

**IN VITRO ACTIVITY OF CEFOPERAZONE WITH AMIKACIN
AGAINST RESISTANT ENTEROBACTERIA AND
PSEUDOMONAS AERUGINOSA**

**THESIS SUBMITTED
UNIVERSITY OF MUMBAI**

**FOR THE DEGREE OF
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IN
MICROBIOLOGY**

**SUBMITTED BY
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**UNDER THE GUIDANCE OF
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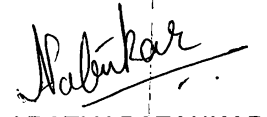
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As required by the University Rule No. 108, I state that the work presented in the thesis, which I intend to submit, is original in nature and, so far, has not been submitted for any degree of this or any other University. Whenever the references have been made to the work of others, it has been clearly indicated with sources of information in the 'Bibliography'.


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INTRODUCTION

Antimicrobial agents are among the wonder drugs of the twentieth century. They have transformed our ability to treat many infectious diseases that were previously killers. (WHO, 1998)

We have now lived through more than a half-century of the antibiotic era. During this time, we have seen the development of an array of antimicrobial agents-drugs that have clearly altered the course of medical history. (Moellering, 1998)

The Problem of Antimicrobial-Drug Resistance - Since their discovery, antimicrobial drugs have proved remarkably effective for the control of bacterial infections. However, it was soon evident that bacterial pathogens were unlikely to surrender unconditionally, because some pathogens rapidly became resistant to many of the first effective drugs (like the β -lactams). Initially, the problem of bacterial resistance to antimicrobial drugs was solved by the discovery of new classes of drugs, such as the aminoglycosides, macrolides and glycopeptides, as well as by the chemical modification of previously existing drugs. Unfortunately, there is no assurance that the development of new antimicrobial drugs can keep pace with the ability of bacterial pathogens to develop resistance.

As more is learnt about the mechanisms and epidemiology of resistance to antimicrobial drugs, it has become clear that bacteria have a remarkable array of tools at their disposal to overcome antibiotics. A single genetic mutation may lead to resistance without altering the pathogenicity or viability of a bacterial strain.

Of potentially greater concern is the fact that bacteria may acquire exogenous genetic material that leads to antimicrobial resistance. Many of the genes that mediate resistance are found on transferable plasmids or on

transposons that can be disseminated among various bacteria by conjugation. Transposons are mobile pieces of DNA that can insert themselves into various locations on the bacterial chromosome, as well as move into plasmids or bacteriophage DNA. Some transposons or plasmids have genetic elements termed integrons that enable them to capture exogenous genes. A number of genes may therefore be inserted into a given integron, resulting in resistance to multiple antimicrobial drugs or possibly allowing the accumulation of both regulatory and structural genes in the same transposon. (Gold, 1996)

Infections caused by resistant microbes fail to respond to treatment resulting in prolonged illness and greater risk of death. Treatment failures also lead to longer periods of infectivity. This increases the numbers of infected people moving in the community, augmenting opportunities for spread of resistance, and exposing the general population to the risk of contracting infection with resistant strains. Prolonged illness increases the costs of treatment, both the direct costs for additional laboratory tests, treatment, hospitalization, and the indirect costs due to loss of income or time away from family. Tragically, the least expensive antibiotic and chemotherapeutic agent often cannot be used to treat common infections because of antimicrobial resistance. The problem of resistance is now worldwide and the ease and frequency of international travel promise an even more rapid spread of newly resistant strains. (Aziz, 1996)

When infections become resistant to 'first-line' antimicrobials, treatment has to be switched to 'second-line' agents, which are usually more costly and may have to be given by injection instead of by mouth. (WHO, 1998) The problem is worse in developing countries because of economic constraints.

Haffkine Institute has been involved in various studies that have examined the status of multiple drug resistance over the last decade. It was observed that there was a steady increase in the resistance of gram-negative bacteria not only to the older antibiotics (streptomycin, tetracycline) but

also to the newly introduced drugs (ciprofloxacin, amikacin). The indiscriminate use of antimicrobial drugs for prophylactic or therapeutic purposes in humans or for veterinary or agricultural purposes provides the selective pressure favoring this accelerated emergence of resistant organisms.

The Solutions - The solutions to the problems of antimicrobial resistance include prevention of bacterial infections, prudent use of antimicrobial agents, appropriate use of antimicrobial combinations, control and prevention of dissemination of antibiotic resistant organisms and lastly discovery and development of new antimicrobials. (Moellering, 1998)

The discovery and development of new antimicrobial drugs that are active against multi-drug resistant organisms is slow due to their prohibitive costs. Moreover, most of the obvious bacterial targets for antimicrobial agents have been discovered, so that it will take new methods and increased efforts to discover novel agents. In addition, the introduction of newer antibiotics is almost immediately followed by a steady increase in resistance against it. Thus, antibiotic combinations are commonly used for patient therapy in the hope of delaying or suppressing the emergence of drug resistant subpopulations or to provide a broad spectrum of activity.

AIMS AND OBJECTIVES

The growing multiple drug resistance among bacteria in hospital practice and the community is posing a serious threat to the successful antimicrobial therapy. In hospital practice, Gram-negative bacteria have surpassed the gram-positive bacteria in the infective process and are posing therapeutic difficulties with simultaneous resistance to a majority of the antimicrobial regimes. (Nema S, 1997) This justifies continuous efforts to provide new therapeutic regimens.

Hence, this study was designed as follows:

1. To study the antibiotic resistance profiles of clinical isolates collected during 1997 and 1998.
2. To identify antibiotic combinations that are effective against these clinical isolates.
3. To study the interactions between the combined antimicrobials by 'checkerboard method' and 'time kill- kinetics'.

LITERATURE REVIEW

History of antibiotics and antibiotic chemotherapy

The first antibiotic, penicillin, was discovered in 1929 by Sir Alexander Fleming who observed inhibition of staphylococci on an agar plate contaminated by *Penicillium* mold. World War II and the inevitable bacterial infections that occurred in war related wounds were an important impetus to study the chemotherapeutic value of penicillin. Penicillin became generally available for treatment of bacterial infections, especially those caused by staphylococci and streptococci by about 1946.

The other important discovery during this period was that of sulphonamides from the red dye Prontosil by Dogmark in 1935. It was the first synthetic chemical with broad antimicrobial activity.

The period of the late 1940s and 1950s saw the discovery and introduction of streptomycin, chloramphenicol, and tetracycline, which ushered the age of antibiotic chemotherapy. These antibiotics were effective against the full array of bacterial pathogens including gram-positive and gram-negative bacteria, intracellular parasites, and the tuberculosis bacillus.

The first resistance mechanism was recorded in 1940 by Abraham and Chain who isolated and characterized an enzyme from *E.coli* that was capable of hydrolyzing penicillin. In 1944, Kirby reported the presence of a similar penicillinase type enzyme in *S.aureus*. Thus even before the widespread use of penicillin around the globe, resistance had already been detected in both gram-negative and gram-positive bacteria. (Tenover, 1996) However, by 1953, during a Shigella outbreak in Japan, a strain of the dysentery bacillus was isolated which was multiply drug resistant, exhibiting resistance to chloramphenicol, tetracycline, streptomycin, and the sulphonamides. There was also evidence mounting that bacteria could pass

genes for multiple drug resistance between strains and even between species. (Todar, 1996)

Antimicrobial Agents

Antibiotics are natural substances produced by microorganisms that kill or inhibit other microorganisms. Chemotherapeutic agents are those antimicrobial compounds, which are chemically synthesized. A hybrid substance is a semisynthetic antibiotic, where a molecular version produced by the microbe is subsequently modified by the chemist to achieve desired properties.

Antibiotics are low-molecular weight substances that are produced as secondary metabolites by certain groups of microorganisms, especially *Streptomyces*, *Bacillus*, and a few molds (*Penicillium* and *Cephalosporium*) that are inhabitants of soils. Antibiotics may have a cidal (killing) effect or a static (inhibitory) effect on a range of microbes. The range of bacteria or other microorganisms that are affected by a certain antibiotic are expressed as its spectrum of action. Antibiotics effective against prokaryotes that kill or inhibit a wide range of Gram-positive and Gram-negative bacteria are said to be broad spectrum.

The most important property of an antimicrobial agent, from a host point of view, is its selective toxicity, i.e., the agent inhibits or kills bacterial pathogens but has little or no toxic effect on the host. This implies that the biochemical processes in the bacteria are in some way different from those in the animal cells, and that the advantage of this difference can be taken in chemotherapy. (Todar, 1996)

Kinds of Antimicrobial Agents and their Mechanisms of action and resistance

Gram-negative bacteria are in general more resistant to a large number of antibiotics and chemotherapeutic agents than gram-positive bacteria. The outer membrane of gram-negative bacteria contributes to this intrinsic resistance by acting as an efficient permeability barrier because of the narrow porin channels and the low fluidity of the LPS leaflet. However, in order to produce clinically significant levels of antibiotic resistance another mechanism is needed in addition to the outer membrane. The nearly ubiquitous periplasmic β -lactamases serves as this second mechanism for resistance to β -lactam of earlier generations and the MIC levels can be explained quantitatively by the synergistic co-operation of the outer membrane barrier and hydrolysis by β -lactamases.

With other classes of antibiotics (tetracycline, chloramphenicol, flouroquinolones), recent studies have showed that multiple drug efflux pumps, sometimes with unusually broad specificity act as this second factor to create the general intrinsic resistance of gram-negative bacteria. When their expression levels are elevated as a consequence of physiological regulation or genetic alteration they can frequently produce impressive levels of resistance to a wide variety of antimicrobial agents. (Nikaido, 1998)

A general overview of the types of antibiotics and a detailed description of those used in this study (their mode of action and resistance) is presented below

I. Cell-wall Synthesis Inhibitors

Cell wall synthesis inhibitors inhibit some step in the synthesis of bacterial peptidoglycan, generally they exert their selective toxicity against eubacteria because human cells lack cell walls.

a) **β-lactams**-The main classes include penicillins (ampicillin, carbenicillin, ticarcillin, piperacillin), cephalosporins (cephalexin, ceftibuten, ceftazidime, cefotaxime, cefoperazone, ceftriazone), monobactams (aztreonam) and carbapenem (imipenem, meropenem).

The β-lactams differ in their gram-positive, gram-negative, and anaerobic spectra and resistance to beta-lactamases. Semisynthetic penicillins like ampicillin have a broadened spectrum against gram-negative organisms and are effective orally. The first-generation and some second-generation cephalosporins are active against gram-negative organisms and against streptococci and staphylococci, while the third-generation agents have expanded gram-negative coverage. Two third-generation cephalosporins, ceftazidime and cefoperazone, are active against *Pseudomonas*. The fourth-generation agent cefepime is most active against gram-positive and gram-negative pathogens, including antibiotic-resistant Enterobacteriaceae. (Kline, 1995)

Mechanism of action- The β-lactam antibiotics are stereochemically related to D-alanyl-D-alanine which is a substrate for the last step in peptidoglycan synthesis, the final cross-linking between peptide side chains. β-lactams bind to and inhibit the carboxypeptidase and transpeptidase enzymes that are required for this step in peptidoglycan biosynthesis. The bacterium dies by an osmotic effect or is digested by autolytic enzymes. (Lambert, 1998)

Mechanisms of resistance- Acquired resistance to β-lactams can occur by three different mechanisms. 1) Inactivation by β-lactamase excreted to the extracellular fluid (gram-positive bacteria) or present in the periplasmic space (gram-negative bacteria). Inactivation occurs by the hydrolysis of the β-lactam ring by the enzyme. Synthesis of β-lactamase is mediated either by plasmids or by chromosomal genes. Chromosomal β-lactams can be constitutively synthesized (*E.coli*, *Shigella* species and

P.mirabilis) or only in the presence of the β -lactam (inducible β -lactamases of *P.aeruginosa*, *Enterobacter* species, *Citrobacter* species and *P.rettgerii*).

2) Resistance could also be due to modifications on Penicillin Binding Proteins (or their substitution for new ones) reducing their affinity for the antibiotic. This mechanism is usually seen in gram-positive organisms 3) The changes in the outer membrane porins of gram-negative bacteria cause reduced penetration of the β -lactam, resulting in low levels of resistance. (Pitout, 1997)

b) Other cell wall inhibitors include bacitracin, cycloserine and glycopeptides (vancomycin)

II. Cell-membrane Inhibitors

They disorganize the structure or inhibit the function of bacterial membranes. The integrity of the cytoplasmic and outer membrane is vital to bacteria and compounds that disorganize the membranes rapidly kill the cells. However, due to the similarities in phospholipids in eubacterial and eukaryotic membranes, this action is rarely specific enough to permit these compounds to be used systemically. The antibacterial antibiotic of clinical importance that acts by this mechanism is Polymyxin. (Todar, 1996)

III. Protein Synthesis Inhibitors

Many therapeutically useful antibiotics owe their action to inhibition of some step in the complex process of protein synthesis. Their attack is always at one of the events occurring on the ribosome and never at the stage of amino acid activation or attachment to a particular tRNA. Most have an affinity or specificity for 70S (and not 80S) ribosomes, and they achieve their selective toxicity in this manner. The most important antibiotics with this mode of action are the aminoglycosides, the tetracyclines, chloramphenicol and the macrolides (e.g. erythromycin)

a) Aminoglycosides-The aminoglycosides are products of *Streptomyces* species and are represented by streptomycin, kanamycin, tobramycin, gentamicin amikacin, and netilmicin. Streptomycin is extensively used as a primary drug in the treatment of tuberculosis. Gentamicin, tobramycin and amikacin are active against many gram-negative and gram-positive bacteria, including some strains of *P. aeruginosa*. An unfortunate side effect of the aminoglycosides that restricts their prolonged use is their nephrotoxicity and ototoxicity.

Mechanisms of action- Aminoglycosides are polycationic substances that combine with the negatively charged bacterial wall LPS and penetrate into the bacteria through an active energy consuming transport mechanism. (Hannock, 1997) These antibiotics exert their activity by binding to bacterial ribosomes and preventing the initiation of protein synthesis. Streptomycin binds to 30S subunit of the bacterial ribosome, specifically to the S12 protein, which is involved in the initiation of protein synthesis. Streptomycin prevents the initiation of protein synthesis by blocking the binding of initiator N-formylmethionine tRNA to the ribosome. It also prevents the normal dissociation of ribosomes into their subunits, leaving them mainly in their 70S form and preventing the formation of polysomes.

Kanamycin, amikacin, and the others bind to the ribosomal 30S subunit and prevent it from joining the 50S subunit during protein synthesis. This leads to the cytoplasmic accumulation of dissociated 30S subunits, which is lethal to the cells. (Todar, 1996)

Mechanisms of resistance – 1) Inactivation by enzymes called aminoglycoside-modifying enzymes, which are usually plasmid or transposon encoded and rarely of chromosomal origin. Their synthesis is constitutive. The three classes of enzymes that modify the aminoglycosides are Aminoglycoside adenylyltransferases (AAD), Aminoglycoside phosphotransferases (APH), and Aminoglycoside acetyltransferases (AAC).

(Shaw, 1993) 2) Alterations of the ribosomes due to chromosomal mutations also give rise to resistance. 3) Resistance can be due to decreased permeability, by mutations affecting membrane transport. (Foster, 1983)

b) Tetracycline- The tetracyclines (tetracycline, chlortetracycline and doxycycline) consist of eight related antibiotics which are natural or semisynthetic products of *Streptomyces*. They are broad-spectrum antibiotics with a wide range of activity against gram-negative and gram-positive bacteria. *P.aeruginosa* is less sensitive but is susceptible to concentrations obtained in the bladder.

Mechanism of action- The tetracyclines act by blocking the binding of aminoacyl tRNA to the A site on the ribosome. Tetracyclines inhibit protein synthesis on isolated 70S or 80S (eukaryotic) ribosomes, and in both cases, their effect is on the small ribosomal subunit. Most bacteria possess an active transport system for tetracycline that will allow intracellular accumulation of the antibiotic at concentrations 50 times as great as that in the medium. This greatly enhances its antibacterial effectiveness and accounts for its specificity of action, since an effective concentration cannot be accumulated in animal cells. Thus a blood level of tetracycline, which is harmless to animal tissues can halt protein synthesis in invading bacteria.

Mechanisms of resistance – There are three types of resistances identified with tetracycline. 1) Plasmid or transposon encoded tetracycline efflux proteins is the major mode of resistance. 2) Plasmid or transposon encoded ribosomal protection factors, which alter the binding site on the 30S ribosomal subunit lowering the affinity for the drugs. (Roberts, 1996) 3) Porin mutations result in low level resistance to tetracycline by reducing their uptake (Chopra, 1992) Tetracycline resistance genes are often found on transmissible elements. 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). (Speer, 1992)

b) **Chloramphenicol**- It has a broad spectrum of activity but exerts a bacteriostatic effect. It is effective against intracellular parasites such as rickettsiae and against typhoid fever. Toxicity, due to bone marrow depression curtails its use in clinical practice. Chloramphenicol was originally discovered and purified from the fermentation of a *Streptomyces*, but currently it is produced entirely by chemical synthesis.

Mechanism of action- Chloramphenicol binds to 50S subunit of ribosomes and inhibits the bacterial enzyme peptidyl transferase thereby preventing the growth of the polypeptide chain during protein synthesis. Chloramphenicol is entirely selective for 70S ribosomes and does not affect 80S ribosomes. (Lambert, 1998)

Mechanism of resistance- 1) Plasmid or transposon encoded chloramphenicol acetyltransferases (CAT) inactivates chloramphenicol by the formation of various acetoxy derivatives which are inactive as they cannot bind to the ribosome. The enzymes are constitutively produced in gram-negative bacteria. 2) Low level resistance arises due to impermeability of the membrane. (Foster, 1983) 3) Resistance can also be due to active efflux proteins (Nikaido 1994)

c) Other protein synthesis inhibitors include Macrolides (erythromycin), Lincomycin and Clindamycin.

IV. Effects on Nucleic Acids

Some chemotherapeutic agents affect the synthesis of DNA or RNA, or can bind to DNA or RNA so that their messages cannot be read.

a) Quinolones- They are synthetic chemotherapeutic agents that have activity mainly against gram-negative bacteria. Nalidixic acid is a quinolone and ciprofloxacin, ofloxacin, trovafloxacin and norfloxacin are fluoroquinolones. The main use of nalidixic acid is in treatment of lower UTI. It is effective against *E.coli*, *K.pneumoniae* and *Proteus* species but is ineffective against *P.aeruginosa* and gram-positive bacteria. The fluoroquinolones show superior activity against the gram-negative bacteria and their spectrum includes staphylococci.

Mechanism of action- The quinolones inhibit DNA gyrase (topoisomerase II) which is essential for DNA replication and allows supercoils to be relaxed and reformed. Binding of the drug inhibits DNA gyrase activity. The drugs are selective, as DNA gyrase is not found in mammalian cells. (Tilloston, 1997)

Mechanisms of resistance- The two main mechanisms include 1) alterations in the structure of DNA gyrase, which has been the most commonly identified resistance mechanism in clinical isolates. 2) Mutations that lead to reduced access of quinolones to DNA gyrase either by efflux systems, which are found in both gram-negative and gram-positive or by alterations in the outer membranes of gram-negative bacteria. (Thomson 1999)

b) Rifamycins – They are products of *Streptomyces*. Rifampicin is a semisynthetic derivative of rifamycin that is active against gram-positive and gram-negative bacteria including *M.tuberculosis*.

V. Competitive Inhibitors

The competitive inhibitors are mostly all synthetic chemotherapeutic agents. Most are 'growth factor analogs' which are structurally similar to a bacterial growth factor but which do not fulfill its metabolic function in the cell.

The Sulphonamides and Trimethoprim are inhibitors of the bacterial enzymes required for the synthesis of tetrahydrofolic acid (THF), the vitamin form of folic acid essential for 1-carbon transfer reactions. Sulphonamides are structurally similar to para aminobenzoic, the substrate for the first enzyme in the THF pathway and they competitively inhibit that step. Trimethoprim is structurally similar to dihydrofolate (DHF) and competitively inhibits the second step in THF synthesis. Since animal cells do not synthesize their own folic acid, these drugs do not affect them.

COMBINATION THERAPY

Therapy with antimicrobial combinations has been used since the time antimicrobials were made available. The simultaneous use of two or more antimicrobial agents has certain rationale and is recommended in specifically defined situations. Since various classes of antimicrobial agents exert different actions on the microorganisms, one drug has the potential to either enhance or inhibit the effect of the second.

The importance of combination theory in clinical practice was realized as early as 1951, when Cates et al (1951) used a daily dose of penicillin and streptomycin to treat enterococcal endocarditis. In three of the four patients, the infection was controlled.

Rationale for combination antibiotic therapy

Combinations of antibiotics are often used to take advantage of different mechanism of actions and toxicity profiles. The use of combinations of various antimicrobial agents has become very common in clinical medicine. Several rationales are cited for the use of combination therapy. (Allan, 1985)

1. Broad coverage - Combination therapy is most often used to provide broad coverage in empiric therapy for patients who are suspected on clinical grounds of having a severe bacterial infection, such as septicemia, when the pathogen has not yet been isolated and the consequences of failing to provide effective therapy are grave. This is particularly important in the setting of severe neutropenia.
2. Polymicrobial infections - Combination therapy is employed for the treatment of polymicrobial infection in which the spectrum of a single agent is inadequate. These infections frequently involve anaerobic organisms and generally arise from mucosal surfaces, for example-intra abdominal or pelvic infections, lung abscess. Synergistic gangrene is another such polymicrobial infection.
3. Emergence of resistance - Combination therapy may prevent or delay the emergence of resistance. A common example of this is in the treatment of pulmonary tuberculosis, in which resistance rapidly develops against single agents. Several reports indicate that when infections due to gram-negative bacilli are treated with a single beta-lactam antibiotic resistance develops during therapy. Therefore, Hilf and colleagues [1989] strongly suggest that combination therapy is more effective than monotherapy for *Pseudomonas aeruginosa* bacteremia.
4. Reduced doses – Combining two agents may allow for a reduction in dose of one or both agents and perhaps a reduction in the incidence of dose related toxicity. For example reduced toxicity of nephrotoxic and ototoxic aminoglycosides due to reduced dosage.
5. Greater inhibition – Antimicrobial agents may also be combined in an attempt to achieve greater inhibition or killing than could be obtained with a single agent.

6. Synergy – when a combination of two different classes of compounds results in bactericidal activity which is significantly greater than the sum of either agent alone, the combination is said to be synergistic.

In addition to these potential benefits, combination therapy may result in several undesirable effects.

1. Antagonism -The combination of two or more antibiotics can result in negative interactions or antagonistic effect, which is defined as less antimicrobial activity with a combination of two or more antibiotics than with the use of either agent alone.
2. Adverse effects -The administration of more than one antibiotic may increase the incidence of adverse drug reactions. These include nephrotoxicity, coagulopathy, diarrhoea, seizures, and hypersensitivity reactions.
3. Superinfection – It is common following broad-spectrum antimicrobial therapy and antimicrobial combinations. Secondary infection is often caused by resistant bacteria and fungi.
4. Costs – Combination therapy is not uniformly more expensive than monotherapy, based on acquisition costs alone. However, when combining the costs of administering several antibiotics, such as preparation of fluids, nursing time and associated toxicity, combination therapy will come out to be more expensive than monotherapy.

Results of Combinations

A combination of two or more antimicrobial drugs may produce the following responses (Scribner, 1982)

Synergism - where at least a four-fold reduction in the effective concentration of both drugs indicates synergy.

Additive effect - where a twofold reduction in the effective concentration of either or both drugs indicates addition.

Indifference - where no change in the effective concentration of the combination indicates indifference.

Antagonism - where four- fold increase in the concentration of either or both antibiotics indicates antagonism.

Jawetz and Gunnison (1952) first examined the concept of antibiotic combinations and devised a scheme, which is still used as the general principle of antibiotic therapy. They observed that bacteristatic antibiotics may antagonize the action of bactericidal drugs, and the latter may be synergistic when used together.

Rahal (1978) revised the Jawetz classification and presented it as follows:

Group 1 (Primarily Bactericidal)

Penicillins, Cephalosporins, Aminoglycosides, Polymyxins, Sulpha-Trimethoprim.

Group 2 (Primarily Bacteristatic)

Tetracyclines, Chloramphenicol, Erythromycin, Lincomycins

Group 1 + Group 1 = Synergistic or Indifferent

Group 2 + Group 2 = Additive

Group 1 + Group 2 = Synergistic, Additive or Antagonistic

Frequent antagonism is seen between Group 1 and Group 2 drugs as most of the bactericidal agents require active cell division or protein synthesis for expression of bactericidal activity and many bacteriostatic drugs of Group 2 inhibit these processes. Drugs in Group 1 may exhibit synergism, by combination of their bactericidal actions.

Clinical significance of synergy

Synergistic combinations are often sought in infections where the development of resistance and /or subsequent failure with monotherapy occurs. Various studies have pointed out that in vitro synergistic results are associated with improved clinical outcomes.

Clinically meaningful synergy is that which results in a reduction of MIC to a serum concentration of the antibiotic achievable with conventional doses.

In a study comparing penicillin plus amikacin with carbenicillin plus amikacin for gram-negative infection cure rate was significantly greater in septicemic patients when in vitro synergy was present against the infecting organism. (Klastersky, 1976)

Reyes et al (1979) examining thirty strains of *P.aeruginosa* obtained from patients with bactericidal endocarditis found that synergism was necessary for, but did not assure medical care with carbenicillin combined with gentamicin or tobramycin.

Klastersky and Zinner (1982) suggested that the use of antibiotic combinations, found to be synergistic in vitro, were associated with a better clinical outcome in patients with decreased immunity eg. In neutropenia and that, the use of synergistic drug combinations should be continued as

empirical therapy in severely neutropenic or otherwise compromised patients.

Klastersky et al (1977) performed a double-blind trial comparing amikacin-penicillin with amikacin-carbenicillin combination for treatment of serious gram-negative infections in non-neutropenic patients. Combinations, synergistic in vitro against the pathogen being treated were associated with significantly better outcomes than those seen with non-synergistic regimens.

According to a study carried out by Klastersky (1986), there is a clear correlation between in-vitro synergism and clinical outcome as synergistic antibiotics resulted in many more favorable clinical responses (80%) than non-synergistic antibiotics (49%).

In the treatment of gram-negative rod infections, of 173 organisms susceptible to both antibiotics of a given combination, those patients whose organisms were synergistically inhibited responded 80% of instances, while a 64% response was noted with nonsynergistic combinations. These effects were particularly striking for *P.aeruginosa* infections, in which 10 of 12 responded to synergistic therapy, while, none of the six responded to nonsynergistic therapy. (Anderson, 1978)

In a review of patients treated between 1979-1980 at their institution, De Jongh (1986) found that synergism has a positive impact on response only among patients with profound persistent neutropenia. Of these 49%, treated with synergistic regimens responded versus none of 13 treated with non-synergistic regimens. Even among patients treated with two antibiotics to which the pathogen was, susceptible synergism was still noted to be an independently beneficial factor.

Although the use of antibiotic drug combinations co-relates with patient survival in *P.aeruginosa* there is no assurance for patient survival. But studies by Klastersky (1972), Anderson (1975) and Young (1978) indicate that patient survival is better when combination of drugs synergistic against *P.aeruginosa* strains are administered.

In a study by Lemer and Reyes (1982) that evaluated *P.aeruginosa* endocarditis, patients with medical cure were those in whom the infecting *P.aeruginosa* was synergistically inhibited.

Several groups have shown that when β -lactams/aminoglycoside combinations demonstrate in vitro synergy against a given organism, administration of these agents to patients resulted in improved clinical results in patients. (Klastersky, 1976 and Murillo and Standiford 1980)

Peterson et al (1984), using a neutropenic rabbit model infected with *P.aeruginosa*, found in vitro synergy data to be predictive of successful therapy for amikacin plus beta-lactam in a synergistic combination.

Combinations of antimicrobials that exhibit synergism

The major mechanisms responsible for antimicrobial synergism against infection are as follows:

1) Cell wall active agents, which enhance the uptake of other drugs

Although resistance to antimicrobials may be the result of drug inactivating enzymes or an insensitive target site, it may also be due to a permeability barrier. In such situations, a given drug could be active if another agent altered the permeability of the bacterial cell in order to permit its entry.

Cell wall active agents (eg. β -lactams) enhance the entry of aminoglycosides in this manner in a number of bacterial species. Work on enterococci (*S.faecalis*) has shown that the uptake of ^{14}C – labeled streptomycin is significantly increased in the presence of penicillin. (Moellering, 1971) Similar observations have been made with *E.coli* (Poltz 1962) and it is assumed that the same mechanism is responsible for the synergism observed against a number of other gram–negative and gram-positive bacteria.

2) Inhibition of protective bacterial enzyme

Bacteria are capable of producing a variety of both chromosomal and plasmid-mediated β -lactamases. The presence of these enzymes frequently renders an organism resistant to a number of β -lactam antibiotics. Several agents have been developed that have little or no antibacterial activity but are potent inhibitors of β -lactamases. The best known of these are clavulanic acid and sulbactam, which are irreversible inhibitors and classified as suicide inactivators. (Bush, 1983)

In general, clavulanic acid and sulbactam primarily inhibit the plasmid-mediated β -lactamases of gram-negative bacilli and the chromosomally mediated class II β -lactamases. They are poor inhibitors of class I β -lactamases, which are inducible, chromosomally mediated cephalosporinases common in many gram-negative bacilli as *P.aeruginosa*, *E.cloacae*, *Serratia* and others. If the resistance of an organism to a given β -lactam is due to the presence of one of the β -lactamases susceptible to inhibition by one of these agents, a combination of one of the inhibitors with the β -lactam may render the organism susceptible to that β -lactam.

English et al (1978) studied the combination of ampicillin and sulbactam with a regime of either agent alone in a mouse model of septicemia. The protective dose was significantly reduced for the combination regimen against ampicillin resistant *H.influenzae*, *K.pneumoniae*, or *Proteus vulgaris*. Boon et al (1982) documented in vivo efficacy in a similar model for clavulanic acid combined with amoxicillin against *E.coli*.

3) Combinations of cell wall active agents

With the development of newer agents exhibiting a remarkable increased activity in gram-negative activity, there has been a renewed interest in the use of such agents as replacements for aminoglycosides in combination with other β -lactams in broad spectrum regimens.

Apart from the advantages of avoiding potential aminoglycoside toxicity many combinations of β -lactam exhibit in vitro synergism against a variety of gram negative bacilli. at least two mechanisms have been evaluated by which such synergism could occur.

a) Competitive inhibition of β -lactamases

This mechanism is similar to that of clavulanic acid or sulbactam, however these agents inhibit β -lactamases by functioning as competitive substrates rather than as irreversible inactivators. In addition most of these agents have significant antibacterial activity of their own, although not against the organism against which synergism is demonstrated.

The best-evaluated group of competitive substrate inhibitors is the isoxazolyl penicillins, such as cloxacillin. When these β -lactams are combined with agents such as penicillin, ampicillin, mezlocillin, cephalothin, and other β -lactams they exhibit synergism against 25 to 75 percent of strains of Enterobacteriaceae and *P.aeruginosa*. (Allan, 1986)

b) Complementary binding to penicillin-binding proteins

Combining agents that have complementary patterns of binding to penicillin-binding proteins may also result in synergism. For example, the compound amdinocillin, specifically binds to penicillin-binding protein 2 of *E.coli* and produces ovoid cells. (Spratt, 1977) At concentrations of amdinocillin sufficient to produce cell death, penicillin-binding proteins other than penicillin-binding protein 2 are not inhibited. Amdinocillin may be combined with other β -lactams that bind preferentially to other penicillin-binding proteins and result in synergistic inhibition and killing. In vitro studies by Neu, (1976) and Grunberg, (1976) documented such synergism between amdinocillin and other penicillins and cephalosporins against a wide variety of gram- negative bacilli.

4) Sequential inhibition of a common biochemical pathway

Trimethoprim /Sulphamethoxazole is one of the most familiar and widely used antimicrobial combinations that is synergistic in its activity against bacteria. Tetrahydrofolic acid is essential in a number of biosynthetic pathways, including DNA synthesis. Most bacteria cannot absorb sufficient exogenous folate and must synthesize tetrahydrofolate from para-aminobenzoic acid and pteridine. Sulfonamides are structural analogues of para-aminobenzoic acid and competitively inhibit the first step in folate synthesis, the formation of 7,8-dihydropteroate. The 7,8-dihydropteroate is then converted to dihydrofolic acid, which is subsequently reduced in the presence of dihydrofolate reductase to tetrahydrofolic acid. The enzyme dihydrofolate reductase is competitively inhibited by trimetoprim. Thus, the combination of trimethoprim and sulphamethoxazole can act on at least two separate steps in the folate pathway. (Then, 1982) and (Salter, 1982)

The gram-negative bacilli often synergistically inhibited by or killed by Trimethoprim /Sulphamethoxazole include *E.coli* and Klebsiella, Proteus, Providencia, Citrobacter, Enterobacter, Serratia, Salmonella, Shigella and Acinetobacter species (Harvey, 1982)

Enterobacteria

They are organisms that inhabit the intestines of humans and animals. They include the species of Escherichia, Klebsiella, Salmonella, Shigella, Proteus, Providencia, and Citrobacter. (Hugo, 1998)

E.coli is the cause of enteritis in young infants, where it can cause diarrhoea and fatal dehydration. It is a common infectant of the urinary tract and bladder in humans. It also causes many pyogenic infections and septicemia. Klebsiella species are found in the gastrointestinal tracts of humans and animals. *K.pneumoniae* causes pneumonia, UTI, pyogenic infections, septicemia and rarely diarrhoea. *S.typhi* is the causative organism of typhoid fever and *S.paratyphi* causes paratyphoid fever. The various shigella species cause bacillary dysentery. *P.vulgaris* and *P.mirabilis*

most commonly cause UTI. They also cause pyelonephritis and pyogenic infections. The *Providencia* and *Citrobacter* species do not cause serious infections but sometimes cause diarrheal diseases and UTI.

Combination Therapy Against Enterobacteria

The most common nosocomial infections in India are those of the UTI, followed by surgical wound, respiratory tract, bloodstream and skin infection. The most common bacterial pathogens are *E.coli*, *P.aeruginosa*, and *S.aureus* followed by *Enterococcus faecalis*, *Enterobacter* species, and *K.pneumoniae*. (Aziz, 1996) Antibiotic combinations are commonly used as empiric therapy to treat patients infected with these organisms. Combinations are used to provide a broad spectrum of activity or to delay or suppress the emergence of drug-resistant subpopulations.

Interactions of β -Lactam and Aminoglycosides against Enterobacteria

In 1962 Poltz and Davis examined interactions between penicillin G and streptomycin against *E.coli*. They treated growing *E.coli* cells with penicillin and found that, it enhanced the uptake of streptomycin and thus hastened killing by streptomycin. However, a brief treatment of these cells by streptomycin failed to affect killing by penicillin. Thus, they proposed the hypothesis that synergism of penicillin with streptomycin depends on the damaging effect of penicillin on the cell wall which increases the access of streptomycin to the intracellular sites.

Hansing et al (1967) reported that endocarditis caused by *E.coli* could be successfully treated with Kanamycin SO₄ administered I.M. and ampicillin sodium administered I.V

Weinstein and Moellering (1973) reported that a combination of penicillin with gentamicin produced enhanced killing, against all strains of enterococci.

Klastersky, Hensgens et al (1976) reported synergism between amikacin and cefazolin for 75% and 62% of the tested cefazolin sensitive & cefazolin resistant *Klebsiella* strains respectively. In vitro results were co-related with serum bactericidal activity sera obtained from patients receiving amikacin plus cefazolin were bactericidal only when synergism was proven in vitro.

D'Alessandary et al (1976) demonstrated synergism between cephalothin and amikacin for 68% of *klebsiella* strains studied.

Neu and Fu (1978) reported synergistic efficacy when azlocillin (7 to 59%) or mezlocillin (25 to 50%) was combined with gentamicin, netilmicin or amikacin against a variety of Enterobacteriaceae. Synergism was most apparent with mezocillin and azocillin – resistant *E.coli*, *Enterobacter* species, and *Proteus* at achievable concentration. However, even organisms resistant to both of the combined antibiotic exhibited synergistic action.

Neu and Aswapokee et al (1979) found amikacin to be superior to other aminoglycosides in producing clinically meaningful synergy i.e. enhanced killing at achievable in vivo during concentrations.

Zinner et al (1981) evaluated the activities of azlocillin, cefotaxime, and amikacin alone and in combination against a variety of Enterobacteriaceae. Of 108 strains tested, 78% synergy was seen with cefotaxime and amikacin and 59% with azlocillin and amikacin. The results were further co-related and confirmed in vivo in neutropenic mice infected with *E.coli*, *Klebsiella pneumoniae* and *Serratia marcescens* as well as in human volunteers after evaluation of the serum bactericidal activity.

Jones and Packer (1982) reported the activity of cefoperazone, cefotaxime, ceftazidime, and moxalactam against 75 species of Enterobacteriaceae that were susceptible to these agents. When these

agents were combined with amikacin 54 to 70% synergistic inhibition occurred.

Glew and Pavuk (1983) demonstrated 90% synergistic interactions against various piperacillin resistant Enterobacteriaceae when piperacillin and amikacin was used and 52% synergy when piperacillin and gentamicin was used.

The synergistic interactions between amikacin and piperacillin, cephalothin, cefotaxitin, cefotaxime, cefamandole or moxalactam against multiply resistant Enterobacteria by time kill method was evaluated by Glew & Pavuk (1984). They reported that cefotaxime-amikacin and piperacillin-amikacin exhibited early synergy in 85% and 81% respectively. Synergy between amikacin and the six β -lactams occurred most often when Enterobacteriaceae strains were susceptible to one or both of the combined antibiotics.

Bayer et al (1984) evaluated the synergistic potential of third generation cephalosporins. They reported an enhanced bactericidal activity for amikacin and gentamicin ranging from 85 to 90%, when these agents were combined with ceftazidime, ceftizoxime, or ceftazidime against various Enterobacteria.

Hooton et al (1984) evaluated the in vitro synergistic activity of combinations of amikacin, gentamicin, tobramycin, or netilmicin with cefoperazone, ceforanide, cefotaxime, cefpiramide, cefsulodin, ceftazidime, cefotaxime, ceftroxone, imipenem, moxalactam, mezlocillin, or piperacillin. Amikacin was significantly more active than the other aminoglycosides with a synergistic activity against 29% of the strains as compared with 22% for netilmicin, 17% for gentamicin and 13% for tobramycin. Among the β -lactams cefoperazone (39%), ceftroxone (38%) and cefpiramide (35%) most often demonstrated inhibitory synergistic activity when combined with any aminoglycoside.

In neutropenic rats with septicemia due to *Klebsiella pneumoniae*, Winston et al (1985) reported in vivo synergism between cefazolin and amikacin.

Arpi et al (1988) observed that the combination of netilmicin-cefotaxime (68%) and netilmicin-piperacillin (61) was synergistically active against enterobacteria.

Ferrara (1989) reported an additive or synergistic effect when meropenem or imipenem were used in combination with an aminoglycoside against gram-negative species (*E.coli*, *Klebsiella species*, *Enterobacter cloacae* and *P.aeruginosa*) while in a few cases antagonism occurred in combination with β -lactams.

Roussel (1995) reported a bactericidal effect with cefotaxime plus sulbactam with the addition of amikacin.

Owen (1997) reported that against pathogenic gram-negative nosocomial isolates a greater percentage of synergy was consistently observed with combination regimes containing tobramycin and piperacillin-tazobactam or ticarcillin-clavulanate than with cephalosporin containing regimes.

Elkhali (1997), using a pig model reported that *K.pneumoniae* resistant to cefepime, ceftazidime and amikacin was susceptible to the combination of these cephalosporins with amikacin.

Interactions of β -Lactam and Ciprofloxacin against Enterobacteria

Haller (1986) examined combinations of ciprofloxacin with a large number of penicillins and second or third cephalosporin against 200 Enterobacteriaceae [and 20 strains of *P.aeruginosa*] by agar dilution checkerboard technique. Reproducible inhibitory synergy in duplicate testing was demonstrated in less than 5% of the tests performed. Antagonism was not encountered.

Chin (1986) demonstrated synergism between ciprofloxacin and azlocillin by checkerboard method against 4 of 39 *K.pneumoniae*, 1 of 35 *Enterobacter species* and none of 23 *E.coli*. Antagonism was observed only against one strain of *K.pneumoniae*.

Moody (1985) found synergism for the combination of ciprofloxacin with azlocillin against one of 30 *S.marcescens* and none of 10 *E.coli*. Antagonism was seen against one *S.marcescens* strain. Similar results, against *E.coli*, Klebsiella, Serratia, and Citrobacter species for either azlocillin or ceftizoxime and ciprofloxacin were reported by Bamberger (1986)

Moody, Gerding et al (1987) examined ciprofloxacin-azlocillin combination against six strains of Enterobacteriaceae. At standard inocula synergism was observed against two and at higher inocula, synergism was observed against four of the six organisms.

Chalkely (1985) reported that synergism between Ciprofloxacin and third generation cephalosporins is encountered only occasionally against Enterobacteriace.

Several studies have examined combinations of ciprofloxacin with imipenem with variable results. Chin and Neu (1987) found this combination to display synergism against seven of thirty-two (21.9%) *Enterobacter* species, but against only one of thirty (3.3%) *S.marcescens* by checkerboard technique.

Kern (1988) observed ciprofloxacin-imipenem synergism against 13 of 48 (25%) mixed gram-negative organisms, of which 34 were *Enterobacter*. Antagonism was also observed against a significant number of strains.

Chow (1988) reported synergism with ciprofloxacin - mezlocillin (89%) and with ciprofloxacin-cefoperazone (67%).

Animal models of infection in which fluoroquinolones were combined with β -lactams and tested against *E.coli*, *Citrobacter freundii*, and *Proteus rettgeri* showed an indifferent effect in most cases. (Neu 1993)

Pohlman (1996) studied ciprofloxacin in combination with aztreonam, ceftazidime, piperacillin/tazobactam and ticarcillin/clavulanic acid against

K.pneumonia, *Enterobacter cloacae*, *S.marcescens* and *P.aeruginosa* by time kill studies. Synergy occurred most frequently at 7 hours and when present was most likely to occur when ciprofloxacin and β -lactams were tested at concentrations equal to their MIC. Antagonism was also noted.

P.aeruginosa

It is a gram-negative rod, belonging to the bacterial family Pseudomonadaceae .It is an oppourtunistic pathogen that causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia and a variety of systemic infection, particularly in victims of severe burns and in cancer and AIDS patients who are immunosuppressed. The bacteria is naturally resistant to many antibiotics due to the permeability barrier afforded by it's outer membrane LPS. (Todar, 1997)

A number of β -lactam antibiotic are available that are active against *P.aeruginosa*, like carbenicillin, ticarcillin, piperacillin, ceftazidime and cefoperazone. Although some of these agents inhibit the growth of this organism at low levels, killing may not occur until much higher concentration are achieved. Emergence of resistance strains has been reported when any one of these agents is used alone against this organism. For these reasons *P.aeruginosa* has been an important consideration in the development of effective combination therapy. Aminoglycosides are active against *P.aeruginosa* as well as many other gram-negative bacilli. Both bacteriostatic as well as bactericidal synergism is commonly achieved when an aminoglycoside is combined with any of the β -lactam active against *P.aeruginosa*. (Allan D 1985)

Combination Therapy Against *Pseudomonas aeruginosa*

The increasing occurrence of and difficulty in managing *P.aeruginosa* infections particularly in immunocompromised hosts and the increase in

resistance to the conventional antipseudomonal antibiotic justify continuing efforts to provide new therapeutic regimens. (Giamarellou H, 1986)

Hilf and colleagues (1989) strongly suggested that combination therapy was more effective than monotherapy for *P.aeruginosa* bacteremia as it prevented emergence of resistance.

Interactions of β -lactams and Aminoglycosides against *P.aeruginosa*

Weinstein et al (1975) demonstrated in comparative interaction studies, that synergy in the presence of amikacin affected a higher percentage of isolates than synergism with gentamicin.

Kelly & Masten (1976) demonstrated a 40-fold increase in activity in the presence of amikacin plus carbenicillin. Marks et al (1976) demonstrated synergy against 92% of gentamicin resistant *Pseudomonas* strains.

Murray (1980) reported contrasting results, when he investigated the efficacy of cefotaxime in combination with gentamicin, tobramycin or amikacin against 50 isolates of *P.aeruginosa*. Despite the fact that a greater no of strains were more effectively inhibited when the combination included gentamicin (60%) than with tobramycin (34%) or amikacin (18%), clinically meaningful synergy, occurred more often with amikacin.

Scribner et al (1982) studied the inhibitory and bactericidal activities of carbenicillin, ticarcillin, moxalactam, cefoperazone, azlocillin, piperacillin or ceftazidime in combination with amikacin, tobramycin or gentamicin against 60 isolates of *P.aeruginosa*, derived from the sputum of patients with cystic fibrosis. All strains were sensitive to each of the drugs. Azlocillin plus amikacin was most often synergistic 65% followed by piperacillin, the lowest degree of synergy observed were those for combinations using ceftazidime.

Hollander et al (1982) investigated the interactions of gentamicin or netilmicin with cefotaxime, moxalactam or ceftazidime against clinical

isolation *P.aeruginosa* and demonstrated synergy against 20 to 40 % of the strains.

Mintz and Drew (1984) assed the activity of carbenicillin and other newer β -lactam (ceperazone, cefotaxime, and moxalactam) in combination with tobramycin against 38 strains of *P.aeruginosa*. 63% synergy occurred with cefotaxime, 26% with carbenicillin, 21% with cefoperazone and 18% with moxalactam.

Buesing and Jorgensen (1984) reported 71% synergy with aztreonam plus amikacin.

Giamarellou, Zissis et al (1984) studied in_vitro interactions between amikacin, netilmicin, tobramycin or gentamicin and various antipseudomonal β -lactams in 30 strains of *P.aeruginosa* resistant to all tested antibiotics. Amikacin produced more frequent and statistically significant synergy at both the total and clinical applicable levels. Clinical relevant synergistic interactions were obtained among the β -lactams in the following order from the most to least productive synergy: ceftazidime, ceftriaxone, moxalactam, aztreonam, cefotaxime, azlocillin, cefoperazone, cefsulodin and carbencillin. The better synergistic results with amikacin was attributed to the inactivation of amikacin by fewer aminoglycoside-inactivating enzymes, whereas variation in the effects between the amikacin and β -lactam combination was explained by differences in the permeability of β -lactams through the bacterial outer-membrane.

Giamarellou et al (1986) concluded that compared with the other aminoglycosides, amikacin exhibits advantageous synergistic in vitro interactions.

Enciso et al (1991) found that ceftazidime plus amikacin exhibited synergy against 53% of the *P.aeruginosa* isolates.

Weiss and Lapointe (1995) reported that a tobramycin ceftazidime combination exerted a synergistic effect against 39% of the test *P.aeruginosa* isolates from patients with cystic fibrosis.

Several studies of experimental infections in animals have provided some in vivo correlation with the in vitro demonstration of synergy.

A model of endocarditis in rabbits was used by Archer et al (1977) to compare a regime of carbenicillin and gentamicin with regimens consisting of each agent separately. A strain of *P.aeruginosa* that was synergistically inhibited in vitro by the combination of carbenicillin and gentamicin was used. The rabbits treated with the combination had higher peak serum bactericidal levels and a lower rate of relapse after two weeks of therapy than did the rabbits treated with a single agent regime.

In an experimental peritonitis model studied by Andriole et al (1971), using a strain of *P.aeruginosa* that was synergistically inhibited by carbenicillin and gentamicin in vitro, rats treated with the combination regime had a much higher survival rate than did rats treated with either agent alone.

In a subsequent study using this same model Andriole VT (1974) compared carbenicillin, cephalothin and cephanone alone or in combination with tobramycin or gentamicin. Only rats treated with a combination exhibiting in vitro synergism survived. Similar results were obtained in *P.aeruginosa* peritonitis models using combinations of ticarcillin and tobramycin in the mouse (Comber 1977) and combination of carbenicillin and gentamicin in the neutropenic rat. (Scott 1976)

Gerber et al (1982) studied *P.aeruginosa* soft tissue infections in the neutropenic mice model that were treated with a combination regimen of carbenicillin and gentamicin or gentamicin alone. The investigators found that the combination regimen was superior to gentamicin alone and the observed in vivo synergism resulted from carbenicillin's suppression of emergence of gentamicin resistant populations.

Interactions of β -lactams with Ciprofloxacin against *P.aeruginosa*

Ciprofloxacin is the most potent of all quinolones. Although ciprofloxacin is active against most strains of *P.aeruginosa*, MIC of the drug is often relatively near the limits of clinically achievable drug concentrations. In addition, multiple drug resistance is common in this bacterial species and resistance to ciprofloxacin can emerge during therapy. As a result, considerable effort has been expended in the study of ciprofloxacin in combination with other antimicrobial agents (β -lactams, aminoglycosides etc) against *P.aeruginosa*.

Synergism between β -lactams and ciprofloxacin against *P.aeruginosa* occur more commonly than with ciprofloxacin-aminoglycosides combinations, but results may vary widely. Antagonism appears to be quite uncommon against these organisms. (Eliopoulos, 1989)

Synergism has been reported by Stratton (1989), between ciprofloxacin and antipseudomonal penicillins and cephalosporins.

Combination of ciprofloxacin with antipseudomonal penicillin, azlocillin demonstrated synergy against 10 to 60% of the isolated tested. (Davies, 1985 and Rudin, 1984)

Combinations of ciprofloxacin or ofloxacin and antipseudomonal penicillin were synergistic against *P.aeruginosa* for 10 to 60% of isolates and had an additive effect for similar percentages. (Chin 1986 and Neu 1985)

Fragg et al (1986) reported that the combination of ciprofloxacin with ceftazidime against *P.aeruginosa* was additive or indifferent.

Giamarellou and Petrikos (1987) reported that cefoperazone and ciprofloxacin showed synergy against 30% of *Pseudomonas*.

Pseudomonas have been reported to be synergistically inhibited by ciprofloxacin and imipenem if the isolates are susceptible (Bustamente et al 1987, Giamarellou and Petrikos 1987)

Chin and Neu (1987) reported that only 8% *Pseudomonas* from cystic fibrosis patients were synergistically inhibited by ciprofloxacin and imipenem.

Shimshido (1991) reported that, 44% of multiply-resistant *P.aeruginosa* were synergistically inhibited by fluoroquinolones and imipenem.

The combination of ciprofloxacin-imipenem-rifampicin was more likely to show synergy than ciprofloxacin-imipenem (Chin and Neu 1987, Kumar 1989)

Peterson (1990) studied the in vitro activity of ciprofloxacin in combination with aztreonam, azlocillin and methicillin against multiple drug resistant *P.aeruginosa* and observed that if the organism was resistant to ciprofloxacin, synergism was observed in more than 50% of the isolates. However, if the isolates were resistant to β -lactams 10% synergism was observed.

Hyatt (1995) observed that the combination of piperacillin with ciprofloxacin and piperacillin-tazobactam with ciprofloxacin was additive against *P.aeruginosa*, but a greater killing was observed with the latter.

Gould (1997) showed clinically achievable synergy between ciprofloxacin and piperacillin-tazobactam, which was bactericidal for 83% of *P.aeruginosa* strains.

Mayer (1999) investigated the synergistic effects of fluoroquinolones (ciprofloxacin, ofloxacin and pefloxacin) in combination with third generation cephalosporins (cefoperazone, ceftriaxone, ceftazidime) against 18 clinical isolates of *Pseudomonas* species. The fluoroquinolone ciprofloxacin in combination with the third generation cephalosporins showed the highest activity.

In a mouse model, using neutropenic mice, that were challenged intraperitoneally with a strain of *P.aeruginosa*, which was synergistically inhibited by the ciprofloxacin-azlocillin combination, Chin et al (1986) found that, the combination therapy offered significant protection against early death, which occurred when either agents were used alone, but a

P.aeruginosa guinea-pig model of respiratory infection did not show increased survival (Kemmerich, 1986)

In the subcutaneous chamber model studied by Bamberger et al (1986) the combination of ciprofloxacin –azlocillin resulted in at least 1,000 fold greater killing than the most active single drug alone against two of six strains of *P.aeruginosa*. The ability to achieve at least 99.9% killing in vitro appeared to co-relate with a better outcome in vivo in this model.

In a neutropenic mouse, thigh infection model, azlocillin alone was ineffective in suppression of infection but did significantly enhanced the activity of high dose ciprofloxacin. Combination therapy lead to an almost 100-fold reduction in titers of viable bacteria compared with those obtained with ciprofloxacin alone. (Haller I, 1987)

In neutropenic, mice challenged intraperitonelly with a *P.aeruginosa* ciprofloxacin-cefpiramide therapy led to significant protection when the drugs were combined at doses that exhibited negligible activity when each drug was used alone. (Fu, 1987)

MATERIALS AND METHODS

Identification of the clinical isolates

Eighty clinical isolates were collected from Kasturba Hospital for Infections Diseases, Mumbai and from Sir JJ Group of Hospitals, Mumbai, during the period of 1997 and 1998. Fifty-four of these clinical isolates were used for the present study.

The clinical isolates were identified on the basis of the following morphological and biochemical characteristics: Gram's nature, colony characteristics, motility, utilization of sugars (Glucose, Sucrose, Lactose and Mannitol), utilization of citrate, Indole test, Methyl Red test, Voges-Proskauer test, PPA test, Oxidase test and Triple Sugar Iron test.(Koneman,1992).

Maintenance of the clinical isolates

The clinical isolates and standard control bacterial cultures were streaked on Nutrient Agar (N.A.) slants containing 100 µg/ml of the requisite antibiotic after confirming their antibiotic resistance pattern. The cultures were stored under refrigeration and were maintained in a viable form by sub-culturing on fresh N.A. slants at regular intervals.

Determination of Antibiotic Resistance

The microbiological laboratory plays an important role both in choosing initial antimicrobial therapy and in monitoring such therapy during the course of treatment. (Zinner, 1986) The accumulated sensitivity tests on all hospital and community isolates in a locality allow early, informed empirical treatment for a serious infection before the results of tests on a patients strain are known. (Mackie and Mc Carteny, 1996)

The antibiotic resistance patterns were determined by two methods:

I. Disc Diffusion Method

This method is also known as the Kirby–Bauer disc diffusion test. (Bauer, 1966) This method involves the use of dried filter paper disc impregnated with a defined concentration of antibiotic on the agar, which is used to culture the test organism. A clear zone of inhibition around the disc indicates the susceptibility of the organism to the antibiotic. The diameter of the zone of inhibition is proportional to the susceptibility of the test organism.

- a) *Medium* - Mueller and Hinton (MH) agar (ph 7.4) was sterilized at 121° c at 15 lbs for 15 mins. This medium was poured into sterile petridishes; the plates were dried and were used to determine the drug sensitivity.
- b) *Antibiotic discs* - Antibiotic discs of the various drugs, containing appropriate concentrations were prepared in our laboratory and were used for this test. (Preparation of discs-Appendix)
- c) *Inoculation* - The test organism was inoculated in MH broth and incubated overnight at 37° C. 0.1ml of the above was then inoculated into fresh MH broth and incubated for 3-4 hours to obtain an active log phase culture.
- d) *Procedure* - Sterile cotton swabs dipped in the test culture were used to swab MH agar plates to obtain a confluent matt growth. Antibiotic discs were impregnated on the plates with sterile forceps. The discs were gently pressed down to ensure that they adhere to the agar surface. The plates were incubated overnight at 37° C.

The zones of inhibition observed after incubation (measured in mm) were compared with the standard NCCLS chart and classified as susceptible, intermediate or resistant

- e) *Control* - Parallel tests were carried out with *E.coli ATTC 10148* which served as the sensitive control.

II. Macro broth dilution method

Although the qualitative disc diffusion method is a sufficient guide to treatment in the majority of infections a quantitative assessment of sensitivity is sometimes required, particularly when the patient needs prolonged therapy with an antimicrobial which exhibits dose related toxicity or where achievable concentrations are close to those required to inhibit growth of the infecting organism. (Stokes, 1993)

The lowest concentration of the antimicrobial that inhibits growth of the organism, as detected by lack of visual turbidity is designated the Minimum Inhibitory Concentration (MIC). (Cruikshank, 1968) The 'MIC' thus represents a quantitative endpoint which is used to compare the susceptibility of a given bacterial strain to inhibition by various antimicrobials or else to estimate for a given antimicrobial agent the relative susceptibilities of series of isolates of a given species or a different species.

- a) *Medium* - Double strength Mueller and Hinton (MH) broth (ph 7.4) was sterilized at 121° c at 15 lbs for 15 mins.
- b) *Inoculation* - The test organism was inoculated in sterile MH broth and incubated overnight at 37° C. 0.1ml of the above was then inoculated into fresh MH broth and incubated for 3-4 hours to obtain an active log phase culture.
- c) *Antibiotic stocks* - Stocks of antibiotics, dissolved in appropriate solvents were made. These stocks were used to prepare a series of two fold dilutions in 4.9 ml volume of broth.

- d) *Procedure* - 0.1 ml of culture was added to tubes containing the range of antibiotics to obtain a final density between 10^5 - 10^6 cfu/ml. The tubes were incubated overnight at 37° C.

The MIC was recorded and compared with the standard NCCLS chart and classified as susceptible or resistant

- e) *Control* - *E.coli* ATTC 10148 served as a sensitive control. A tube containing the highest concentration of the antibiotic in broth served as a negative control to check the turbidity of the medium. A tube containing 0.1 ml culture in broth served as a positive control.

Tests for investigating the inhibitory interactions between combined antimicrobials

Disc Approximation Test

It is a preliminary identification test for determination of antibiotic interactions. It determines the bacteriostatic interactions between two antibiotics. This test uses the same standard inoculum and MH agar as the Kirby-Bauer disc diffusion test. (Krogstad and Moellering, 1986)

- a) *Medium* - Sterile MH agar was poured into sterile petridishes. The plates were dried and then used for this test.
- b) *Test organisms* – Nine clinical isolates that were resistant to all the antibiotics used in this study were used. Three of which belonged to the *Proteus* group, three to *Escherchia*, two to *Klebsiella* and one to *Pseudomonas*. These test organisms were inoculated in MH broth and incubated overnight at 37° C. 0.1ml of the above was then inoculated into

fresh MH broth and incubated for 3-4 hours to obtain an active log phase culture.

- c) *Procedure* - Sterile cotton swabs were dipped in the cultures and used to swab the plates to obtain a confluent matt growth. Two discs (one for each antimicrobial) were placed on the seeded surface with their centers separated by a distance (measured in mm) that was equal to or slightly greater than half the sum of their individual zones of inhibition when obtained separately. The plates were incubated overnight at 37⁰ C.

The pattern observed with additive or indifferent combination is that of two independent circles. With synergistic combinations, enhancement or bridging is observed at or near the junction of the two zones of inhibition. With antagonistic combinations, truncation is observed near the junction of the two zones.

Checkerboard Technique

For a more precise investigation of the interaction between two antimicrobials, the checkerboard technique was undertaken. It is a modification of the broth dilution technique for the determination of MIC and asses inhibitory effects of the two antibiotics in combination. In this technique, the test isolate is exposed to a series of concentrations of two antimicrobials in broth, alone and in all the possible combinations with each other. (Krogstad and Moellering, 1986)

- a) *Medium* – The medium used for the test was sterile double strength MH broth.
- b) *Test cultures* – Eight clinical isolates that exhibited synergism for the Cefoperazone with Amikacin combination by the disc approximation test

were used. The test organisms were inoculated in sterile MH broth and incubated overnight at 37° C. 0.1 ml of the above was then inoculated into fresh MH broth and incubated for 3-4 hours to obtain an active log phase culture.

- c) *Antimicrobial agents* - Stock solutions of Cefoperazone and Amikacin were made with sterile double strength MH broth.
- d) *Protocol* – In a square template various combinations of the two antimicrobials being tested were prepared from appropriate stock solutions. Their range included from 1/16 to twice the MIC as shown in the figure.

Drug B µg/ ml	64		32:1	16:1	8:1	4:1	2:1	1:1
	32		16:1	8:1	4:1	2:1	1:1	1:2
	16		8:1	4:1	2:1	1:1	1:2	1:4
	8		4:1	2:1	1:1	1:2	1:4	1:8
	4		2:1	1:1	1:2	1:4	1:8	1:16
	2		1:1	1:2	1:4	1:8	1:16	1:32
	0							
		0	2	4	8	16	32	64
		Drug A µg/ml						

Arrangement of the Drug Dilutions in the Checkerboard Test

The first row and column of tubes with only one drug served to confirm the individual MIC values of the test isolates. The first tube, which contained no antibiotic, served as a positive control.

In a final volume of 5.0 ml in each tube, antimicrobial drugs (diluted in broth) were added from appropriate stock solutions. For example-to achieve a 1:1 ratio of Cefoperazone and Amikacin with their individual concentrations at 4 µg/ml, 0.2 ml from stock solutions (100 µg/ml) of both drugs are added to the tube. 0.1 ml of the test organism in the log phase was added to obtain a final density between 10^5 to 10^6 cfu/ml. 4.5 ml of sterile broth was added to make a final volume of 5.0 ml. The tubes, incubated overnight at 37° C were inspected for growth.

Results are depicted by drawing an isobologram, which is a line joining the points that represent all combinations with the same effect including the equally effective concentrations of the antibiotics used alone. A concave curve indicates synergism, a convex curve indicates antagonism, and a straight line indicates an additive effect.

Elion et al (1954) devised a method for quantifying the MIC results obtained in terms of Fractional Inhibitory Concentration (FIC) Index, defined as the sum of the FIC values of the two drugs in combination.

It is calculated as follows:

$$\text{FIC index} = \text{FIC of drug A} + \text{FIC of drug B}$$

$$\text{FIC of drug A} = \frac{\text{MIC of drug A in combination with B}}{\text{MIC of drug A}}$$

Interpretation:

Synergy = FIC <0.5

Partial synergy/ addition = FIC 0.5-0.7

Indifference = FIC 0.76-2.75

Antagonism = FIC >2.75

Time Kill Kinetics

The most informative method for investigating the bactericidal interactions of antimicrobial combinations is the Time kill Kinetics technique. Apart from confirming the checkerboard test results, it offers an advantage over the checkerboard test in that both the rate and extent of killing can be assessed. In this technique the rate at which two antimicrobials kill an inoculum of the test isolate, individually is compared with the rate at which they kill in combination by plotting the number of surviving bacteria against time. (Krostad and Moellering, 1986)

- a) *Medium* – Sterile double strength MH broth was used to prepare preliminary cultures as well as for monitoring the growth of the test organisms for kinetics studies. MH broth was also used as a diluent for antibiotic socks.
For performing the viable counts of the test organisms, MH agar was used.
- b) *Test isolates* – Two test isolates, namely J3 (*E.coli*) and J9 (*P.aeruginosa*) were used to study the time kill kinetics.
- c) *Inoculation* – Over-night cultures of the test organisms were diluted with sterile MH broth to obtain a density of $10^6 - 10^7$ cfu/ml. This served as the Preliminary culture.

- d) *Concentrations of drugs and their combinations* – Sub-inhibitory concentrations of the individual drugs (1/2 of MIC) and their combinations were prepared using appropriate stock solutions. Based on the MIC values for each drug in combination, different ratios (1:2, 1:4 etc) were used.
- e) *Control* – A flask containing only the test culture and no antibiotic served as a positive control.
- f) *Protocol* – 1.0 ml of the preliminary culture was added (to obtain a final density between 10^5 – 10^6 cfu/ml) to all the flasks containing different sub inhibitory concentrations of the individual drugs as well as their combinations, in the medium and the flask containing the medium alone. Sterile MH broth was added to make the total volume 50.0 ml.

The flasks were incubated at 37° C for 24 hrs under shaker conditions, during which samples were drawn at intervals in order to obtain viable counts. The time intervals were 0, 1, 2, 3, 4, 6 and 24 hours. Viable counts were obtained by surface spreading 0.1 ml of the sample on sterile MH agar plates after appropriate dilutions in saline. The plates were incubated overnight at 37° C for 24 hrs and colony counts were obtained. The logs of viable counts were plotted against time. Synergism is observed when the (cfu)/ml count for 24 hours for the antibiotic combination is greater than or equal to a 2 log cfu/ml reduction more than either agent alone. (Krogstad and Moellering, 1986)

RESULTS AND DISCUSSION

Identification of the clinical isolates:

The clinical isolates collected in 1997 and 1998, from the hospitals were isolated and identified. Fifty-four clinical isolates from these were used for this study. All these isolates were gram-negative coccobacilli or bacilli. The Enterobacteria [so referred to as they inhabit the intestines of humans and animals] (Hugo 1998) included *Escherichia*, *Klebsiella*, *Salmonella*, *Shigella*, *Proteus*, *Providencia*, and *Citrobacter* species. The other group of organisms was that of *Pseudomonas aeruginosa*.

Based on their morphological and biochemical characteristics the 54 isolates were identified up to the genus/species level. There were 17 *Escherichia coli*, which showed smooth pink colonies on Mac Conkey's agar. 11 *Klebsiella* [8 *K.pneumoniae*, 3 *K. oxytoca*], which showed characteristic pink mucoid colonies on Mac Conkey's agar. 6 *Proteus* [3 *P.vulgaris*, 3 *P.mirabilis*] which were PPA positive and showed characteristic swarming on the plates. 7 *Salmonella* [6 *S.typhi*, 1 *S.paratyphi*] since serological tests were not performed for *Salmonella* they could not be further identified to their specific serotype. 1 *Shigella dysenteriae*, 1 *Citrobacter freundii* and 1 *Providencia retgerii*. 10 *Pseudomonas aeruginosa*, were oxidase positive and produced a distinct bluish green pigment called pyocyanin-characteristic of this organism.

TABLE 1- Standard Biochemical Chart
for the Identification of the Isolates

GENUS/ SPECIES	BIOCHEMICAL REACTIONS														
	Gl	Su	La	Ma	In	MR	VP	Ci	Sl	TSI		H ₂ S	Mt	ppa	Ox
E. coli	AG	AG	AG	AG	+	+	-	-	AC	AC	+	-	+	-	-
K. pneumoniae	AG	AG	AG	AG	-	-	+	+	AC	AC	+	-	-	-	-
K. oxytoca	AG	AG	AG	AG	+	-	+	+	AC	AC	+	-	-	-	-
P. vulgaris	AG	AG	-	-	+	+	-	-	AL	AC	+	+	+	+	-
P. mirabilis	AG	-	-	-	-	+	-	-	AL	AC	+	+	+	+	-
S. typhi	A	-	-	A	-	+	-	-	AL	AC	-	+	+	-	-
S. paratyphi	AG	-	-	AG	-	+	-	-	AL	AC	+	(+)	+	-	-
Shigella dysenteriae	A	-	-	-	+	+	-	-	AL	AC	-	-	-	-	-
C. freundii	AG	(A)	(A)	A	-	+	-	+	AL	AC	-	-	+	-	-
P. retgerii	A	-	-	A	+	+	-	+	AL	AC	-	-	+	+	-
P. aeruginosa	(A)	-	-	-	-	-	-	+	AL	NC	-	-	+	-	+

LEGEND: **AG** = Acid +Gas, **A** = Acid only, **+** = Positive, **-** = Negative,
() = Variable, **AL** = Alkaline, **AC** = Acidic , **NC** = No change

Antibiotic Resistance Profile

The antibiotic resistance profile for all the clinical isolates was determined by the Disc-diffusion method. The Broth dilution method was used to confirm this profile and to determine the levels of resistance to the antibiotics.

The antibiotics, against which the clinical isolates were tested were as follows: Ciprofloxacin (Cip), Nalidixic acid (Nal), Tetracycline (Tet), Chloramphenicol (Chl), Streptomycin (Str), Gentamicin (Gen), Amikacin (Amk), Ampicillin (Amp), Cephalexin (Cfl), Ceftibuten (Cfb), Cetazidime (Cfz), Cefotaxime (Cfx), Cefoperazone (Cfp) and Ceftriaxone (Cft).

Table 2A shows the drug resistance pattern, obtained by the disc diffusion method and Table 2B shows the levels of drug resistance, obtained by the broth dilution method. Table 3A and 3B are the standard NCCLS charts. The percentage of resistance against individual antibiotics is shown in Table 4.

On analysis, the drug resistance profiles revealed the following:

- 1) Among the quinolones, 81% of resistance was observed against the potent flouroquinolone ciprofloxacin, whereas 96% resistance was observed against the older quinolone nalidixic acid. All the ciprofloxacin resistant strains were also simultaneously resistant to nalidixic acid. This could be due to cross resistance, often observed with ciprofloxacin.
- 2) Among the aminoglycosides, amikacin proved most effective with 61% resistance as compared to gentamicin (81%) and streptomycin (100%). The better activity of amikacin could be due to the inactivation of amikacin by fewer aminoglycoside –inactivating enzymes.

TABLE 2A - Drug Resistance Profiles

Isolates	Cip	Nal	Tet	Chl	Str	Gen	Amk
K2	-	-	10	-	-	11	16
K3	22	-	-	-	-	12	17
K4	25	-	-	-	-	14	18
K5	-	-	-	-	-	-	9
K6	23	-	-	-	-	13	15
K7	21	-	-	-	-	17	18
K9	-	-	-	-	-	-	8
K10	8	-	-	-	-	7	10
K11	19	-	-	-	-	8	10
K13	20	-	-	-	-	17	22
K15	16	-	-	-	-	-	-
K16	16	-	-	-	-	-	-
K17	19	-	-	-	-	6	9
K18	10	-	-	-	-	-	-
K23	13	-	-	-	-	10	12
K25	-	-	-	-	-	-	10
K27	-	-	-	10	-	-	9
K28	-	-	-	-	-	-	14
K29	-	-	-	-	-	-	5
K30	-	-	-	5	-	-	-
K31	-	-	-	-	-	-	-
K36	-	-	-	-	-	-	15
K38	11	-	-	-	8	15	17
K39	-	-	-	-	-	-	16
K42	-	-	-	-	-	-	11
E.coli 10148	30	20	17	29	16	20	24

Cip – Ciprofloxacin, Nal – Nalidixic acid, Tet – Tetracycline, Chl – Chloramphenicol, Str – Streptomycin, Gen – Gentamicin, Amk – Amikacin
Zones measured in mm.

TABLE 2A-Drug Resistance Profiles

(…Continued)

Isolates	Amp	Cfl	Cfb	Cfz	Cfx	Cfp	Cft
K2	-	18	20	19	23	19	27
K3	-	16	26	18	24	8	24
K4	-	21	25	18	23	23	21
K5	-	17	24	18	23	18	23
K6	-	19	25	19	22	22	20
K7	-	20	22	19	28	23	32
K9	-	-	11	17	26	17	10
K10	-	-	9	-	11	5	-
K11	-	-	-	-	10	19	13
K13	-	19	24	20	25	22	22
K15	-	7	20	-	18	17	15
K16	-	13	21	16	19	19	14
K17	-	-	7	14	9	20	11
K18	-	-	15	5	-	15	10
K23	-	-	10	-	11	18	-
K25	-	16	23	11	23	27	22
K27	-	-	10	9	11	10	-
K28	-	14	15	16	22	17	-
K29	-	-	-	-	12	22	17
K30	-	-	-	-	10	19	19
K31	-	-	7	-	8	20	21
K36	-	17	23	16	26	26	17
K38	-	-	17	17	24	17	14
K39	-	15	24	15	20	18	19
K42	-	-	9	11	13	13	-
E.coli101							
48	17	17	20	26	24	23	25

Amp – Ampicillin, Cfl –Cephalexin, Cfb – Ceftibuten, Cfz – Ceftazidime,

Cfx – Cefotaxime, Cfp – Cefoperazone, Cft – Ceftriazone

Zones measured in mm.

TABLE 2A- Drug Resistance Profiles

(…Continued)

Isolates	Cip	Nal	Tet	Chl	Str	Gen	Amk
J2	-	-	-	11	-	-	-
J3	9	-	-	7	-	-	11
J8	20	-	-	30	-	15	19
J9	-	-	-	-	-	-	-
J15	26	-	14	25	-	13	30
J16	-	-	-	-	-	-	18
J17	24	-	-	-	-	22	23
J18	-	-	-	-	-	19	16
J19	12	-	-	-	-	-	8
J21	26	20	-	8	-	19	14
J23	11	-	-	12	-	-	11
J24	21	-	9	-	-	11	13
J25	-	-	-	-	-	-	14
J26	12	-	15	-	-	-	17
J27	23	-	-	-	-	18	11
J28	-	-	6	6	-	-	9
J30	-	-	10	7	-	-	8
J31	-	-	-	-	-	-	15
J32	-	-	13	-	-	-	12
J33	-	-	-	-	-	-	-
J34	-	-	-	-	-	-	9
J35	-	-	-	7	-	-	-
J36	25	19	13	18	9	16	19
J37	-	-	-	13	-	-	16
J38	-	14	-	16	-	20	14
J39	-	-	-	-	-	-	15
J40	10	-	-	-	-	-	12
J41	-	-	-	-	-	-	14
J42	-	-	-	-	-	-	11

Cip – Ciprofloxacin, Nal – Nalidixic acid, Tet – Tetracycline, Chl –

Chloramphenicol, Str – Streptomycin, Gen – Gentamicin, Amk – Amikacin.

Zones measured in mm.

TABLE 2A- Drug Resistance Profiles

(…Continued)

Isolates	Amp	Cfl	Cfb	Cfz	Cfx	Cfp	Cft
J2	-	-	-	-	9	21	9
J3	-	6	15	10	16	9	-
J8	-	-	-	6	23	18	18
J9	-	-	-	12	-	11	-
J15	11	-	16	16	17	13	8
J16	10	15	20	15	20	21	25
J17	-	-	10	15	25	22	26
J18	-	17	20	14	25	25	23
J19	-	18	25	-	25	24	27
J21	-	20	24	25	26	25	22
J23	-	14	24	20	24	23	19
J24	-	-	10	15	17	22	8
J25	-	16	23	-	25	23	26
J26	-	19	24	17	24	22	23
J27	-	18	21	20	30	24	15
J28	-	-	14	12	-	-	-
J30	-	18	24	12	22	18	17
J31	-	-	19	9	17	15	13
J32	-	10	11	6	18	6	10
J33	-	12	22	13	24	23	26
J34	-	11	18	-	24	19	17
J35	-	-	-	20	19	17	16
J36	-	16	22	17	26	21	19
J37	-	-	18	-	23	23	23
J38	-	-	6	11	10	12	5
J39	-	6	18	12	19	10	8
J40	-	12	19	-	23	17	16
J41	-	9	20	-	16	19	19
J42	-	-	-	12	13	13	26

Amp – Ampicillin, Cfl –Cephalexin, Cfb – Ceftibuten, Cfz – Ceftazidime,

Cfx – Cefotaxime, Cfp – Cefoperazone, Cft – Ceftriazone

Zones measured in mm.

TABLE 2B - Levels of drug resistance

Isolates	Cip	Nal	Tet	Chl	Str	Gen	Amk
K2	128	1024	128	128	>1024	64	16
K3	1	>1024	256	512	>1024	8	16
K4	≤0.25	>1024	512	256	>1024	4	8
K5	128	>1024	512	512	>1024	1024	128
K6	0.5	>1024	256	128	>1024	4	16
K7	4	1024	256	256	>1024	2	8
K9	32	>1024	128	512	>1024	512	256
K10	16	>1024	256	256	>1024	512	128
K11	32	>1024	64	512	>1024	512	64
K13	8	>1024	512	256	>1024	16	≤0.25
K15	8	>1024	128	256	>1024	>1024	64
K16	16	>1024	256	256	>1024	>1024	32
K17	16	>1024	64	1024	>1024	512	64
K18	32	>1024	256	512	>1024	>1024	64
K23	32	>1024	128	512	>1024	128	64
K25	128	>1024	512	512	>1024	256	32
K27	64	>1024	128	128	>1024	>1024	128
K28	32	>1024	526	512	>1024	512	16
K29	128	>1024	128	512	>1024	>1024	32
K30	128	>1024	>1024	1024	>1024	>1024	256
K31	128	>1024	1024	1024	>1024	>1024	128
K36	64	>1024	512	64	>1024	1024	16
K38	32	>1024	256	512	>1024	4	16
K39	64	>1024	128	512	>1024	256	16
K42	32	>1024	128	128	>1024	>1024	64
E.coli 10148	≤0.25	1	1	2	16	4	1

Cip – Ciprofloxacin, Nal – Nalidixic acid, Tet – Tetracycline, Chl – Chloramphenicol, Str – Streptomycin, Gen – Gentamicin, Amk – Amikacin.
MIC values in µg/ml.

TABLE 2B- Levels of drug resistance

(...Continued)

Isolates	Amp	Cfl	Cfb	Cfz	Cfx	Cfp	Cft
K2	512	16	1	8	2	8	≤0.25
K3	>1024	16	≤0.25	4	1	512	≤0.25
K4	1024	2	≤0.25	8	1	0.5	2
K5	512	16	0.5	8	0.5	8	2
K6	>1024	4	≤0.25	≤0.25	4	2	8
K7	1024	2	0.5	0.5	16	1	≤0.25
K9	>1024	>1024	256	16	≤0.25	8	128
K10	>1024	>1024	256	512	32	>1024	256
K11	1024	>1024	512	1024	8	4	128
K13	>1024	4	≤0.25	2	32	1	1
K15	>1024	256	4	512	32	8	64
K16	>1024	64	2	32	64	4	64
K17	512	>1024	512	64	64	4	256
K18	>1024	>1024	1024	512	128	128	256
K23	>1024	>1024	256	>1024	512	512	512
K25	512	32	≤0.25	256	0.5	≤0.25	≤0.25
K27	>1024	>1024	256	256	32	256	1024
K28	>1024	64	32	32	8	8	512
K29	>1024	>1024	512	>1024	32	2	8
K30	>1024	>1024	512	>1024	256	8	8
K31	>1024	>1024	512	>1024	128	4	4
K36	>1024	16	<0.25	16	≤0.25	<0.25	8
K38	>1024	>1024	32	32	0.5	16	16
K39	512	16	<0.25	32	≤0.25	4	16
K42	>1024	>1024	256	128	64	128	512
E.coli 10148	4	4	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25

Amp – Ampicillin, Cfl –Cephalexin, Cfb – Ceftibuten, Cfz – Ceftazidime,

Cfx – Cefotaxime, Cfp – Cefoperazone, Cft – Ceftriazone

MIC values in µg/ml.

TABLE 2B- Levels of drug resistance

(…Continued)

Isolates	Cip	Nal	Tet	Chl	Str	Gen	Amk
J2	32	>1024	128	128	>1024	>1024	128
J3	32	>1024	128	256	>1024	1024	32
J8	0.5	>1024	256	4	>1024	16	8
J9	128	>1024	512	512	>1024	>1024	128
J15	0.5	>1024	32	8	>1024	64	1
J16	128	>1024	256	256	>1024	256	16
J17	1	>1024	128	1024	>1024	1	1
J18	128	>1024	128	512	>1024	4	16
J19	32	>1024	256	256	>1024	>1024	128
J21	≤0.25	2	64	128	>1024	4	4
J23	16	>1024	64	256	>1024	1024	32
J24	1	>1024	64	1024	>1024	128	64
J25	128	>1024	256	256	>1024	>1024	32
J26	16	512	32	256	>1024	8	4
J27	0.5	512	1024	128	>1024	4	64
J28	64	>1024	512	512	>1024	>1024	64
J30	64	>1024	128	256	>1024	1024	64
J31	128	>1024	128	256	>1024	>1024	16
J32	256	>1024	64	512	>1024	>1024	64
J33	128	>1024	>1024	512	>1024	>1024	128
J34	128	>1024	128	512	>1024	>1024	64
J35	128	>1024	128	512	>1024	>1024	128
J36	≤0.25	8	32	16	>1024	4	2
J37	128	>1024	128	64	>1024	1024	16
J38	128	256	128	64	>1024	1	32
J39	64	>1024	512	512	>1024	>1024	16
J40	16	>1024	512	512	>1024	>1024	64
J41	128	>1024	1024	256	>1024	1024	32
J42	128	>1024	128	512	>1024	1024	128

Cip – Ciprofloxacin, Nal – Nalidixic acid, Tet – Tetracycline, Chl –

Chloramphenicol, Str – Streptomycin, Gen – Gentamicin, Amk – Amikacin

MIC values in µg/ml.

TABLE 2B- Levels of drug resistance

(…Continued)

Isolates	Amp	Cfl	Cfb	Cfz	Cfx	Cfp	Cft
J2	>1024	>1024	1024	>1024	>1024	8	128
J3	>1024	512	64	128	32	512	512
J8	>1024	>1024	>1024	512	32	16	16
J9	>1024	>1024	>1024	256	512	128	256
J15	>1024	1024	512	32	32	64	256
J16	512	32	1	32	4	4	0.5
J17	1024	512	128	16	≤0.25	2	≤0.25
J18	512	16	1	16	0.5	0.5	2
J19	>1024	16	≤0.25	512	0.5	0.5	≤0.25
J21	128	2	≤0.25	0.5	≤0.25	0.5	4
J23	512	32	≤0.25	1	≤0.25	2	8
J24	>1024	>1024	256	32	64	4	128
J25	>1024	32	≤0.25	1024	≤0.25	2	≤0.25
J26	>1024	8	≤0.25	32	1	2	0.5
J27	>1024	8	2	4	≤0.25	2	16
J28	>1024	>1024	32	128	512	>1024	1024
J30	>1024	8	≤0.25	128	32	8	16
J31	>1024	512	4	256	64	64	32
J32	>1024	128	256	256	64	>1024	64
J33	1024	64	0.5	128	1	2	≤0.25
J34	>1024	128	4	>1024	2	8	8
J35	>1024	>1024	>1024	4	64	8	16
J36	>1024	32	0.5	16	≤0.25	4	8
J37	>1024	>1024	8	512	8	1	1
J38	>1024	>1024	512	128	128	128	512
J39	>1024	256	32	128	16	128	256
J40	>1024	128	8	>1024	4	16	8
J41	>1024	256	1	>1024	32	16	8
J42	>1024	>1024	1024	128	128	64	512

Amp – Ampicillin, Cfl –Cephalexin, Cfb – Ceftibuten, Cfz – Ceftazidime,

Cfx – Cefotaxime, Cfp – Cefoperazone, Cft – Ceftriazone

MIC values in µg/ml.

TABLE 3A- ZONE DIAMETER INTERPRETIVE STANDARDS

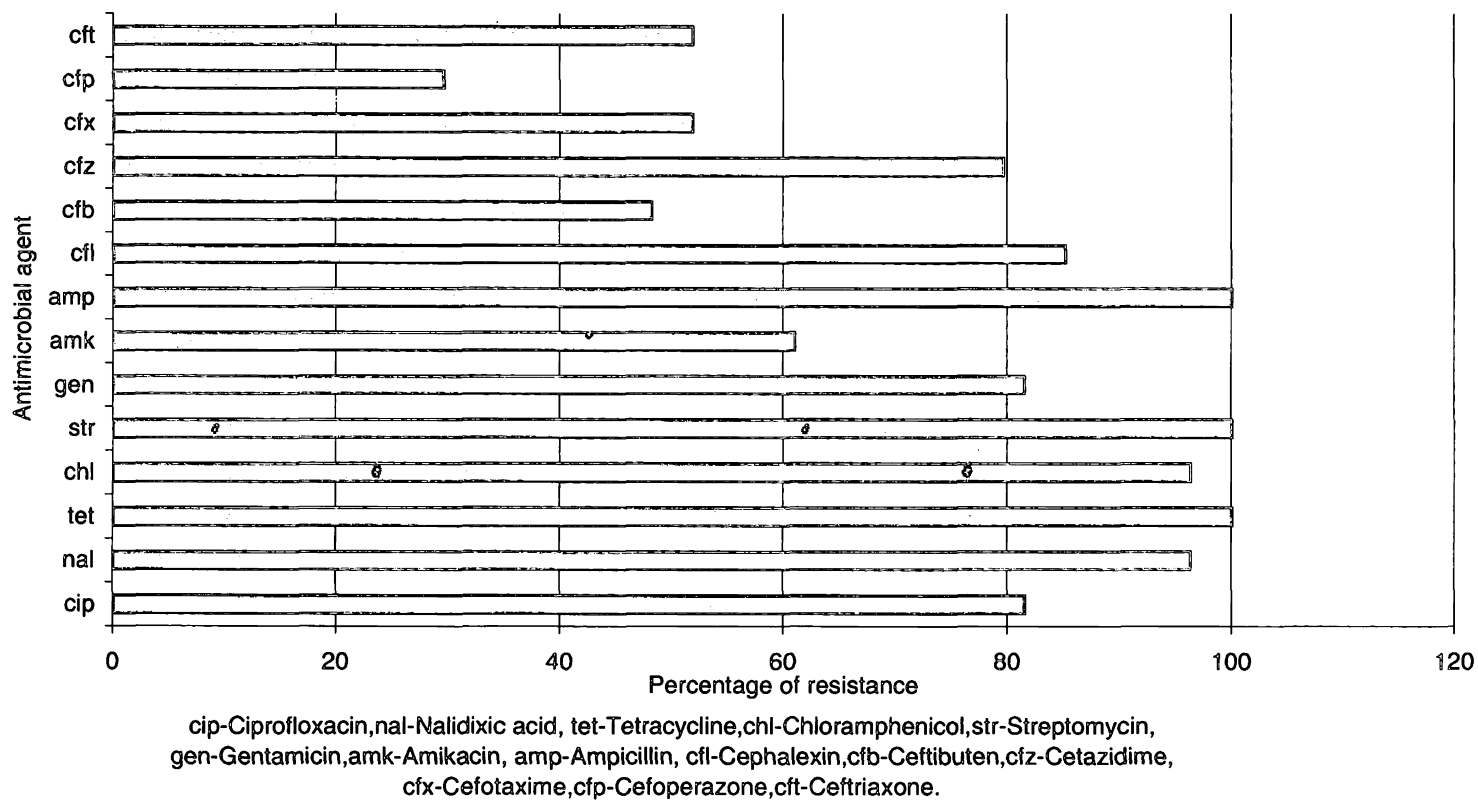
ANTIMICROBIAL AGENTS	DISC CONTENT (µg/ Disc)	ZONE DIAMETER IN mm.		
		RESISTANT	INTERMEDIATE	SUSCEPTIBLE
AMIKACIN	30	≤ 14	15-16	≥ 17
AMPICILLIN	10	≤13	14-16	≥ 17
CEPHALEXIN	30	≤14	15-17	≥18
CEFTIBUTEN	30	≤14	15-17	≥18
CEFTAZIDIME	30	≤14	15-17	≥ 18
CEFOTAXIME	30	≤ 14	15-22	≥ 23
CEFTRIAZONE	75	≤ 13	14-20	≥ 21
CEFOPERAZONE	30	≤ 15	16-20	≥ 21
CHLORAMPHENICOL	30	≤ 12	13-17	≥18
CIPROFLOXACIN	5	≤ 15	16-20	≥ 21
GENTAMICIN	10	≤ 12	13-14	≥ 15
STERPTOMYCIN	10	≤ 11	12-14	≥ 15
TETRACYCLINE	30	≤14	15-18	≥ 19
NALIDIXIC ACID	30	≤ 14	14-18	≥ 19

TABLE 3B- MIC BREAKPOINTS

ANTIMICROBIAL AGENTS	RESISTANT	SENSITIVE	SOLVENTS
AMIKACIN	≥32	≤8	D/W
AMPICILLIN	≥32	≤8	D/W
CEPHALEXIN	≥32	≤8	D/W
CEFTIBUTEN	≥32	≤8	PO ₄ Buffer pH-8
CEFTAZIDIME	≥32	≤8	D/W
CEFOTAXIME	≥64	≤8	D/W
CEFTRIAZONE	≥64	≤8	D/W
CEFOPERAZONE	≥64	≤16	D/W
CHLORAMPHENICOL	≥32	≤8	Ethanol+D/W
CIPROFLOXACIN	≥4	≤1	NaOH+D/W
GENTAMICIN	≥8	≤4	D/W
TETRACYCLINE	≥16	≤4	D/W
NALIDIXIC ACID	≥32	≤8	NaOH+D/W

D/W = Distilled Water

Percentage of clinical isolates(54) resistant to individual antibiotics



- 3) Among the β -lactams, 100% resistance was observed against the older penicillin ampicillin. In the cephalosporin group, highest resistance was observed against the 1st generation cephalosporin cephalexin (85%). The least amount of resistance was observed against the 3rd generation cephalosporin cefoperazone (29%). This could be due to the fact that cefoperazone was introduced in India as late as 1994.
- 4) High-level resistance, which was sometimes observed against broad-spectrum cephalosporins, could be due to β -lactamase activity specified by plasmids. In addition, most if not all gram-negative organisms express low levels of chromosomally encoded β -lactamase, which may contribute to intrinsic resistance. (Foster, 1983)
- 5) There was a higher incidence of resistance to the older antibiotics like ampicillin (100%), tetracycline (100%) and streptomycin (100%) than the newer antibiotics.
- 6) High level resistance (when present), was observed against streptomycin, nalidixic acid and ampicillin (mode MIC $\geq 1024\mu\text{g/ml}$), tetracycline (mode MIC $=128\mu\text{g/ml}$) and chloramphenicol (mode MIC $=512\mu\text{g/ml}$). [mode-the value most frequently obtained]
- 7) The 3rd generation cephalosporins in general proved to be the most effective group of antibiotics.

Preliminary identification of interactions by the disc approximation test

As animal and human data are difficult to obtain decisions regarding the selection of optimal combinations are based on in vitro information (Giamarellou, 1986)

There are various studies which suggest that the use of synergistic antibiotic combinations co-relate better with better patient survival (Refer to Literature review - *Clinical significance of synergy*)

Interactions of three main classes of antibiotics namely β -lactams with aminoglycosides or fluroquinolones were screened to determine their efficacy in vitro against multiply resistant organisms.

Cephalosporins are among the most frequently prescribed antibiotics as a result of their broad spectrum of microbiologic activity, favorable pharmacokinetics, low incidence of adverse reactions, and proven clinical efficacy for a wide variety of infections. (Kline, 1995)

Cephalosporins have a wide range of activity, which includes gram-negative, gram-positive, and anaerobic organisms. Clinical experience demonstrates that only 5-10% of penicillin-allergic patients have similar reactions to cephalosporins, making cephalosporins good alternative drugs (Saxon, 1983).

Cephalosporins are divided into various generations (classes) based on their side chain modifications and increased range of activity.

For this study, cephalosporins from three generations were used. 1st generation- Cephalexin, 2nd generation- Ceftibuten, 3rd generation- Cefazidime, Ceftriazone, Cefotaxime and Cefoperazone.

Hooton et al (1984) evaluated the in vitro synergistic activity of combinations of amikacin, gentamicin, tobramycin, or netilmicin with a wide range of cephalosporins, imipenem, moxalactam, mezlocillin, or piperacillin. Amikacin was significantly more active than the other aminoglycosides with a synergistic activity against 29% of the strains as compared with 22% for netilmicin, 17% for gentamicin and 13% for tobramycin. Among the β -lactams cefoperazone (39%), ceftriaxone (38%) and cefpiramide (35%) most often demonstrated inhibitory synergistic activity when combined with any aminoglycoside.

Giamarellou et al (1984) studied in vitro interactions between amikacin, netilmicin, tobramycin or gentamicin and various antipseudomonal β -lactams in 30 strains of *P.aeruginosa*. Amikacin produced more frequent and statistically significant synergy at both the total and clinical applicable levels. The better synergistic results with amikacin were attributed to the inactivation of amikacin by fewer aminoglycoside-inactivating enzymes.

Pickering and Rutherford (1981) demonstrated that among the aminoglycosides (gentamicin, tobramycin, netilmicin, and amikacin) amikacin was the least inactivated aminoglycoside when combined with β -lactams.

Hence, for this study the aminoglycoside used was Amikacin.

Eliopoulos (1989) reported that combinations of ciprofloxacin with β -lactams are infrequently synergistic against Enterobacteriaceae and gram-positive bacteria. Against *P.aeruginosa*, combinations of ciprofloxacin with β -lactams vary over a wide range.

Neu (1991) reported that combinations of fluoroquinolones with aminoglycosides, β -lactams, imidazoles, and clindamycin infrequently show synergism against Enterobacteria.

Neu (1993) also reported that anti-pseudomonal penicillin, ceftazidime, or imipenem in combination with fluoroquinolones are synergistic and may be useful for treating infections in immunocompromised patients.

Mayer (1999) reported that quinolones (primarily ciprofloxacin) in combination with β -lactams exerts enhanced activity against clinical isolates of *P.aeruginosa*.

Hence, the fluoroquinolone used in this study was Ciprofloxacin.

Thus for the primary identification of synergistic interactions the following combinations were used: Amikacin or Ciprofloxacin with Cephalexin, Ceftibuten, Cefazidime, Ceftriazone, Cefotaxime and Cefoperazone.

Nine clinical isolates, K27 (*Proteus mirabilis*), K42 (*E.coli*), J9 (*Pseudomonas aeruginosa*), J3 (*E.coli*), J39 (*E.coli*), K23 (*Proteus mirabilis*), K10 (*Poteus mirabilis*), K32 (*Klebsiella pneumoniae*), and K28 (*Klebsiella pneumoniae*), resistant to all the fourteen antibiotics against which they were tested were used to evaluate the interactions. The results were as follows:

- 1) Ciprofloxacin in combination with Ceftibuten and Cefotaxime exhibited a synergistic effect against three (J42, J39, J32) and five (K27, J42, J3, J39, J32) of the isolates tested respectively. Thus, infrequent synergism was observed for Ciprofloxacin with Cephalosporins.
- 2) Amikacin in combination with Cefotaxime, Cetriaxone and Ceftibuten exhibited synergism against five (K27, J42, J3, K23, K10), five (K27, J42, J3, K23, K10) and six (K27, J42, J3, K23, J39, K10) isolates respectively.
- 3) The combination of Amikacin with Cefoperazone exhibited synergism against eight isolates (all isolates tested except J28) including a *Pseudomonas* and was hence used to evaluate it's potential as a broad-spectrum combination.
- 4) Neither synergism nor antagonism was observed with any of the other combinations.

Checkerboard Technique

The most frequently used method to study antimicrobial interactions involves formation of a checkerboard array representing all possible combinations of two antibiotics serially diluted within a desired range (Krogstad and Moellering, 1986)

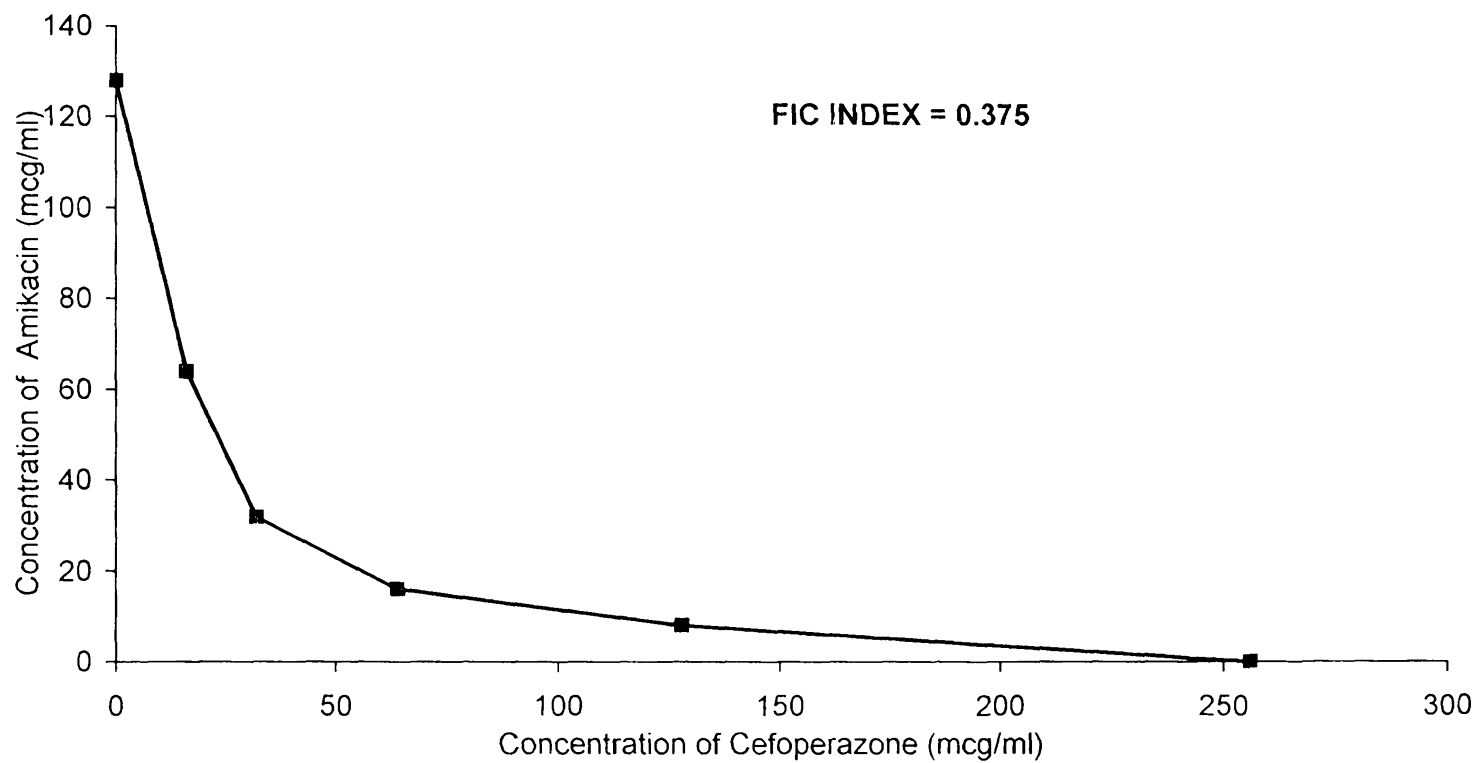
The checkerboard assay was performed for all the eight clinical isolates that demonstrated synergism by the disc-approximation method. The results were analyzed geometrically in the form of isobolograms and algebraically with the Fractional Inhibitory Concentration Indices.

The isobolograms are shown from pages 64 to 71 and the FIC indices are shown in Table 5

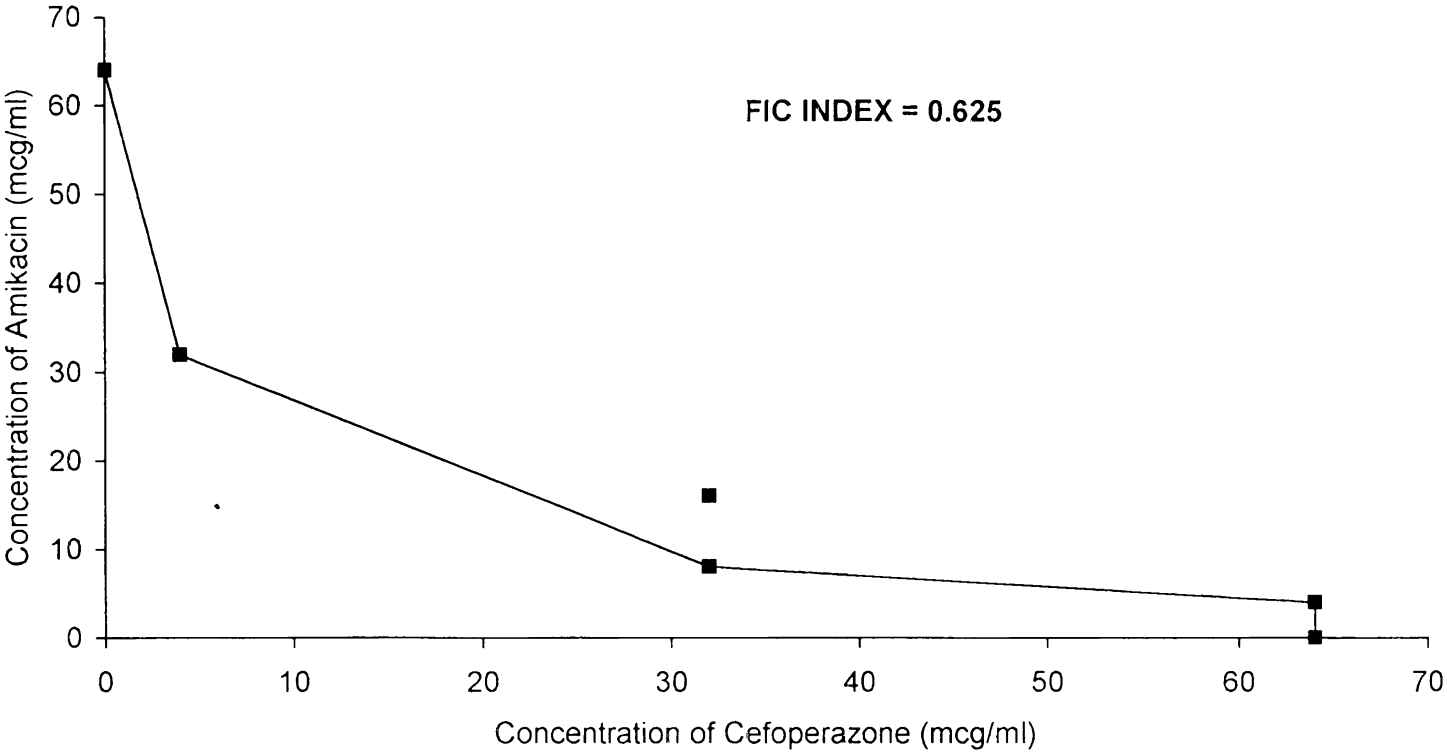
Observations of the results show that:

1. All the isobolograms showed concave curves, which indicated synergism.
2. The most frequent effective ratios for the combination of amikacin and cefoperazone were 1:4 and 1:8.
3. The FIC indices for all the tested isolates were ≤ 0.7 , which indicated synergism.
4. The FIC indices of K10 and J32 could not be determined as their MIC values were not determined over 1024 μ g/ml. However, synergism (defined as at least a four-fold reduction in the individual MIC values of either or both antibiotics) was observed in both the isolates.
5. Effective ratios of the antibiotics in combinations were of clinically achievable levels in case of seven isolates. In the case of K10, the concentration of amikacin in combination is clinically achievable, but the concentration of ceoperazone in combination is too high to be achieved clinically.

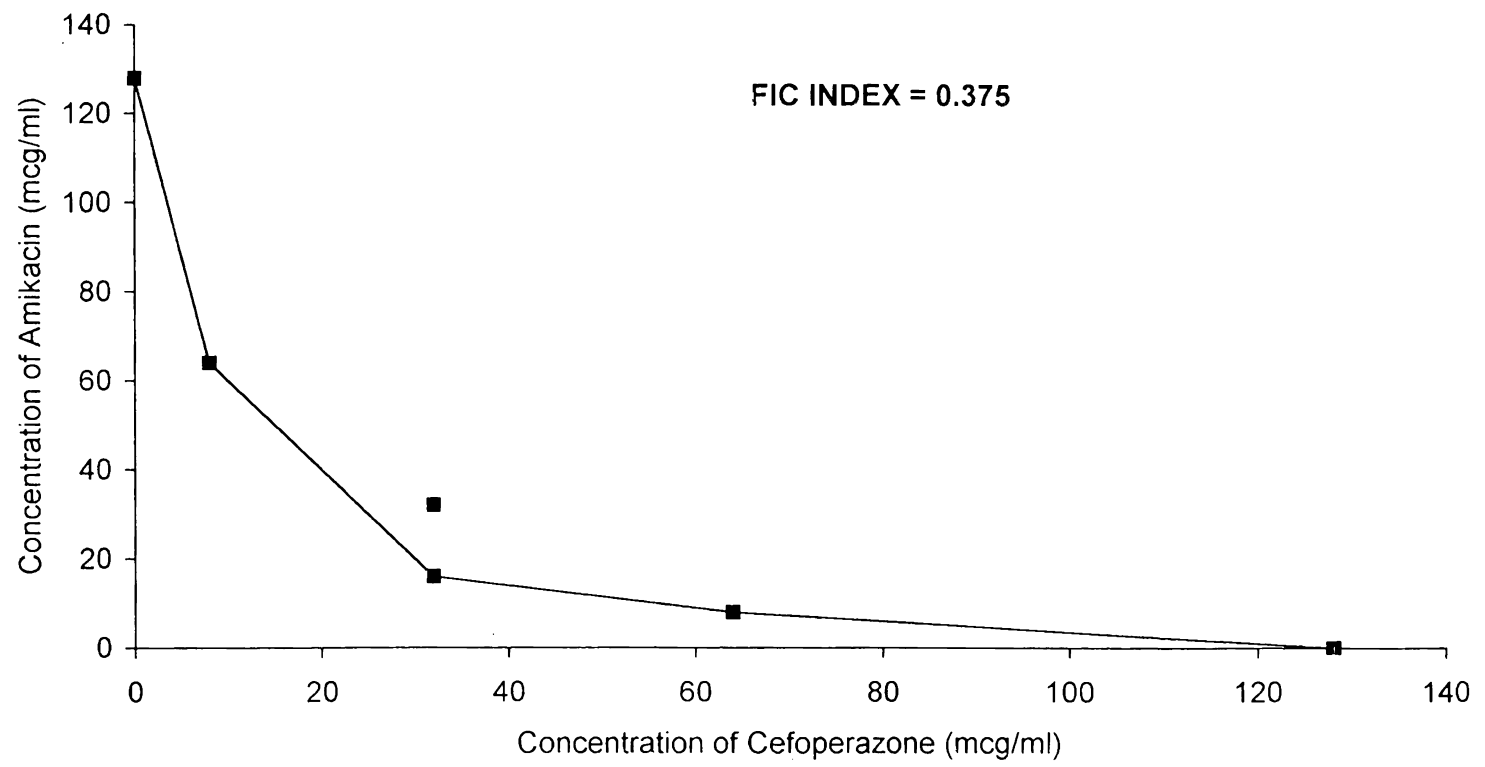
CHECKERBOARD ANALYSIS: ISOBOLOGRAM- K27



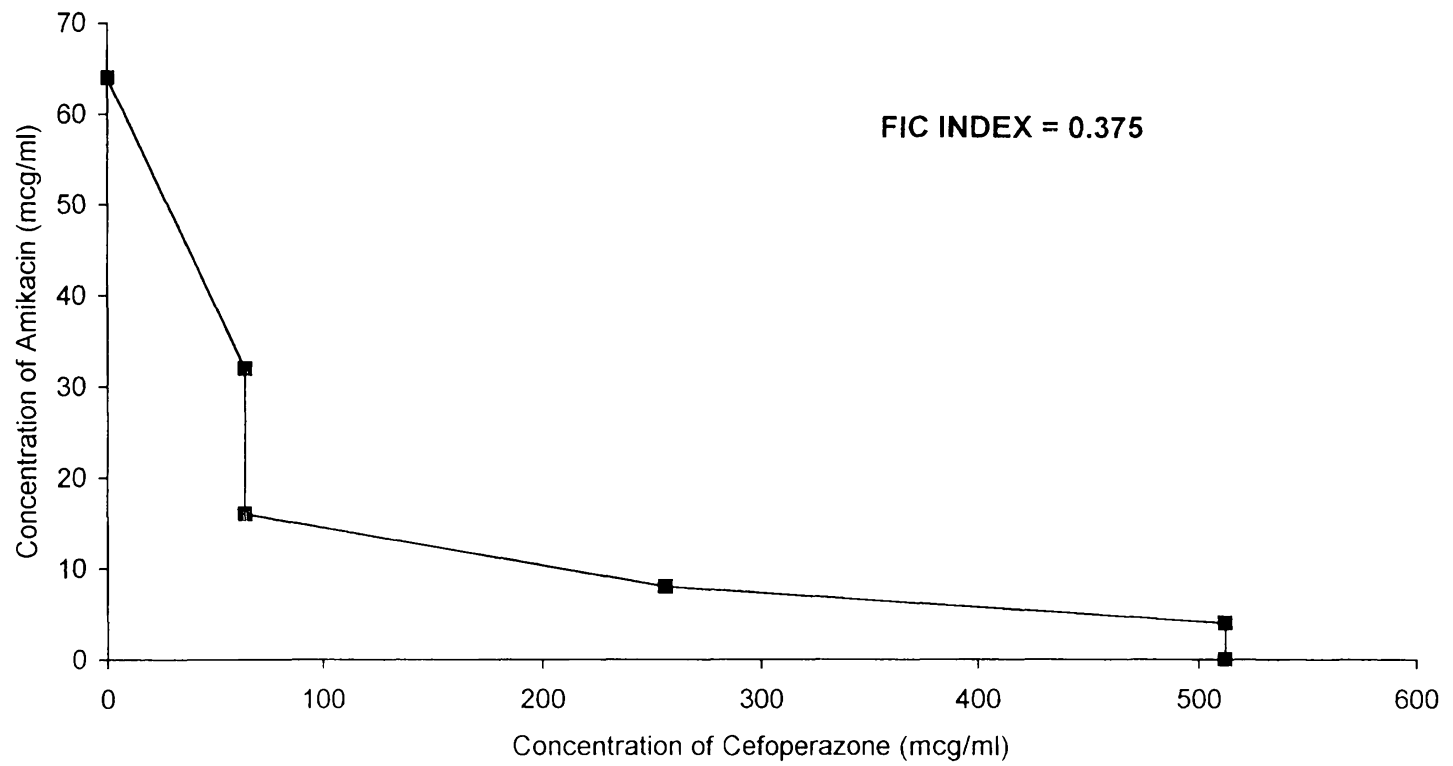
CHECKERBOARD ANALYSIS: ISOBOLOGRAM- J42



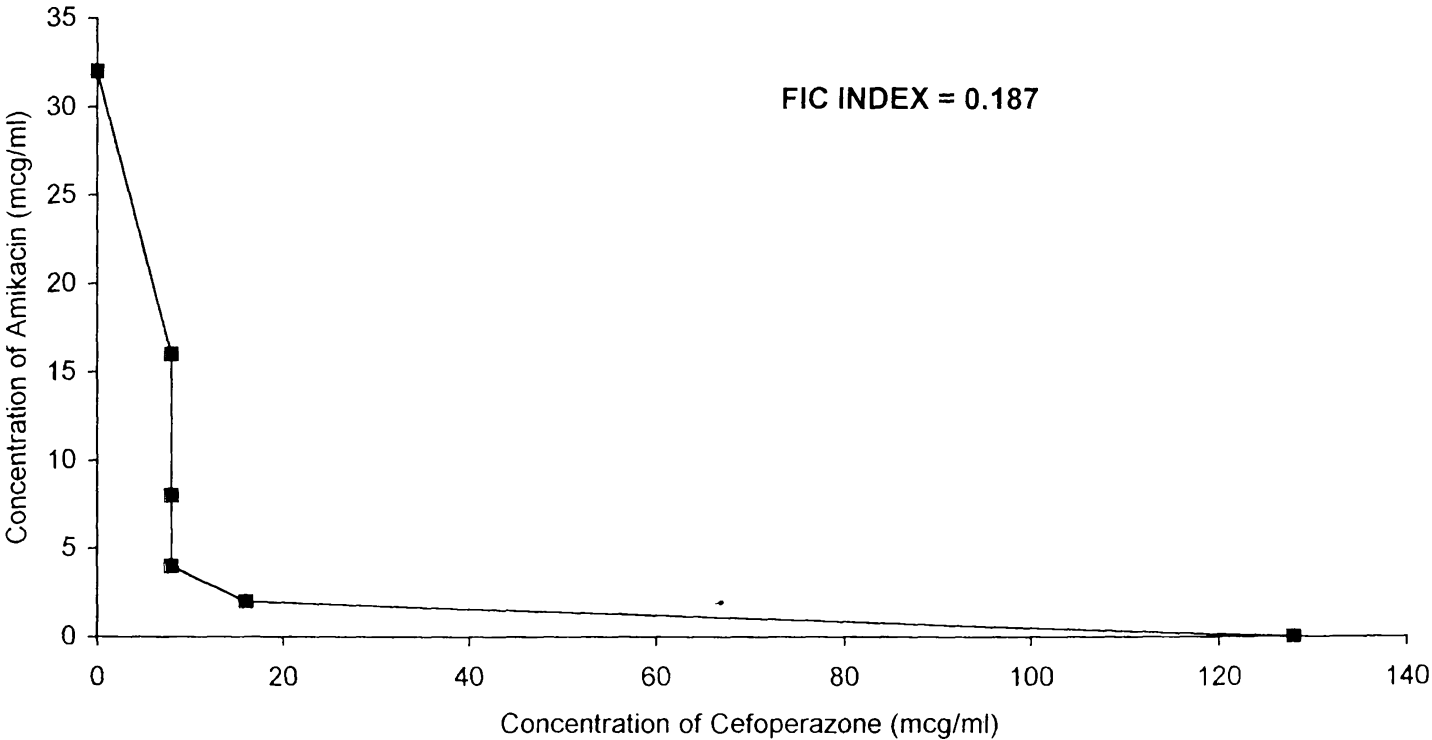
CHECKERBOARD ANALYSIS: ISOBOLOGRAM- J9



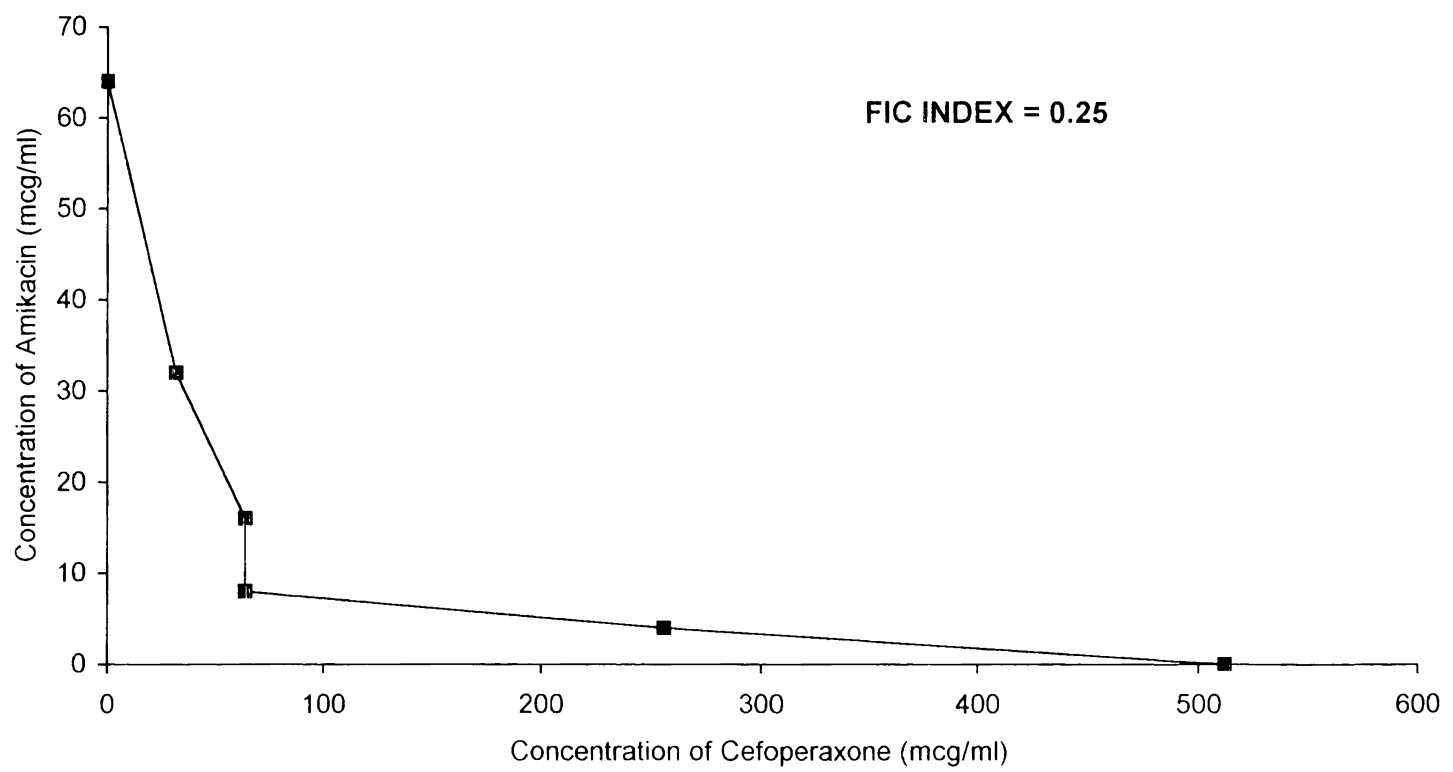
CHECKERBOARD ANALYSIS: ISOBOLOGRAM- J3



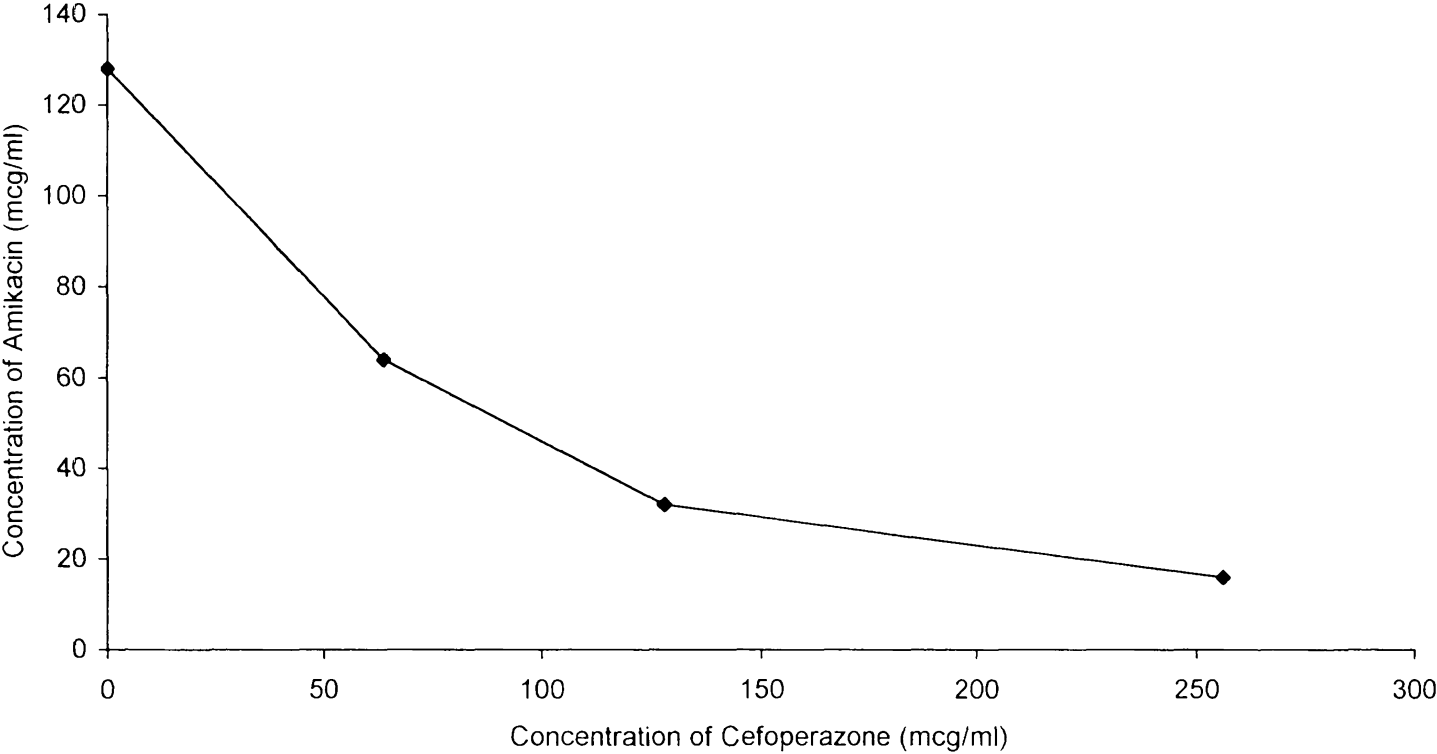
CHECKERBOARD ANALYSIS: ISOBOLOGRAM- J39



CHEKERBOARD ANALYSIS: ISOBOLOGRAM- K23



CHECKERBOARD ANALYSIS: ISOBOLOGRAM- K10



CHECKERBOARD ANALYSIS: ISOBOLOGRAM- J32

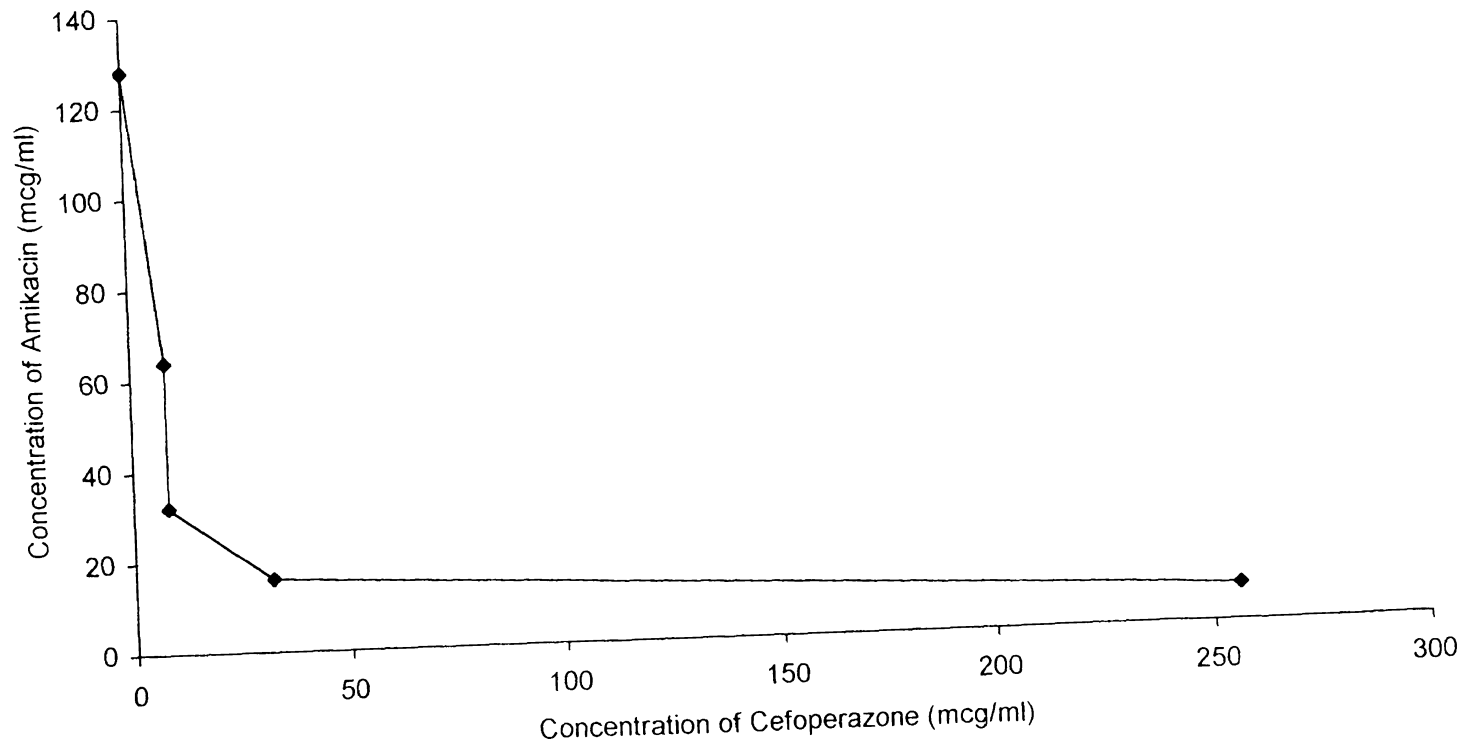


TABLE 5
DETERMINATION OF THE FIC INDICES

CLINICAL ISOLATE	MIC OF AMK	MIC OF CFP	MIC OF AMK IN COMBN	MIC OF CFP IN COMBN	FIC OF AMK	FIC OF CFP	FIC INDEX
K27	128	256	16	64	0.125	0.25	0.375
K42	64	64	8	32	0.125	0.5	0.625
J9	128	128	16	32	0.125	0.25	0.375
J3	64	512	16	64	0.25	0.125	0.375
J39	32	128	2	16	0.0625	0.125	0.1875
K23	64	512	8	64	0.125	0.125	0.25
K10	128	>1024	16	256	-	-	-
K32	128	>1024	16	64	-	-	-

AMK - Amikacin, CFP - Cefoperazone, COMBN. - Combination,
MIC values in μ g/ml.

Time kill Kinetics

The time kill technique involves repeated sampling of flasks containing the individual drugs and their combinations to determine the reduction of a fixed inoculum over 24 hours exposure. (Krogstad and Moellering, 1986)

Time kill studies were carried out for two organisms, namely J3 (*E.coli*) and J9 (*P. aeruginosa*).

The concentrations of the antibiotics, used alone or in combination for the study were as follows:

Organism	Sub-inhibitory conc.		Combinations
	Amk µg/ml	Cfp µg/ml	Amk: Cfp µg/ml
J3	32	256	-
	-	-	1:4 (16: 64 µg/ml)
	-	-	1:8 (16: 128 µg/ml)
J9	64	64	-
	-	-	1:4 (16: 32 µg/ml)
	-	-	1:2 (16: 64 µg/ml)

Amk-Amikacin, Cfp- Cefoperazone

Though, the checkerboard analyses of the drug combinations are shown in the form of isobolograms (pages 64 to 71), the checkerboard results of the isolates used in the Time kill study are presented, in Table 6 and Table 7 for convenience.

The Time kill curves are shown on pages 77 and 78.

TABLE 6

CHECKERBOARD RESULTS- J3

Amikacin $\mu\text{g/ ml}$	128	-	-	-	-	-	-	
	64	-	-	-	-	-	-	
	32	+	+	-	-	-	-	
	16	+	+	-	-	-	-	
	8	+	+	+	+	-	-	
	4	+	+	+	+	+	-	
	0	+	+	+	+	+	-	
		0	32	64	128	256	512	1024
Cefoperazone $\mu\text{g/ ml}$								

TABLE 7

CHECKERBOARD RESULTS- J9

256	-	-	-	-	-	-	-
128	-	-	-	-	-	-	-
64	-	-	-	-	-	-	-
32	+	+	-	-	-	-	-
16	+	+	-	-	-	-	-
8	+	+	+	+	-	-	-
0	+	+	+	+	+	-	-
	0	8	16	32	64	128	256

Cefoperazone $\mu\text{g/ ml}$

The Time-kill kinetics results for J3 showed that:

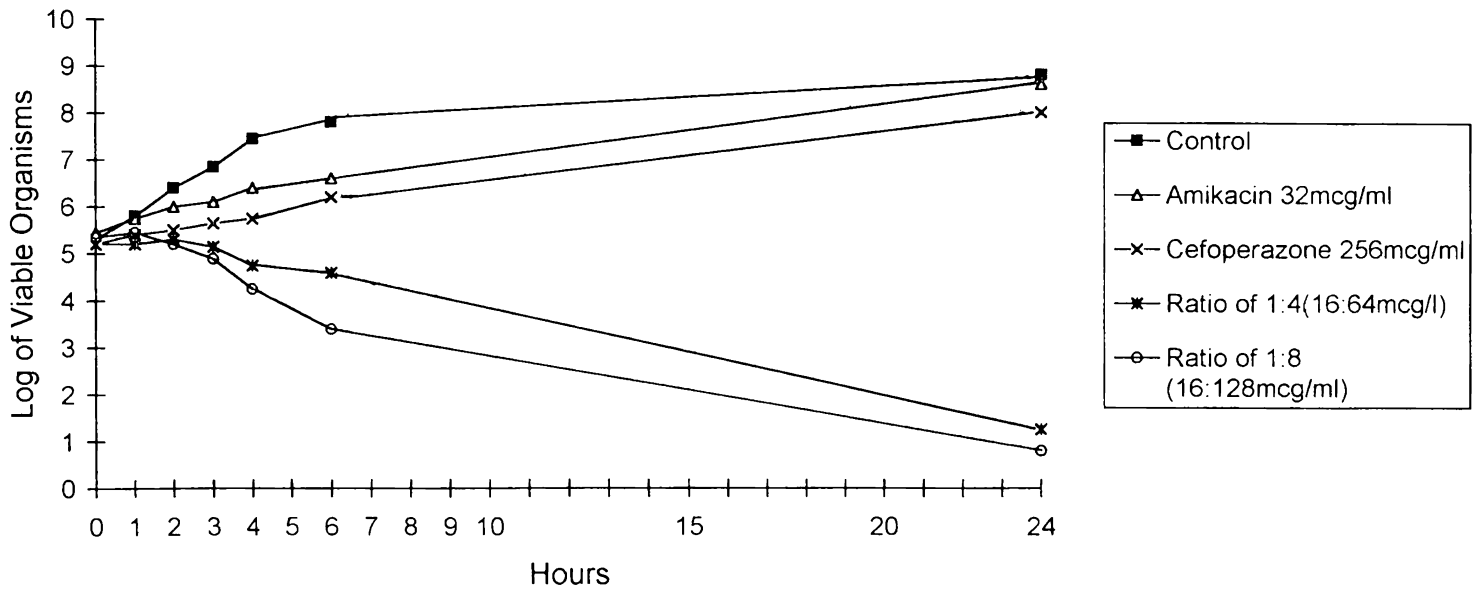
1. A short lag period of about two hours was observed before the onset of inhibition for the inhibitory concentrations.
2. Both the sub-inhibitory concentrations did not show inhibition, as the effective cidal concentrations could be much higher than the inhibitory concentrations obtained by the MIC.
3. There was at least a 6 log reduction in the viable count with both the ratios tested, which indicates synergism (defined as a reduction of ≥ 2 log cfu/ml count)
4. Clinically meaningful synergy is that which results in a reduction of MIC to a serum concentration of the antibiotic achievable with conventional doses. For example, the borderline serum levels achievable in vivo for amikacin is 32 $\mu\text{g/ml}$ and that for cefoperazone is 64 $\mu\text{g/ml}$.

Though the ratio 1: 8 demonstrated synergism and a higher rate of killing than the 1:4 ratio, it did not demonstrate clinically significant synergism. Hence, the 1:4 ratio is more relevant.

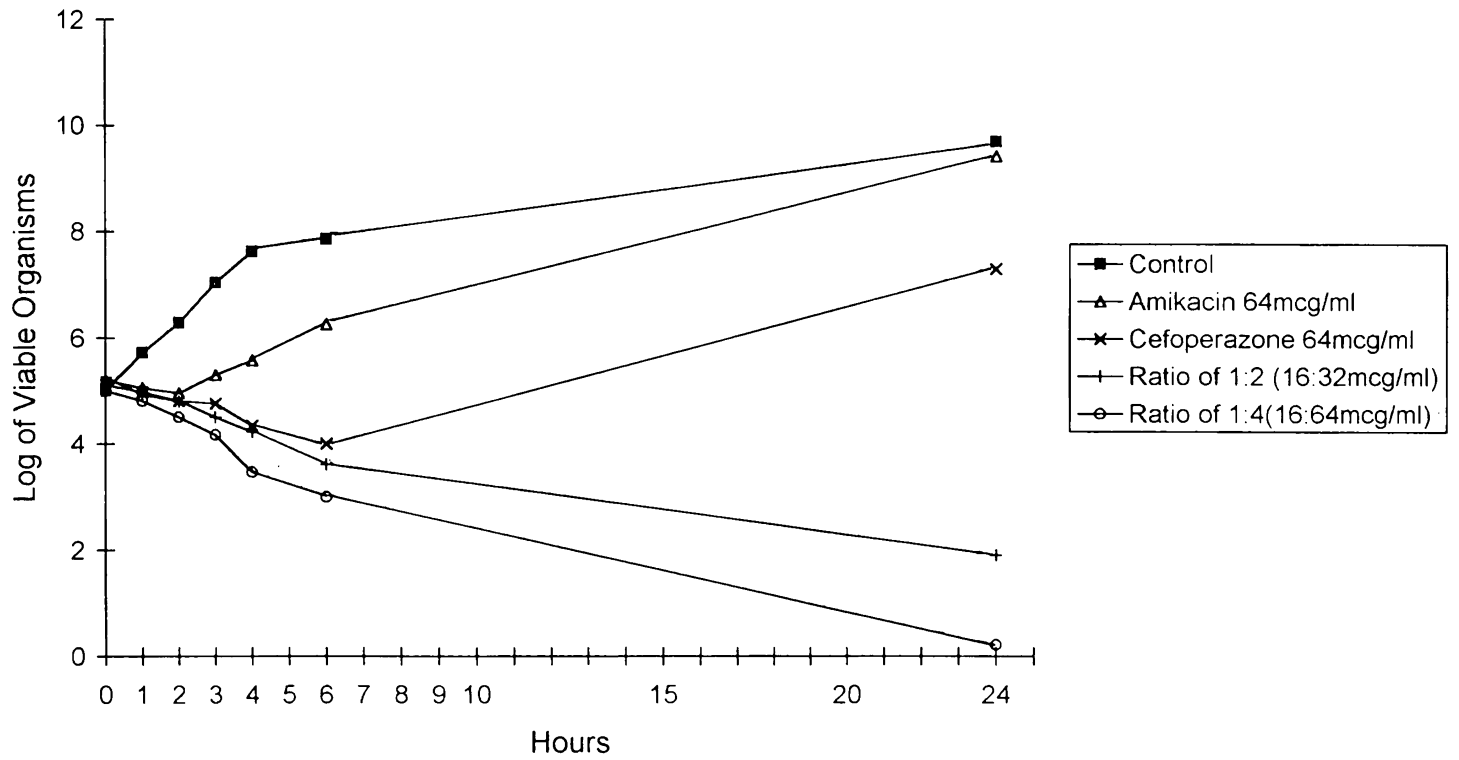
The Time-kill kinetics results for J9 showed that:

1. There was no lag period for the onset of inhibition for the inhibitory concentrations.
2. The sub-inhibitory concentration of cefoperazone could maintain bactericidal activity only for about 6 hours and sub-inhibitory concentration of amikacin could maintain bactericidal activity for about an hour.
3. There was at least a 5log reduction in the viable counts with both the combinations, which indicates synergism.

Time-Kill Curves (J3)



Time-Kill Curves (J9)



4. Both the ratios tested (1:2 and 1:4) were clinically significant, but the ratio of 1:4 demonstrated a higher rate of killing.

Thus, the ratio of 1:4 seemed generally more effective in the case of both the clinical isolates.

Advantages of the combination Cefoperazone with Amikacin

The administration of an aminoglycoside in combination with an antipseudomonal β -lactam constitutes an effective treatment regimen in patients with serious or life threatening infections due to gram-negative bacteria. Because of the seriousness of these infections in most patients, it is most important to ensure that patients are getting full benefit from the drugs that are administered. (Giamarellou, 1986) The combination of Cefoperazone with Amikacin has the following advantages:

1. Both the antibiotics are bactericidal.
2. They act on different target sites, such that action of one aids the other (action of the β -lactam on the cell-wall facilitates the uptake of the aminoglycoside)
3. Cefoperazone is active against gram-negative, gram-positive, and anaerobic organisms whereas amikacin is active against gram-negative and gram-positive organisms. Both these antibiotics have enhanced anti-pseudomonal activity.

Summary

Despite the introduction into clinical practice of highly potent antibiotics such as third generation cephalosporins, fluoroquinolones and aminoglycosides, infections due to multiresistant isolates pose a major therapeutic problem worldwide. In addition, gram-negative organisms are generally more resistant to antimicrobial drugs than gram-positive, due to the protection offered by their outer membrane. The use of antimicrobial combination therapy in patients takes the advantage of synergistic antibiotic interactions to enhance the efficacy of the treatment and to decrease the development of resistance during therapy.

For the past 10 years, Haffkine Institute has monitored the drug resistant profiles of gram-negative organisms through various studies. The isolates used in this study were collected between 1997 and 1998 from two local hospitals in Bombay (Kasturba Hospital for Infectious Diseases and The J.J. Group of Hospitals).

- The clinical isolates were identified, and their drug resistance profiles and levels of resistance were determined by the 'Disc diffusion method' and the 'Macro Broth Dilution Method'. The antibiotic profile showed that all the clinical isolates (54) were resistant to Tetracycline, Streptomycin, and Ampicillin. Amongst the aminoglycosides, least resistance was shown to Amikacin. Amongst the cephalosporin family, highest resistance was shown against the first generation cephalosporin, Cephalexin, while least resistance was shown to the third generation cephalosporin, Cefoperazone, not only in this group but also amongst the other antibiotics tested.

- The present study investigated the synergistic effect of the aminoglycoside-Amikacin and fluoroquinolone-Ciprofloxacin in combination with various cephalosporins (Cephalexin, Ceftibuten, Ceftazidime, Cefotaxime, Ceftriazone and Cefoperazone).
- The preliminary screening of the effects of these drugs was done by the 'Double-disc diffusion technique'. The combination of Cefoperazone with Amikacin proved to be most effective compared to the other combinations studied and was further evaluated by the 'Checkerboard assay' and 'Time kill kinetics'.
- The Checkerboard assay revealed that cefoperazone with amikacin exhibited synergism against all the isolates. Concave isobolograms were obtained for all the isolates, indicating synergy. The Fractional Inhibitory Concentration Indices also indicated synergy (FIC index ≤ 0.7).
- The Time Kill method demonstrated bactericidal synergy after 24 hours. The most effective ratio of the combination was 1:4.
- Clinically meaningful synergy was obtained for all the isolates with both the methods. Antagonism was not found with this combination.

Appendix

1. Nutrient agar (NA)

Peptone	10.0 g
Sodium chloride	5.0 g
Meat extract	3.0 g
Distilled water	1000.0 ml
pH	7.4
Agar	20 g

The medium was sterilized by autoclaving at 121°C / 15 psi /15 mins.

2. Mueller-Hinton Broth and Agar (MH)

Casein acid hydrolysate	29.0 g
Beef extract	10.0 g
Potato starch	2.5 g
Distilled water	1000.0 ml
pH	7.4

2% agar was added to obtain a solid medium. The medium was sterilized by autoclaving at 121° C / 15 psi/ 15 mins.

Preparation of antibiotic discs

Discs of about 5 mm diameter were punched with a leather punch from Whatmann's filter paper they were separated and spread out singly on a sterile petridishes, so that each disc is more than 2 mm away from the other discs. The discs were then sterilized in the oven at 160° C for 1 hour. On cooling, the antibiotic solution of required concentration was dropped on the discs with standard pipettes. The discs were dried in the incubator with the lids of the petridishes open. The dried discs were transferred aseptically to sterile glass bottles and stored under refrigeration.

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THE DEGREE OF MASTER OF SCIENCE
IN
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Pseudomonas aeruginosa.
2. NAME OF THE CANDIDATE: ARATI V. PATANKAR
3. NAME OF THE GUIDE: Dr. S. V. GADRE
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INTRODUCTION

Since their discovery, antimicrobial drugs have proved remarkably effective for the control of bacterial infections. However, it was soon evident that bacterial pathogens were unlikely to surrender unconditionally, because some pathogens rapidly became resistant to many of the first effective drugs. Initially, the problem of bacterial resistance to antimicrobial drugs was solved by the discovery of new classes of drugs and by the chemical modification of previously existing drugs. Unfortunately, there is no assurance that the development of new antimicrobial drugs can keep pace with the ability of bacterial pathogens to develop resistance. As more is learnt about the mechanisms and epidemiology of resistance to antimicrobial drugs, it has become clear that bacteria have a remarkable array of tools at their disposal to overcome antibiotics. A single genetic mutation may lead to resistance without altering the pathogenicity or viability of a bacterial strain. (Gold, 1996)

One of the solutions to the problem of antimicrobial resistance is the appropriate use of antimicrobial combinations. A combination of two antimicrobial drugs may produce the following responses:

Synergism where at least a four-fold reduction in the effective concentration of either or both drugs indicates synergy.

Additive effect where a two fold reduction in the effective concentration of either or both drugs indicates addition.

Indifference where no change in the effective concentration of the combination indicates indifference.

Antagonism where a four- fold increase in the concentration of either or both antibiotics indicates antagonism.

Synergistic combinations are often sought as empiric treatment to provide a broad coverage and to prevent the emergence of resistant organism. In clinical practice, the significance of synergism is primarily limited to beta-lactam containing combinations. These drugs can be synergistically affected by a variety of other compounds but their most important synergistic interaction results from their combined action with aminoglycosides. (Klastersky, 1986)

Cefoperazone belongs to the extended spectrum 3rd generation cephalosporin antibiotic. It is a bactericidal drug which interferes with cell wall biosynthesis by binding to proteins-penicillin binding proteins-which by transpeptidation produces cross linkage of peptidoglycan chains that forms the bacterial cell wall. It is an anti-pseudomonal cephalosporin. Pathogenic bacteria susceptible to the action of cefoperazone include a wide range of species comprising –gram positive, gram negative and anaerobic organisms. (Shah 1995)

Amikacin is an aminoglycoside. It is a bactericidal drug which interferes with protein synthesis by binding irreversibly to the ribosomes. It is less susceptible to enzyme inactivation and hence active against gentamicin and other aminoglycoside resistant organisms. Bacteria susceptible to amikacin include gram positive and gram negative but not anaerobic bacteria. (O' Grady, 1993)

AIMS AND OBJECTIVES

1. To study the antibiotic resistance profiles of clinical isolates collected during 1997-98.
2. To identify antibiotic combinations that are effective against these clinical isolates.
3. To study the interactions between the combined antimicrobials by 'checkerboard method' and 'time kill- kinetics'.

MATERIALS AND METHODS

Identification of the clinical isolates:

80 clinical isolates were collected from Kasturba Hospital for Infections Diseases, Mumbai and from Sir JJ Group of Hospitals, Mumbai, during the period of 1997-98. These isolates were subjected to identification based on their morphological, cultural & biochemical characteristics as per standard methods. (Koneman, 1992).

54 of these clinical isolates were used for the present study.

Determination of multi-drug resistance profiles

a) Disc diffusion test

The multi –drug resistance profiles of the clinical isolates were determined by carrying out the Kirby-Bauer disk diffusion test. (Bauer, 1966)

The test culture in the log phase was seeded on the Mueller-Hinton (MH) agar plates with sterile cotton swabs. Antibiotic discs of appropriate concentrations were impregnated on the agar. After incubation for 24 hrs. at 37°C the zones of inhibition were measured in (mm) and interpreted as sensitive, intermediate or resistant on the basis of the NCCLS chart.(NCCLS,1988)

E.coli ATTC 10148 served as a sensitive control.

b) Minimum inhibitory concentration

The minimum inhibitory concentrations (MIC) of the clinical isolates were determined by The Broth Dilution method. Serial two fold dilutions were made in sterile MH broth from appropriate antibiotic stock solutions .0.1ml of the test culture in the log phase was added to get the final density between 10^5 – 10^6 cfu/ml.

The tubes were incubated at 37°C for 24 hrs. The MIC was determined by observing the tubes for growth of the test organism. E. coli ATTC 10148 served as a sensitive control. (Cruikshank & Stokes, 1993)

Tests for investigating the inhibitory interactions between combined antimicrobials

a) Disk approximation test

Sterile MH agar plates were seeded with the log phase test culture with sterile cotton swabs. To test the possible interactions between two antimicrobials, two discs (one for each antimicrobial) were placed on the surface of the seeded agar with their centers separated by a distance (measured in mm) that is equal to or slightly greater than half the sum of their individual zones of inhibition when obtained separately.

After incubation for 24 hrs at 37°C, an extended inhibition zone indicated synergy and a flattened or reduced inhibition zone indicated antagonism. (Stokes, 1993, Krogstad, 1986)

b) Checkerboard technique

The checkerboard technique is used to evaluate the combination of two antimicrobials, potentially exhibiting synergism. It is a modification of the broth dilution technique for the determination of MIC. In this technique the test isolate is exposed to a series of concentrations of two antimicrobials in broth, alone and in all the possible combinations with each other, using an 8x8 square template. After incubation for 24hrs at 37°C, the tubes were examined for growth. (Stokes, 1993) Each MIC end point for the antimicrobial in combination is plotted in the form of a curve & further analyzed by determining the Fractional Inhibitory Concentration (FIC) for each individual as follows (Elion, 1954)

$$\text{FIC index} = \text{FIC of drug A} + \text{FIC of drug B}$$

$$\text{FIC of drug A} = \frac{\text{MIC of drug A in combination with B}}{\text{MIC of drug A}}$$

Interpretation:

Synergy = mean FIC < 0.5

Partial synergy/ addition = mean FIC 0.5-0.7

Indifference = mean FIC 0.76-2.75

Antagonism = mean FIC > 2.75

Time-kill kinetics

A log phase culture of the test organism was diluted to 10^5 – 10^6 cfu/ml and added to various flasks containing sterile MH broth, with or without sub inhibitory concentrations of the individual drugs & their combinations. The inoculated flasks were incubated for 24 hrs at 37°C under shaker conditions & samples drawn out at hourly intervals. Viable counts were obtained by surface spreading 0.1ml of the drawn sample on sterile MH agar plates after appropriate dilutions. Logs of the viable counts were plotted against time.

. Synergism is observed when the (cfu)/ml count for 24 hours for the antibiotic combination is greater than or equal to a 2 log cfu/ml reduction more than either agent alone.

(Krogstad, 1986)

RESULTS

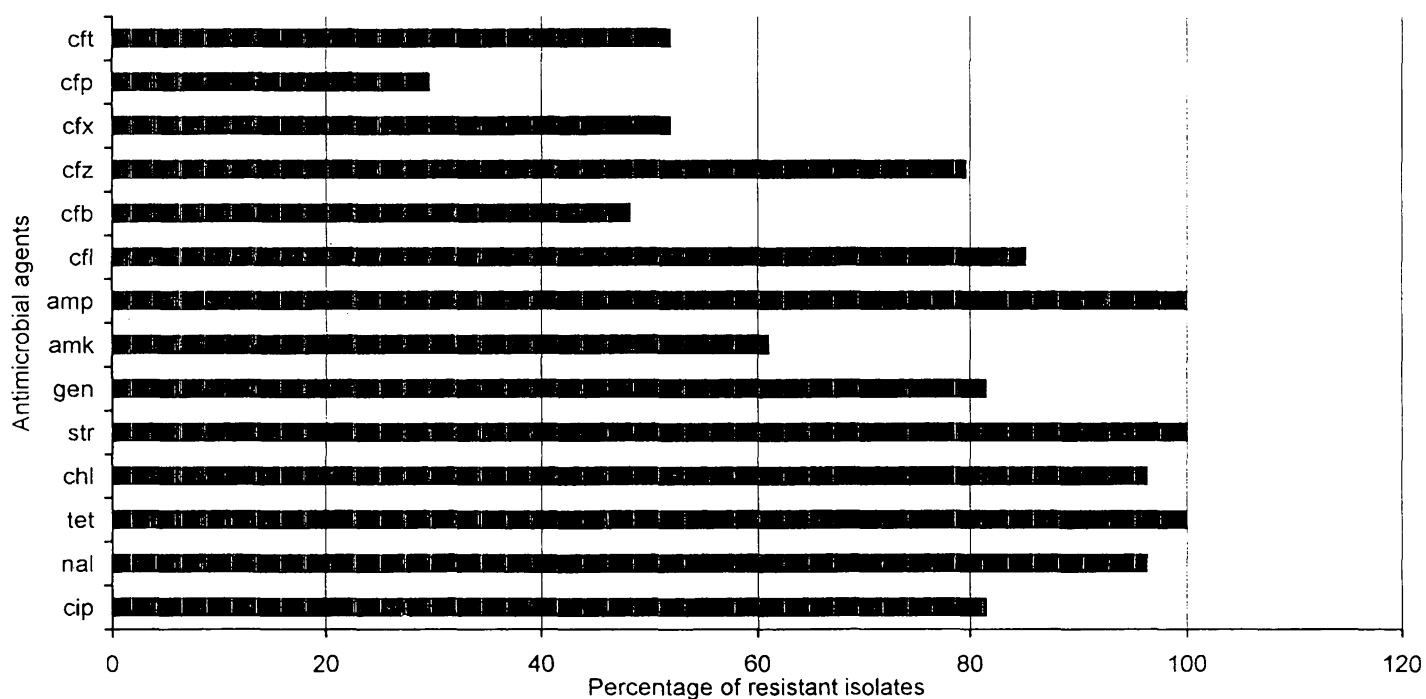
1. The clinical isolates belonged to the following genera: *Escherichia* (17), *Klebsiella* (11), *Proteus* (6), *Salmonella* (7), *Shigella* (1), *Providencia* (1), *Citrobacter* (1) and *Pseudomonas* (10).
2. The antibiotic profile showed that all the clinical isolates (54) were resistant to Tetracycline, Streptomycin, and Ampicillin. Amongst the aminoglycosides, least resistance was shown to Amikacin. Amongst the cephalosporin family, highest resistance was shown against the first generation cephalosporin, Cephalexin, while least resistance was shown to the third generation cephalosporin, Cefoperazone, not only in this group but also amongst the other antibiotics tested.
3. For effective treatment of infections due to multi-resistant isolates, clinicians often resort to the most potent fluoroquinolones or their combinations with beta-lactams, where synergy has been demonstrated. The other most widely documented synergy is to be found when beta-lactams are combined with aminoglycosides. (Mayer, 1999)

Hence for the identification of antibiotic interactions the following combinations were used: Amikacin/Ciprofloxacin with Cephalexin, Ceftibuten, Cefotaxime, Ceftazidime, Ceftriaxone and Cefoperazone. These combinations were tested against those isolates, which were resistant to all the antibiotics, used.

4. Ciprofloxacin in combination with Ceftibuten and Cefotaxime exhibited a synergistic effect against 3 and 5 of the isolates tested respectively. Amikacin in combination with Cefotaxime, Ceftriaxone, and Ceftibuten showed synergism against 5, 5 and 6 isolates respectively. The combination of Amikacin with cefoperazone showed synergy with 8 isolates including a *Pseudomonas* and was hence used for further studies.
5. The checkerboard assay was performed for all the isolates showing synergy for the combination of Amikacin with Cefoperazone, according to which a ratio of 1:4 or 1:8 was most frequently effective against all the samples. The FIC indices of all the samples were below 1.0 indicating synergy.
6. Time kill studies were carried out for two organisms, namely J3 (*E.coli*) and J9 (*P. aeruginosa*). The antibiotics Amikacin and Cefoperazone were used individually at half their respective MIC's for their sub inhibitory doses. The sub inhibitory concentrations of the individual drugs could not exhibit continued bactericidal activity.
Combination ratios of 1:4 and 1:8 were used against J3 and ratios of 1:2 and 1:4 were used against J9. All ratios of the antibiotics in combination were synergistic but the ratio of 1:4 gave a higher rate of killing, after 24 hours.

All the above results will be presented and discussed, in detail, in the thesis.

Percentage of resistant clinical isolates(54)



cip-Ciprofloxacin,nal-Nalidixic acid, tet-Tetracycline,chl-Chloramphenicol,str-Streptomycin,
gen-Gentamicin,amk-Amikacin, ampAmpicillin, cfl-Cephalexin,cfb-Ceftibuten,cfz-Cetazidime,
cfx-Cefotaxime,cfp-Cefoperazone,cft-Ceftriaxone.

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