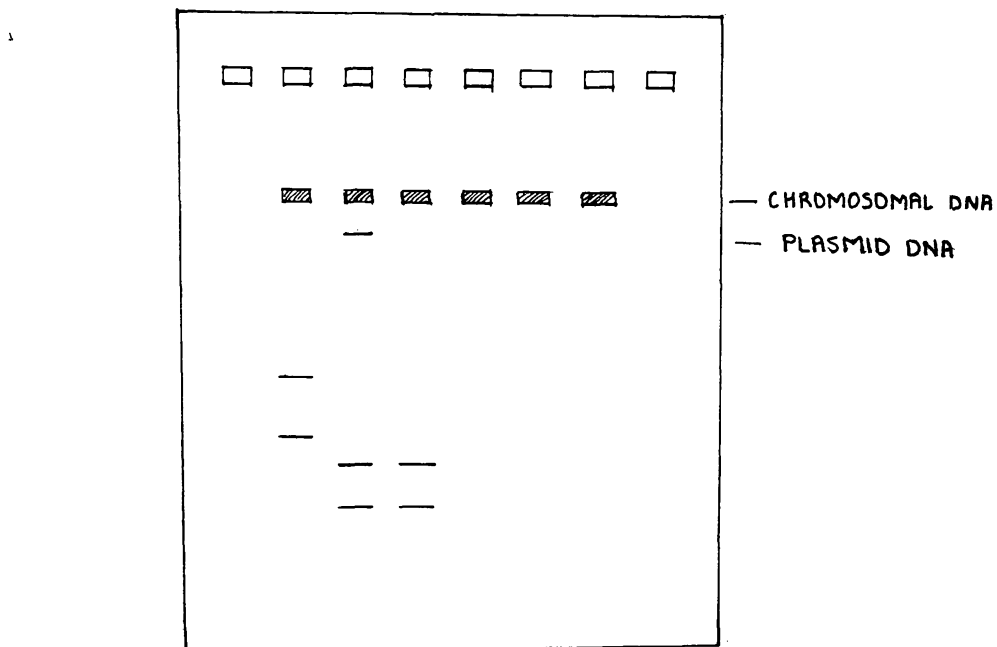
- ❖ Strains of J1 and J2 that were cured of all its resistance markers did not show the presence of the large molecular weight plasmid DNA that was present in the original resistant strains. They however showed the presence of the two small molecular weight plasmid DNAs that were present in the original resistant strain.
- ❖ All the transconjugants failed to show plasmid bands on agarose gel electrophoresis.

— CHROMOSOMAL DNA

— PLASMID DNA

- 71 -

GEL ELECTROPHORESIS PATTERN



LANE 2: *E.coli* pBR 322

LANE 3: J2 (*Salmonella typhi*)

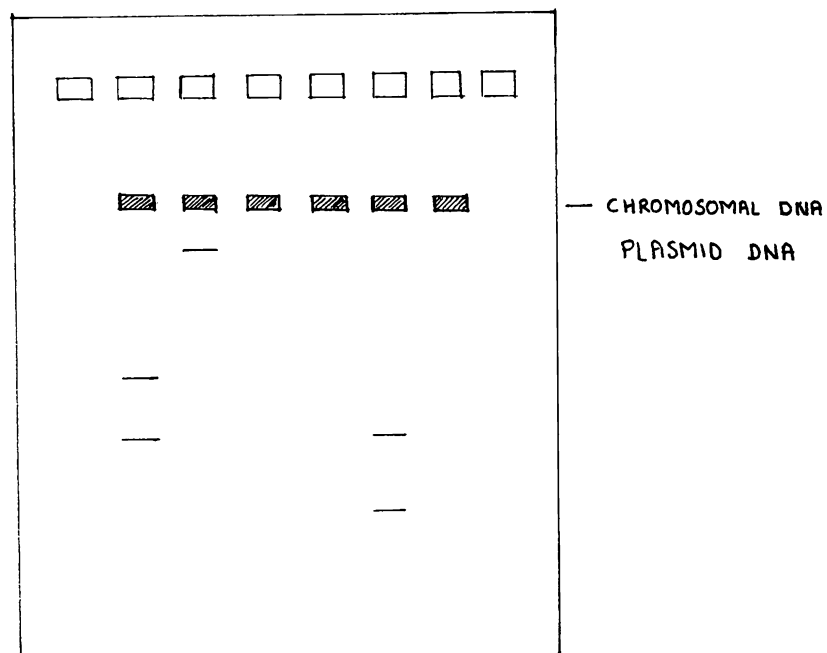
LANE 4: J2 CURED OF ALL ANTIBIOTIC RESISTANCE MARKERS

LANE 5: *E.coli* K12

LANE 6: TJ2 (J2 X *E.coli* K12)

LANE 7: J36 (*Citrobacter freundii*)

GEL ELECTROPHORESIS PATTERN



- LANE 2: *E.coli* pBR 322
LANE 3: K15 (*Klebsiella pneumoniae*)
LANE 4: *E.coli* K12
LANE 5: TK15 (K15 X *E.coli* K12)
LANE 6: J21 (*Shigella dysenteriae*)
LANE 7: TJ21 (J21 X *E.coli* K12)

DISCUSSION

[In the last two decades, bacterial drug resistance in the form of extrachromosomal genetic elements like plasmids and transposons account for most of the high level resistance that is found in India. Antimicrobial resistance among gram negative organisms has been a long term and well recognized problem here in India. Resistance has been observed in multiple genera including Escherichia, Enterobacter, Klebsiella, Proteus, Salmonella, Serratia and Pseudomonas. A recent study investigating the resistance patterns of gram negative clinical isolates causing hospital infections in India, stated that most of the isolates were resistant to common antibiotics like Ampicillin, Chloramphenicol, Cotrimoxazole, Gentamicin, and the first and second generation cephalosporins. They also found that 70% of their clinical isolates of E.coli were resistant to Ciprofloxacin (Nema 1997).

Our study of the resistance patterns of the 61 clinical isolates collected from the various hospitals in Bombay from the year 1997 to 1998 showed 100% resistance to the older antibiotics Ampicillin, Tetracycline and Streptomycin. 80% of the isolates were resistant to the Aminoglycoside Gentamicin while 83% of the isolates were resistant to Kanamycin. Resistance to the semisynthetic Aminoglycoside Amikacin was

73%. 92% of the isolates were resistant to the older quinolone Naladixic acid while the fairly recent Fluoroquinolone Ciprofloxacin showed only 70.5% resistance. Resistance to Chloramphenicol was 93% and 93.5% of the isolates were resistant to the combination of Trimethoprim and Sulphamethoxazole. The least amount of resistance was seen to the third generation Cephalosporins. 69% of the isolates were resistant to Ceftazidime, while only 52.5% (incorrectly reported in the synopsis as 67%) of the isolates were resistant to Cefotaxime. From these results we can come to the conclusion that Streptomycin, Tetracycline and Ampicillin are no longer effective against gram negative pathogenic bacteria. The most effective antibiotics are the third generation Cephalosporins, the fluoroquinolone Ciprofloxacin and to an extent semisynthetic Aminoglycoside Amikacin.

***Shigella dysenteriae* (J21):**

Observations and Inferences:

❖ *Shigella dysenteriae* is the main cause of severe diarrheal disease in the developing world. The clinical isolate of *Shigella dysenteriae* (J21) used in my study, was resistant to, Chloramphenicol, Tetracycline, Streptomycin, Ampicillin and Trimethoprim+ Sulphamethoxazole.

There is a big problem with multiple resistance of *Shigella* species, which is mostly plasmid-borne. A study on the R factor of *Shigella* species isolated in Lucknow in 1997 (Agarwal SK 1997) showed that most of the strains isolated showed variable resistance to Streptomycin, Chloramphenicol, Tetracycline and Ampicillin.

❖ J21 did not lose any of its resistance markers on treatment with ethidium bromide or acridine orange. Exposing the resistant cells to U.V prior to treatment with ethidium bromide also did not result in the loss of any of the resistance determinants. However, the inability to cure a strain of its resistant markers should never be used as evidence against plasmid involvement. . These curing agents are individually effective only against some plasmids and their likely response is unpredictable. Plasmids may be refractory to curing for a number of reasons.

- i) The plasmids may simply be refractory to the curing agents or may appear to be so if it carries functions other than antibiotic resistance that are vital for cell viability (Stanisich 1988).
- ii) The efficiency of curing agents also varies widely depending on the plasmid and the particular host carrying it (Stanisich 1988, Riva 1973, Bastarrachea 1968). An R factor sensitive to acriflavine in one strain can become resistant in another host. The reason for this is not clear, but it certainly limits the practical effectiveness of these curing agents.
- iii) Efflux pumps are seen amongst gram negative bacteria. Homologs of the Acr system in E.coli are seen among gram negative bacteria which make them resistant to dyes, detergents and antibiotics (Dzwokai 1994)
- iv) In the curing process, the number of plasmids must become lower than the number of chromosomes. This situation can possibly be obtained if the number of copies of plasmid per chromosome is between one or two. In some cases this number is considerably higher, and the emergence of plasmid variants will require a complete and immediate inhibition of the replication of all plasmids in all cells.
- v) When the plasmid copy number is one or two, the rate of replication of the plasmid could be close to that of the

chromosome and hence it would be difficult to cure the strain of only the plasmid without damaging the chromosome.

Finding an effective agent for a specific plasmid is a long and empirical task. However, where curing cannot be demonstrated, transfer of the resistance genes to other bacteria, and plasmid isolation can be used to demonstrate the role of the plasmid in conferring resistance (Stanisich 1988).

- ❖ J21 transferred its resistance determinants *en bloc* to Recipient E.coli K12 via conjugation. Plasmid DNA isolation also revealed the presence of two low molecular weight plasmid DNA. This indicates that the resistance genes may be borne on a conjugable or a self-transmissible plasmid.

***Salmonella typhi* (J1 and J2):**

Observations and Inferences:

- ❖ Both the clinical isolates J1 and J2 that were chosen for the study were resistant to Ampicillin, Streptomycin, Tetracycline, Chloramphenicol, and Trimethoprim + Sulphamethoxazole. Chloramphenicol, Ampicillin and Trimethoprim were the antibiotics that were used against *Salmonella typhi*, the causative organism of typhoid fever. However, since 1990, *Salmonellae* resistant to these three

antibiotics was recognized in various parts of India (Sudar Sena 1992, Rathish 1994). In a recent study conducted by Sanghavi (Sanghavi 1999) of the antibiotic sensitivity of *Salmonella* serotypes isolated from 1991 till May 1998, it was observed that 54 % of the isolates collected, were resistant to Chloramphenicol, Ampicillin, Streptomycin, Tetracycline and Trimethoprim.

Presently, third generation Cephalosporins and fluoroquinolones are the only effective antibiotics against *Salmonella*.

- ❖ J1 and J2 transferred all their resistance determinants *en bloc* to the recipient *E.coli* K12 via conjugation at 28°C but no transconjugants were observed when conjugation was carried out at 37°C. Similar results were observed by Shanahan and co workers. In their study they found that the transfer of antibiotic resistance genes in the *salmonella* species occurs more readily at lower temperatures (Shanahan 1998).

J1 transferred its markers at a frequency of 4.97×10^{-1} and J2 transferred at a frequency of 4.74×10^{-1} . Similar results were reported by Hermans (Hermans 1996). From their study they concluded that the *S. typhi* strains that they isolated from Dhaka, transferred resistance to Tetracycline, Ampicillin, Trimethoprim + Sulphamethoxazole,

Chloramphenicol, Streptomycin at a frequency of 5×10^{-2} at 28° C.

❖ On treatment with Ethidium bromide, two types of strains were obtained: one that had lost all its resistance markers and one that had lost resistance only to Tetracycline. Such segregation of antibiotic resistance determinants has previously been reported in E.coli, Shigella and Salmonella on treatment with aminoacridines (Hahn 1976).

❖ Plasmid DNA isolation showed that the strain that was cured of all its resistance markers did not show the presence of a large molecular weight plasmid which was observed in the original resistant strain.

These results indicate that a large molecular weight plasmid codes for the antibiotic resistance genes in the clinical isolates of J1 and J2. Similar findings have been reported in recent investigations conducted by Shanahan et al (Shanahan 1998) where they suggest that the plasmids that encode for resistance to Chloramphenicol, Ampicillin, Streptomycin, Tetracycline and Trimethoprim are large plasmids (165kb -180kb).

***Klebsiella pneumoniae* (K15):**

Observations and Inference:

- ❖ The clinical isolate of *Klebsiella pneumoniae* (K15) that was used in my study was intermediately resistant to Ciprofloxacin and the third generation cephalosporin Cefotaxime. However, it showed high level resistance to Ceftazidime another third generation cephalosporin, Chloramphenicol, Trimethoprim+ Sulphamethoxazole and all the Aminoglycosides that it was tested against.

Most of the resistance to the third generation cephalosporins is due to the extended spectrum beta lactamases (ESBLs). These ESBLs confer moderate or high level resistance to Ceftazidime, but the level of resistance to Cefotaxime may be low. Among the Enterobacteriaceae, these ESBLs are most prevalent in *Klebsiella pneumoniae*. The location of the ESBL genes on plasmids also explains the problem of coresistance, given that such plasmids also often carry genes that code for resistance to Aminoglycosides, Chloramphenicol and Trimethoprim+ Sulphamethoxazole (Nordmann 1998).

- ❖ K15 transferred all its resistance markers to recipient E.coli K12 except makers for resistance to Gentamicin and Tetracycline.

- ❖ Plasmid DNA analysis of the original resistant strain showed the presence of a high molecular weight plasmid.

- ❖ Attempts made to cure K15 of its resistance markers using Ethidium Bromide or Acridine Orange were unsuccessful. Exposure of the resistant strain to U.V prior to treatment with ethidium bromide was also unsuccessful in curing it of its resistance determinants.

One of the main reasons for this is probably the large size of the plasmid. Plasmid isolation and its demonstration by agarose gel electrophoresis indicate that the plasmid bands just below the chromosome. The larger the plasmid DNA the more refractory it is to curing as the chances of curing the strain of only the plasmid without damaging the chromosome decrease with the increase in the size of the plasmid. This large plasmid may also code for functions other than antibiotic resistance, that are vital for cell viability. Some of the other reasons suggested for the unsuccessful attempts in curing J21 of its resistance determinants also hold good for K15.

The role of the plasmid in conferring drug resistance can be determined by the results of the conjugation experiments and by the physical demonstration of the plasmid DNA.

- ❖ Resistance to the third generation cephalosporins in K15 is probably due to the presence of ESBLs. As suggested by

Nordmann (Nordmann 1998), the genes that code for these ESBLs may be present on a large plasmid along with genes for resistance to the other antibiotics. This is further confirmed by plasmid isolation and by the fact that K15 transferred most of its resistance genes to *E.coli* K12. The genes that code for resistance to Gentamicin and Tetracycline were probably not transferred, as they may be present on a chromosomally integrated transposon.

***Escherichia coli* (J27) :**

Observations and Inference:

- ❖ The resistant strain of *E.coli* (J27) used in my study had the following antibiotic resistance pattern: It was resistant to Ampicillin, Amikacin, Kanamycin, Streptomycin, Naladixic acid, Tetracycline and Chloramphenicol. It was sensitive to the third generation Cephalosporins, Gentamicin, Ciprofloxacin and Trimethoprim + Sulphamethoxazole.
- ❖ Attempts to cure *E.coli* (J27) of its antibiotic resistance markers using mutagens such as ethidium bromide and acridine orange proved to be unsuccessful. Exposure of the resistant strain to U.V prior to treatment with ethidium bromide also proved to be unsuccessful.

The main reason for this could be the Acr efflux system that has been identified in clinical isolates of *Escherichia coli*. These efflux pumps make it resistant to a broad range of inhibitors such as basic dyes, detergents and hydrophobic antibiotics. It is suggested that the Acr proteins in *E.coli* actively pump acriflavine and any other basic dye out of the cell using energy from the proton motive force (Dzwokai ma 1994). Another reason for no loss of the resistance determinants on treatment with mutagens could be that the resistance determinants are coded for by chromosomally integrated transposons (Dr. Stuart Levy - personal correspondence).

- ❖ J27 also did not transfer its resistance markers to recipient *E.coli* K12 via conjugation.
- ❖ Plasmid DNA isolation and physical demonstration by gel electrophoresis do not indicate the presence of any plasmid bands.
- ❖ The results of the investigation clearly indicate that the drug resistance genes in J27 are not borne on a plasmid. Resistance to the antibiotics could be due to the following possibilities:

1. Mutations in the *mar* locus:

Multiple antibiotic resistant (Mar) mutants of *Escherichia.coli* express chromosomally mediated resistance to a broad range of

structurally unrelated antibiotics. When Cohen et al (Cohen S.P 1988) investigated the mechanisms of chromosomal multiple antibiotic mutants in *E.coli*, they were found to show decreased expression of OmpF porin. Clinical isolates of *E.coli* with decreased amounts of OmpF have been previously linked to resistance to β lactams (Harder 1981), tetracycline (Pugsley 1978), chloramphenicol (Reeve 1968), and hydrophilic quinolones (Hirai 1986). Besides causing a decreased expression of the Ompf porin, the *marRAB* genes also activate the expression of antibiotic efflux pumps (Dzwokai Ma 1994). Maneewannakul and Levy (Maneewannakul 1996) examined multidrug resistant clinical *E.coli* isolates for mutations in the *marRAB* operon of the *mar* locus and through their findings, proved that *mar* operon mutations contribute to quinolone and multidrug resistance in clinical *E.coli* isolates.

2. Acr Efflux systems:

Free living *E.coli* lives in a hostile environment where it is constantly exposed to growth inhibitors, antibiotics etc. It is therefore not unreasonable to assume that it may produce efflux pumps to protect itself from its hostile environment. The ACR efflux system in *E.coli* has been reported to render it more resistant to a broad range of inhibitors. There are many other efflux transporters involved in multidrug efflux, e.g Emr AB, the first multi drug pump discovered in *E.coli* (Lomovskaya 1992, Dzwokai Ma 1994).

3. Chromosomally integrated transposons:

The results of my experiments very strongly suggest that resistance in J27 is due to chromosomally integrated transposons or one transposon containing several integrons that code for resistance to multiple antibiotics (Suggested by Dr. Stuart Levy- personal correspondence).

***Citrobacter freundii* (J36):**

Observations and Inferences:

- ◆ The clinical isolate of *Citrobacter freundii* that was used in my study was resistant to only four out of the 12 antibiotics that were tested against it. It is resistant to all the older antibiotics like Ampicillin, Kanamycin, Streptomycin and Tetracycline. It is sensitive to the relatively newer antibiotics.
- ◆ Attempts to cure J36 by treating it with Ethidium Bromide, Acridine Orange or U.V and Ethidium Bromide proved to be unsuccessful.
- ◆ J36 did not transfer any of its resistance markers to the Recipient E.coli K12

- ♦ Plasmid isolation did not indicate the presence of any plasmid DNA.
- ♦ All the above results indicate that the resistance determinants in the clinical isolate of *Citrobacter freundii* (J36) are present on chromosomally integrated transposons or one transposon containing several integrons coding for antibiotic resistance.

Transconjugants:

1. TK15 : *Klebsiella pneumoniae* (K15) X *E.coli* K12:

TK15 received all the antibiotic resistance markers from K15 except the markers for resistance to Gentamicin and Tetracycline. However plasmid DNA analysis of TK15 did not show the presence of the large molecular weight plasmid DNA present in the donor K15.

2. TJ21 : *Shigella dysenteriae* (J21) X *E.coli* K12

TJ21 received all the antibiotic resistance markers en bloc from J21. Plasmid DNA analysis of TJ21 did not show the presence of either of the two small molecular weight plasmid DNAs present in the donor J21.

3. TJ1: *Salmonella typhi* (J1) X *E.coli* K12

TJ1 received all the antibiotic resistance markers en bloc from the donor J1. Plasmid DNA analysis of TJ1 however, did not show the presence of either the two small molecular weight plasmid DNAs or the large molecular weight plasmid DNA present in the donor J1.

4. TJ2: *Salmonella typhi* (J2) X *E.coli* K12

TJ2 received all the antibiotic resistance markers en bloc from the donor J2. However, plasmid DNA analysis of TJ2, did not show the presence of either the two small molecular weight plasmid DNAs or the large molecular weight plasmid DNA present in the donor J2.

All the above results indicate that antibiotic resistance has been transferred to the recipient *E.coli* K12. However plasmid analysis does not reveal the presence of a plasmid in any of these transconjugants. Further investigations would be required to explain this observation and hence no definite explanation can be provided for this.

SUMMARY AND CONCLUSION

In the last two decades, we have seen an increase in bacterial resistance to commonly used antibiotics all over the world. When resistant strains of bacteria first appeared, the most commonly heard explanation was that resistance was due to spontaneous mutation of chromosomal genes. However the spontaneous mutation hypothesis was inconsistent in explaining the appearance of multiple antibiotic resistance in bacterial populations. Molecular analysis of resistant strains established that the genes were present on a plasmid. Plasmids conferring resistance, i.e. R plasmids have since been discovered in numerous gram negative and gram positive bacteria (Mosher 1992).

This study of multiple antibiotic resistance among gram negative bacterial pathogens isolated between 1997 and 1998, brings to light the following observations:

1. All the isolates collected in the study, were multi drug resistant and were resistant to at least four out of the twelve antibiotics used. 100% resistance was seen to the older antibiotics like Tetracycline, Streptomycin and Ampicillin.
2. Twenty (32.8 %) of the 61 isolates were resistant to all twelve of the antibiotics including the third generation Cephalosporins and the Flouroquinolones which are

currently the most effective antibiotics available against gram negative bacterial pathogens.

3. The clinical isolate J21(*Shigella dysenteriae*) used in the study was resistant to five of the antibiotics tested against it. The markers for resistance to these antibiotics are most probably located on a self-transmissible plasmid.
4. The clinical isolates of *Salmonella typhi* (J1 and J2) were both resistant to five of the antibiotics tested against it. The resistance markers that code for resistance to these antibiotics are located on a high molecular weight self transmissible plasmid in both J1 and J2.
5. J27, the multi drug resistant clinical isolate of *Escherichia coli* was resistant to seven of the antibiotics and all the results strongly indicate that the resistance determinants are present on chromosomally integrated transposons or a single chromosomally integrated transposon, containing several integrons coding for resistance to the antibiotics.
6. The MDR clinical isolate of *Klebsiella pneumoniae* (K15) was either completely resistant or intermediately resistant to all 12 of the antibiotics tested against it. The resistance markers are most probably present on a self-transmissible, large molecular weight plasmid.

7. The clinical isolate J36 (*Citrobacter freundii*) was resistant to only four of the antibiotics. The results obtained strongly indicate that the resistance determinants are present on a chromosomally integrated transposon or a single chromosomally integrated transposon, containing several integrons coding for resistance to the antibiotics

The results discussed above indicate that multi drug resistance in the clinical isolates of *Citrobacter freundii* and *Escherichia coli* are not plasmid coded, but may be coded for by chromosomally integrated transposons. On the other hand, antibiotic resistance in the clinical isolates of *Salmonella typhi* (J1), *Salmonella typhi* (J2), *Klebsiella pneumoniae* and *Shigella dysenteriae*, may be coded for by a plasmid.

APPENDIX

A1. MEDIA

1. LURIA-BERTANI BROTH AND AGAR

Tryptone	10.0 gm
Yeast extract	5.0 gm
Sodium chloride	5.0 gm
Distilled water	1000.0 ml
pH = 7.6	

2% agar-agar was added to the above broth to obtain a solid medium. The medium was sterilised by autoclaving at 121°C/15 lbs/15 mts.

2. MAC' CONKEY'S AGAR

Peptone	20.0 gm
Sodium taurocholate	5.0 gm
Sodium chloride	5.0 gm
Distilled water	900.0 ml
pH = 7.2	

To the above ingredients the following were added:

Lactose	10.0 gm
Agar-agar	20.0 gm

The medium was boiled to digest the agar and 1 % Aq. Neutral Red solution was added. The total volume was made to 1000.0 ml. The medium was sterilised by autoclaving at 11°C/10 lbs/10 mts.

3. MUELLER-HINTON BROTH AND AGAR (MH)

Casein acid hydrolysate	29.0 gm
Beef extract	10.0 gm
Potato Starch	2.5 gm
Distilled water	1000.0 ml
pH = 7.6	

The medium was boiled and filtered through Whatman filter paper No. 1. 2% agar-agar was added to obtain a solid medium. The medium was sterilised by autoclaving at 121°C/15 lbs/15 mts.

4. NUTRIENT AGAR (NA)

Peptone	10.0 gm
Sodium chloride	5.0 gm
Meat extract	3.0 gm.
Distilled water	900.0 ml
pH = 7.2	

2% Agar-agar was added to obtain a solid medium. The medium was sterilized by autoclaving at 121°C/15 lbs/15 mts.

A2. REAGENTS

1. ACRIDINE ORANGE (10 mg /ml. HI-MEDIA)

Acridine orange	100.0 mg
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Distilled water	10.0 ml
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pH = 7.6

The stock solution was stored in a cool, dark place.

2. AGAROSE (0.7 % SIGMA)

Agarose	0.14 gm
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Glass distilled water	18.0 ml.
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10X TAE buffer	2.0 ml.
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Agarose was digested in a boiling water bath and the solution cooled to 50°C and TAE buffer added. Ethidium Bromide at a concentration of 0.5 µgms/ml is added to this.

3. ETHANOL

Double distilled ethanol using complete glass unit and cooled to -40°C was used.

4. ETHIDIUM BROMIDE (5 mg/ml. SIGMA)

Stock Solution for Curing

Ethidium bromide	50 mg.
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Glass distilled water	10 ml.
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Storage was done at refrigeration temperature.

5. ETHIDIUM BROMIDE (1 mg/ml. SIGMA)

Stock Solution for Gel Electrophoresis

Ethidium bromide	10 mg.
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Glass distilled water	10 ml.
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Storage was done at refrigeration temperature.

6. ETHYLENE DIAMINE TETRA ACETIC ACID (EDTA 0.5 M)

EDTA	18.61 gm
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Glass distilled water	80.0 ml
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The solution was stirred vigorously on a magnetic stirrer and the pH adjusted to 8.0 with NaOH pellets. The total volume was then made up to 100 ml. The solution was sterilised by autoclaving at 121°C/15 lbs/15 mts and stored at refrigeration temperature.

7. CHLOROFORM – ISOAMYL ALCOHOL MIXTURE

Double distilled chloroform and isoamyl alcohol were mixed in the ratio 24: 1 (v/v) respectively. This mixture was stored in a brown coloured bottle at refrigeration temperature.

8. LYSOZYME (2 mg/ml. SIGMA)

Chilled lysozyme solution was made fresh each time in sterile Tris – EDTA – Glucose buffer (Solution 1 pH = 8.0) at a working concentration of 2 mg / ml.

9. LYSING SOLUTION:

SDS (3%)	3gms
Tris Base	0.605 gms
Glass distilled water	100 ml

Adjust the pH to 12.6 using 2N NaOH.

10. PHENOL- CHLOROFORM

Equal volumes of unbuffered phenol and chloroform are mixed together and stored in a brown coloured bottle at refrigeration temperature.

11. SODIUM ACETATE (3 M. QUALIGENS)

Sodium Acetate	40.81 mg.
Glass distilled water	80.0 ml

The pH was adjusted to 5.2 with glacial acetic acid and the volume was then made up to 100 ml with glass distilled water. The solution was sterilised by autoclaving at 115° C / 10 lbs. / 10 mts.

12. SODIUM HYDROXIDE – SDS MIXTURE

1 N NaOH Solution:

NaOH pellets 4.0 gm.

Glass distilled water 100.0 ml

Sterilised by autoclaving at 115o C / 10 lbs. / 10 mts.

SodiumDodecyl Sulphate (SDS 10 % SIGMA):

SDS 10.0 gm.

Glass distilled water 100.0 ml

1 % SDS in 0.2 N NaOH (working stock):

10 ml of 10 % SDS and 20 ml of 1 N NaOH were added to 70 ml of sterile glass distilled water. The alkaline SDS solution thus prepared was stored at room temperature.

13. TRIS HYDROXYMETHYL AMINO METHANE (Tris-HCl. 1 M SIGMA)

Trizma base 12.11 gm.

Glass distilled water 80.0 ml

The pH was adjusted to 8.0 with conc. HCl. The total volume was made up to 100 ml with glass distilled water. The solution was sterilised by autoclaving at 110°C/10 lbs./10 mts.

A3. BUFFERS

1. GEL LOADING BUFFER

0.25 % bromophenol blue is added to 40 % (w/v) sucrose.

Bromophenol blue	25.0 mg.
Glass distilled water	10.0 ml
Sucrose	4.0 mg.

The buffer was stored at 4° C.

2. TRIS-EDTA (TE) BUFFER

1 M Tris (pH = 8.0)	1.0 ml
0.5 M EDTA (pH = 8.0)	0.2 ml
Glass distilled water	98.8 ml

The final concentration of Tris and EDTA are 10 mM and 1 mM respectively.

3. TRIS BORATE EDTA BUFFER

Tris Base	5.4 gms
Boric Acid	2.75 gms
EDTA (0.5M)	2 ml
Glass distilled water	98 ml
pH – 8.0	

4. E-BUFFER

Tris Base	2.42 gms
Glacial Acetic Acid	0.571 ml
EDTA (0.5M)	2 ml
Glass distilled water	8 ml
PH – 8.0	

A4. Preparation of Antibiotic Discs:

Discs of about 5mm diameter are cut with a leather punch from good quality blotting paper. They are separated and spread out singly in 15-cm petriplates, so that each disc is not less than 2 mm from its neighbors. They are sterilized in the oven at 160°C for one hour. When they are cooled 0.01 ml of a sterile solution of the required antibiotic is dropped from a standard dropping pipette on to each disc. The discs are then dried and can be stored at 4°C.

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Internet Site Addresses

1. (<http://vet.purdue.edu/depts/bms/courses/chmr/aminogl.html>)
2. (<http://vet.purdue.edu/depts/bms/courses/chmr/aminogl.html>)
3. (<http://biosafety.ihe.be/AR/aminoglycosides.html>)

A SYNOPSIS OF THE THESIS
TO BE SUBMITTED TO
THE UNIVERSITY OF MUMBAI
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
MICROBIOLOGY

TITLE OF THE THESIS: MULTI DRUG RESISTANCE IN GRAM-
NEGATIVE BACTERIAL PATHOGENS

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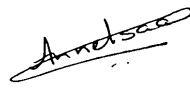
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INTRODUCTION

Ever since antibiotics became widely available in the 1940s, they have been hailed as miracle drugs—magic bullets able to eliminate bacteria, without doing much harm to the cells of the treated individuals. When antibiotics were first introduced for the treatment of common microbial infections, the bacterial geneticists of the day, suggested that the development of antibiotic resistance, during therapy was unlikely because the frequency of mutation to resistance in bacteria was too low. Yet, with each passing decade, bacteria that defy not only single but also multiple antibiotics have become increasingly common.

The first resistance mechanism was recorded in 1940 by Abraham and Chain (Abraham 1940) who characterized an enzyme from *E coli* that was capable of hydrolyzing penicillin. In 1959, resistance to multiple drugs was recognized in strains of *Shigella dysenteriae* in Japan (Akiba.T 1960), and it was soon discovered that all of the resistance traits in that organism could be transferred to a recipient cell of *E coli* via cell to cell matings. In the late 1960s and the early 1970s, multi-resistance again emerged, first in *S aureus*, then in a variety of gram negative bacilli, particularly those causing nosocomial outbreaks(Tenover 1991, Neu 1992, Murray 1991). Hence, the introduction of clinically effective antimicrobial agents has regularly been followed by the rapid emergence of multi drug resistant strains of bacteria. How did we end up in this worrisome & worsening situation?

In order to answer this question we must fully understand the molecular basis of microbial drug resistance.

Genetic Basis of Resistance:

Antibiotic resistance is classified into two broad types: intrinsic and acquired (Hugo and Russel).

Intrinsic resistance is the natural resistance possessed by most strains of a bacterial species and is part of their genetic make up, encoded on the chromosome.

Acquired resistance may be due either to mutations affecting genes on the bacterial chromosome or due to acquisition of gene conferring resistance upon a previously sensitive organism. Three genetic elements are responsible for acquired resistance: chromosomes, plasmids and transposons(Lewis 1989).

1. *Chromosomal mutations:*

Resistance to certain antibiotics can arise as a consequence of mutations to chromosomal genes because of changes in the DNA sequence.

2. *Plasmids:*

Plasmids appear to be the most common means in nature for transferring resistance as they have the ability to transfer within and between bacterial species. Plasmids are transmissible by conjugation, transformation and transduction. Conjugation is the most common method of resistance transfer in clinically important bacteria. Plasmids that can mediate their own transfer are termed conjugative plasmids. Non conjugative plasmids can transfer to other bacteria if they are mobilised by other conjugative plasmids present in the same cell

3. *Transposons:*

Transposons or “ jumping genes” as they are commonly referred to, are mobile genetic elements capable of transferring or transposing independently from one DNA molecule to another (Shapiro1983). They may encode genes for resistance to a wide variety of antibiotics, as well as many other metabolic properties. They need not share extensive regions of homology with the replicon into which they insert, as is required in classical genetic recombination and are therefore a major cause of the transfer and spread of antibiotic resistance genes among different bacterial species. It is possible for bacteria to acquire a series of transposons coding for different antibiotic resistances by insertion in existing plasmids or the chromosome. Conjugative transposons can mediate their own transfer and hence make the spread of resistance genes more likely since they do not depend on insertion into conjugative plasmids for their mobilization (Salysers1995).

The present study was inspired by the fact that for the past 20 years the prevalence of resistance to commonly used antibiotics has been steadily increasing in nosocomial isolates of gram negative coliforms. The problem is due to the wide distribution of plasmids and transposons encoding multiple drug resistance and their dissemination among different coliforms. The project therefore attempts to detect and characterize drug resistance in the clinical isolates of gram negative Coliforms collected from 1997 to 1998.

AIMS AND OBJECTIVES

1. To monitor the antibiotic resistance patterns of the clinical isolates collected from 1997 to 1998.
2. To determine the role of the plasmid in conferring drug resistance by the following experiments:
 - a) Curing the resistant isolates of R- plasmids, if any, and demonstrating the consequent loss of resistance to several antibiotics.
 - b) Isolation and demonstration of the R- plasmid.
3. To study the transferability of the resistance factors by conjugation.

MATERIALS AND METHODS

Collection & Identification of Clinical Isolates:

61 clinical isolates were collected from various specimens like urine, pus, blood & stool from Kasturba Hospital of Infectious Diseases & from Sir J.J Group of Hospitals, Mumbai , during the period of 1997-98.

The isolates were subjected to identification on the basis of their morphological, cultural & biochemical characteristics as per standard methods (Cowan and Steel 1974, Stokes 1987)

Antibiotic Resistance patterns of the isolates

Antibiograms of the 61 isolates were determined on Mueller Hinton (M.H) agar plates by the Kirby-Bauer disk diffusion method (Bauer and Kirby 1966), against 12 antibiotics :-

Ampicillin(Am), Chloramphenicol(Cm), Gentamicin(Gm), Kanamycin(Km), Amikacin(Ak), Streptomycin(Sm), Cefazidime(Cz), Cefotaxime(Cf), Naladixic acid(Na), Ciprofloxacin(Cp), Tetracycline(Tc) and Trimethoprim+ Sulphamethoxazole(T+S).

Protocol:

- A loopful of the culture was inoculated in fresh M.H broth & incubated overnight at 37°C. A loopful of this overnight culture was then inoculated into fresh M.H broth for 3-4 hours in order to adjust the culture density to 10^5 to 10^6 c.f.u.
- Sterile cotton swabs were soaked in the inoculum & used to surface spread on MH agar plates, in order to get a confluent growth.
- Antibiotic disks were then placed on the surface spread plates. The plates were then incubated at 37°C for 24hours.
- After overnight incubation, the zone diameters are measured.

The results were compared against the standard charts provided by NCCLS (NCCLS 1988).

E.coli ATCC 10148 served as a sensitive control.

Curing of the Plasmid

The phenomenon of the loss of a plasmid is called “curing” & this can be augmented by compounds like acridine dyes, ethidium bromide, rifampicin etc..

Curing experiments using intercalating dyes like Ethidium Bromide (Bouanchaud 1969) and Acridine Orange (Hirota 1960) were carried out. In order to increase the efficiency of curing, the resistant organisms were first exposed to UV before treatment with Ethidium Bromide (Watanabe 1961). The following 6 clinical isolates were subjected to treatment with the above mentioned mutagens.

Clinical Isolate	MDR Pattern
<i>Klebsiella pneumoniae</i> (K 15)	Resistant to all 12 antibiotics
<i>Shigella dysenteriae</i> (J 21)	Am-Sm Tc-Cm
<i>Escherichia coli</i> (J 27)	Am-Ak-Km-Sm-Na-Cp-Tc-Cm
<i>Citrobacter freundii</i> (J 36)	Am-Km-Sm
<i>Salmonella typhi</i> (J1)	Am-Sm-Tc-Cm
<i>Salmonella typhi</i> (J2)	Am-Sm-Tc-Cm

The control strain used was *Salmonella typhimurium* TA 100 carrying a curable Amp^r plasmid.

Treatment with Acridine Orange/ Ethidium Bromide:

1. Mid log phase cultures were inoculated in Luria Bertani broth containing various concentrations of Ethidium Bromide/Acridine Orange. The flask was then incubated at 37°C in the dark, under shaken conditions for 24 hours.
2. The next day, plate dilutions were done on L.B agar plates & incubated at 37°C/24 hours.
3. Isolated colonies grown on L.B agar plates were patched on L.B agar containing the requisite amount of the antibiotic, & on plain L.B plates. These were incubated at 37°C/24 hours.

Treatment with UV and Ethidium Bromide:

The resistant strains were first grown in Luria Bertani broth and at the exponential phase of their growth, 5 ml of this culture was transferred to a sterile petri dish and subjected to UV radiation. The duration of exposure to UV was decided for each sample by determining the dosage that gave 1% survivors. The exposed cultures were then incubated in the dark at 37°C for 60 minutes subsequently treated with Ethidium Bromide according to the above mentioned protocol.

Transferability of Antibiotic Resistance Markers :

Cojugation experiments were carried out in liquid culture by the method described by Willets(1988). A total of six donor strains (K15, J1, J2, J21, J27, J36) were crossed individually with *E.coli* K12(Lac⁺ F⁻ Nal^r) .

1. Donor & recipient strains were grown in L.B broth overnight at 37°C for 24 hours to a density of 10⁸ cells/ml.
2. 0.2 ml of the donor strain was mixed with 1.8ml of the recipient strain. The tube was incubated at 37°C /24 hours.
3. A loopful of the culture was plated out on Mac conkey's agar with appropriate amounts of the requisite antibiotic. The plates were incubated at 37°C/24 hours.

Isolation of the plasmid and its demonstration by Gel Electrophoresis.

The plasmid extraction procedure of Brimboin and Doly(1979) were followed with slight modifications.

1. Inoculate the organism in 5 ml of Luria Bertani broth containing 100µgms/ml of the Ampicillin stock solution. Incubate at 37°C for 24 hours.
2. Spin down the cells at 12,000 rpm for 10 minutes.
3. Suspend the solution in 100µl of Solution1 .
4. Add 200µl of Solution 2 to the plasmid preparation. Close the tube and mix gently by inverting 5 times.
5. Keep on ice for two minutes.
6. Add 150µl of ice cold solution 3. Close the tube and vortex gently.
7. Keep the tube on ice for 5 minutes.
8. Centrifuge the tube at 12,000 rpm for 5 minutes at 4°C.
9. Transfer 400µl of the supernatant to a fresh tube.
10. An equal volume of Chloroform:isoamylalcohol (24:1) was added to the supernatant and gently mixed.
11. Centrifuge the tube at 13,000 rpm for 2 minutes at 4°C to remove any contaminating proteins remaining in the supernatant.
12. The upper aqueous phase was transferred to a fresh tube and two volumes of ice cold ethanol were added. This was kept at room temperature to precipitate the plasmid DNA.
13. Centrifuge the tube at 4°C for 30 minutes .
14. Discard the supernatant and air dry the pellet for 10 minutes.
15. The pellet is then resuspended in 50µl of Tris- EDTA buffer (10 mM ,Ph 8.0).

SOLUTION 1	SOLUTION2	SOLUTION3
Glucose 50mM	SDS 2%	Na Acetate 3M
Tris.Cl 25mM	NaOH 5N	
EDTA 10mM		

Gel Electrophoresis:

The presence of the plasmid was demonstrated by preparing a horizontal agarose gel and viewing it on an UV transilluminator.

1. Agarose gel electrophoresis was carried out using 0.7% agarose.
0.21 grams of Agarose was added to 24ml of distilled water. To this 6 ml of 5x Tris Borate EDTA buffer and Ethidium Bromide at a concentration of 0.5µgms/ml were added . This was then digested completely in a water bath. The solution was cooled and poured into a cast and set to form a gel.
2. 20µl of the plasmid preparation was mixed with 5µl of the loading buffer and loaded into the wells of the gel.
3. Electrophoresis was carried out at 12v /cm for 2 hours.
4. The gel was then viewed on an UV transilluminator.

RESULTS

1. Of the 61 isolates collected there were 17 *Escherichia coli*, 11 *Klebsiella*, 14 *Salmonella*, 10 *Pseudomonas*, 6 *Proteus*, 1 *Shigella*, 1 *Citrobacter* and 1 *Providencia*.
2. Nineteen different antibiotic patterns were obtained. 32.8% of the isolates were resistant to all the 12 antibiotics tested and 73.8% of the 61 isolates showed resistance to 10 antibiotics. The multi drug resistant patterns of all 61 isolates is discussed in Table 1.

3. Isolates J1 and J2 were successfully cured of all their resistance markers on treatment with Ethidium Bromide. Attempts to cure K15, J21, J1, J2, J27, and J36 proved to be unsuccessful.

The Standard strain of *Salmonella typhimurium* TA 100 carrying a plasmid for Ampicillin resistance cured at a frequency of 75%.

4. J21, J2 and J1 transferred their resistance markers *en bloc* to the recipient *E. coli* K12 and *E. coli* AB1157. K15 transferred all its resistance markers to recipient *E. coli* K12, except the markers for Gentamicin and Tetracycline resistance.

5. Six Clinical isolates, 4 transconjugants, a standard strain of *E. coli* containing plasmid PBR322 (4.3kb), and Kas40 a strain known to contain 3 large plasmids were used for plasmid analysis.

E. coli PBR 322 clearly showed the presence of the 4.3 kb plasmid.

Kas 40 showed the presence of three large plasmids.

Four of the isolates K15, J21, J1, and J2 showed the presence of plasmids.

However, J27, J36 and transconjugants T1, T2, T15, T21 failed to show any plasmid bands on the agarose gel.

Further experiments are being presently carried out and all the results will be discussed in detail, in the thesis, in the light of our findings.

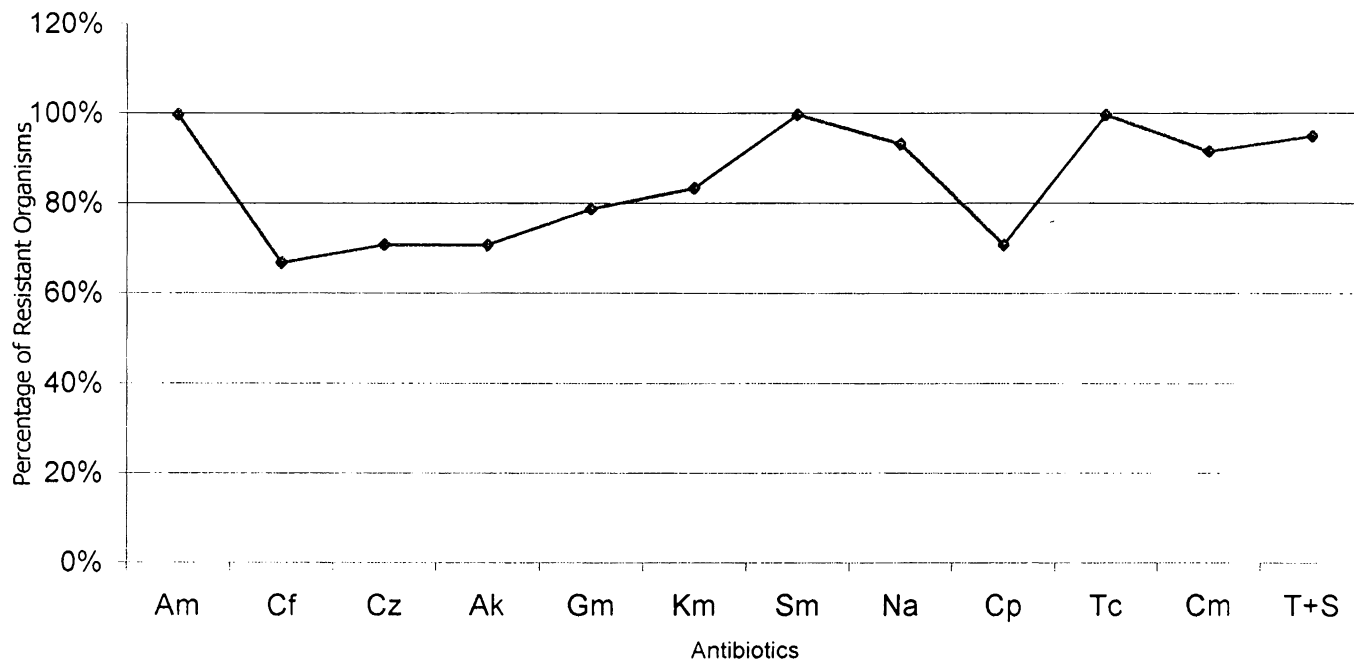
MULTIPLE DRUG RESISTANT PATTERN

Table 1

NO: OF ANTIBIOTICS	MDR PATTERN	NO: OF RESISTANT ISOLATES
12	Am-Cf-Cz -Ak-Gm-Km-Sm-Na-Cp-Tc-Cm-T+S	20
11	Am-Cz -Ak-Gm-Km-Sm-Na-Cp-Tc-Cm-T+S	15
	Am-Cf-Cz -Gm-Km-Sm-Na-Cp-Tc-Cm-T+S	1
	Am-Cf-Cz -Ak-Gm-Km-Sm-Na-Cp-Tc-Cm	1
	Am-Cf-Cz -Ak-Gm-Km-Sm-Na-Tc-Cm-T+S	3
	Am-Cf-Ak-Gm-Km-Sm-Na-Cp-Tc-Cm-T+S	1
10	Am-Cf-Gm-Km-Sm-Na-Cp-Tc-Cm-T+S	2
	Am-Ak-Gm-Km-Sm-Na-Cp-Tc-Cm-T+S	1
	Am-Cf-Ak-Gm-Km-Sm-Na-Tc-Cm-T+S	1
8	Am-Cf-Gm-Km-Sm-Na-Tc-T+S	2
9	Am-Cf-Cz -Ak-Km-Sm-Na-Cp-Tc	1
7	Am-Cz-Km-Sm-Na-Tc-T+S	1
	Am-Sm-Na-Cp-Tc-Cm-T+S	2
5	Am-Sm-Tc-Cm-T+S	2
4	Am-Km-Sm-Tc	1

**Ampicillin(Am), Chloramphenicol(Cm), Gentamicin(Gm),
Kanamycin(Km), Amikacin(Ak), Streptomycin(Sm), Ceftazidime(Cz),
Cefotaxime(Cf), Naladixic acid(Na), Ciprofloxacin(Cp), Tetracycline(Tc)
and Trimethoprim+ Sulphamethoxazole(T+S).**

Percentage of Resistant Organisms to Individual Antibiotics



**Ampicillin(Am), Chloramphenicol(Cm), Gentamicin(Gm),
Kanamycin(Km), Amikacin(Ak), Streptomycin(Sm), Cefazidime(Cz),
Cefotaxime(Cf), Naladixic acid(Na), Ciprofloxacin(Cp), Tetracycline(Tc)
and Trimethoprim+ Sulphamethoxazole(T+S).**

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