

STUDY OF EFFECT OF DIVERSE IN-VIVO FACTORS
ON RESISTANCE OF BACTERIA TO ANTIBIOTICS
EMPLOYING IN-VITRO TECHNIQUES

THESIS SUBMITTED
UNIVERSITY OF BOMBAY

FOR THE DEGREE OF
MASTER OF PHILOSOPHY
IN
MICROBIOLOGY

SUBMITTED BY
S.N. JOGDAND

UNDER THE GUIDANCE
DR. R. S. KAMAT

HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
BOMBAY

365

1989

Disclaimer

This research work belongs to

Mr. S. N. Jogand

No content of this work can be
circulated or used for any commercial
purpose without the consent of
respective researcher



STUDY OF EFFECTS OF DIVERSE IN-VIVO FACTORS ON THE
SUSCEPTIBILITY OF BACTERIA TO ANTIBIOTICS EMPLOYING
IN-VITRO TECHNIQUES

A DISSERTATION SUBMITTED TO
THE UNIVERSITY OF BOMBAY

In Partial Fulfilment of the Requirements for the Degree of

MASTER OF PHILOSOPHY

In

MICROBIOLOGY

By

S.N. JOGDAND

577.18
JOG

HAFFKINE INSTITUTE
BOMBAY

(1989)

HAFFKINE INSTITUTE LIBRARY
BOMBAY.

Call No. 577.18
Price

Acc No.
Source Purchased.
Donated

365

ACKNOWLEDGEMENT

I express my thanks and gratitude to **Dr. R.S.Kamat**, M.D., Asst. Director, Haffkine Institute, Head Depts. of Immunology and Clinical Pathology, Parel, Bombay - 400 012, for his valuable guidance and for the time he spared from his busy schedule to discuss and plan the course of my Dissertation.

I also thank **Dr. S.R. Sengupta** M.D., Director, Haffkine Institute for the facilities provided for this work.

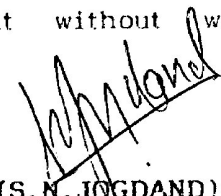
I am extremely grateful to **Dr. S.V. Gadre** Head, Dept. of Bacteriology and **Dr. R.P. Bhamaria** for providing standard cultures required by me during this work.

I am also obliged to **Dr. S.V. Athlekar** of Clinical Pathology, Haffkine Institute and **Dr. Mrs. S.S. Shende** of Railway Hospital, Byculla, Bombay for providing the multiresistant pathogens.

I also thank **Prof. (Mrs.) A.N. Joshi**, Head, Dept. of Microbiology of Modern College, Vashi, New Bombay for cooperation and encouragement.

I am deeply grateful to **Dr. V.D. Patil**, Principal, Modern College, Vashi, New Bombay for giving permission and facilities in this college laboratory to carry on this Dissertation work.

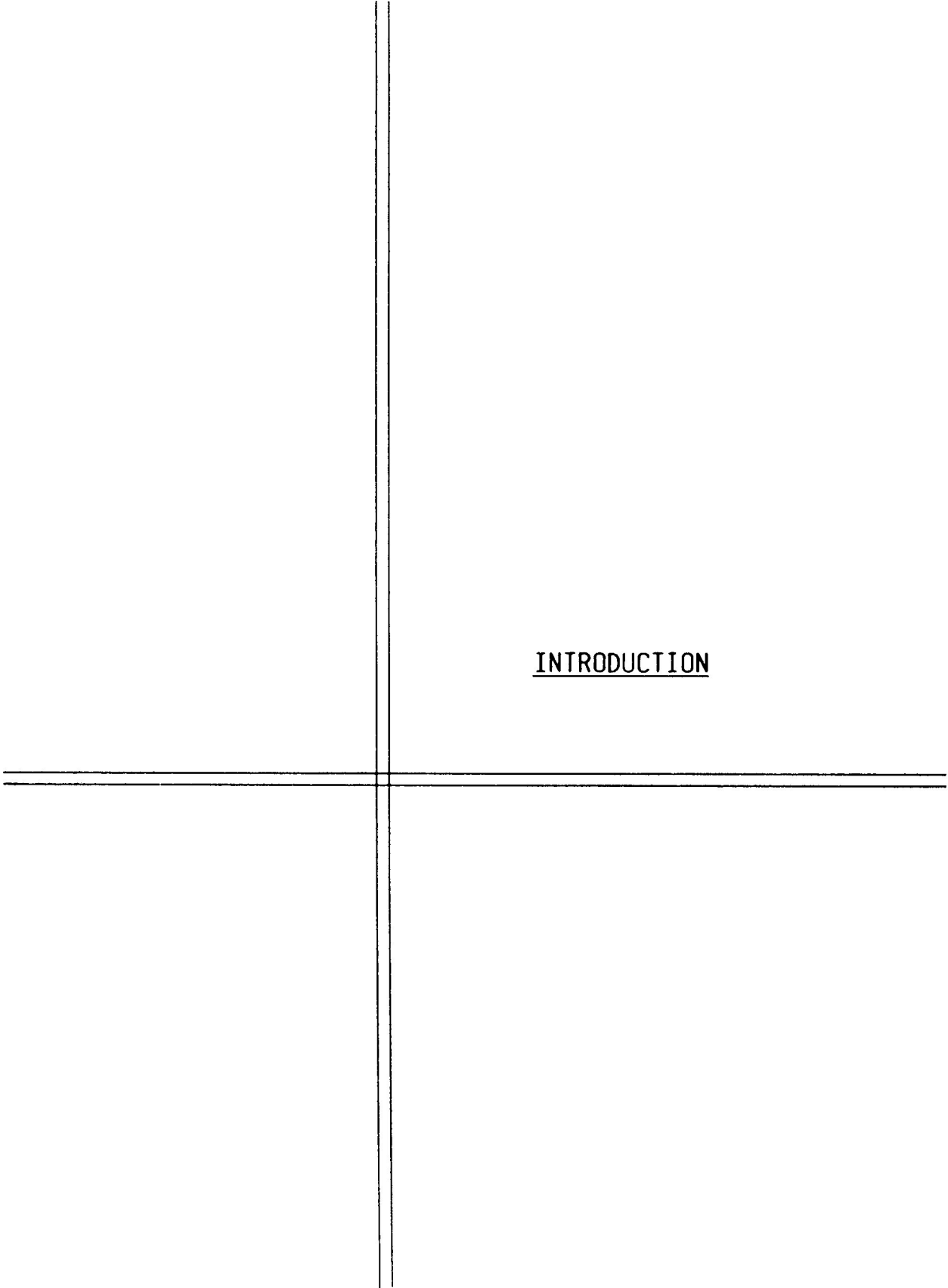
Lastly I am deeply indebted to all the members of my family for their patience and constant encouragement without which this Dissertation would never have reached completion.


(S.N. JOGDAND)

CONTENTS

		<u>Page No.</u>
I	INTRODUCTION	1
II	REVIEW OF LITERATURE	4
III	MATERIALS AND METHODS	25
IV	RESULTS	35
V	DISCUSSION	80
VI	SUMMARY AND CONCLUSION	88
VII	APPENDIX	90
VIII	BIBLIOGRAPHY	91





INTRODUCTION



INTRODUCTION

Drug resistance is the state of decreased sensitivity of micro-organisms to drugs that ordinarily cause growth inhibition or organismal death.

In 1907, first detailed account, of drug resistance was given by Paul Ehrlich (1). He observed bacterial resistance to sulfonamides and other antibiotics of those times. Since then it is a widespread phenomenon and everpresent threat to chemotherapy.

Earlier there was controversy over whether drug resistance is phenotypic adaptation of cells to inhibitory agent or genotypic selection.

Drug resistant strains can be at a disadvantage in drug free environment, since existence of non-essential mechanisms involved in drug resistance may depress their growth potential. Therefore if selective pressure applied by inhibitory agent is removed, microbial population often reverts to drug sensitive genotype (1). The genotypic alterations giving rise to drug resistant variants are spontaneous mutation, transformation, transduction and conjugation. Conjugation is the most threatening means of acquired resistance (1).

Acquired drug resistance is an important limitation to the use of antibiotics and many other antimicrobial and anticancerous agents.

In-vitro tests are routinely carried out to determine sensitivity of pathogens towards antibiotics. But these in-vitro tests do not take into account the following factors :

- 1) 'Penetration of the drug' effect, as is experienced in erradi-

cation of hepatobiliary infections.

2) 'Concentration of the drug' effect, as is observed in treatment of urinary tract infections.

3) Antagonistic actions of various ions as is experienced by effect of calcium and magnesium ions in urine on action of various antibiotics.

4) Supportive role of some body fluids, ions and enzymes which is important in gastrointestinal tract because of actions of bile acids, proteolytic enzymes etc.

5) Acidic or alkaline environment in-vivo, as is experienced in efficacy of gentamycin in urinary infections.

6) Aerobic or anaerobic environment in-vivo e.g. treatment of intra-abdominal abscess is difficult, due to anaerobic conditions although causative organism is otherwise susceptible.

These factors are responsible for the limitations of conventional in-vitro susceptibility testing methods. Hence results of in-vitro susceptibility tests cannot be extrapolated directly to the efficacy of drug in-vivo.

Thus one often encounters discrepancies between in-vitro results and outcome of the treatment. The discrepancy observed can be of two types, namely -

1) Organism appears resistant by in-vitro test but therapy of that particular antibiotic is successful.

2) Organism shows susceptibility by in-vitro test but exhibits resistance to the treatment with that particular drug in-vivo.

Some of the host factors that modify the susceptibility of the organism can be considered and duplicated during in-vitro testing. In such tests chances of correlation between in-vitro test results and clinical outcome of therapy are expected to be very high.

Hence it is logical to examine susceptibility patterns of organisms using conventional in-vitro susceptibility tests and modified in-vitro tests employing conditions designed to mimic the host factors.

In the work presented here we have studied factors responsible for the discrepancy between in-vitro susceptibility testing and in-vivo efficacy of the drug. Such a study can be useful in designing more reliable in-vitro tests which can guide us in appropriate drug therapy during infection.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

With emergence of organisms that are resistant to every existing and new drugs coming in use, drug resistance has become a serious problem in chemotherapy. Surveys of emergence and survival of resistant population following the use of antibiotics have made it clear that selection always plays a major part in the process. Resistant strains always survive in a given niche at the expense of sensitive companions of same or other species. But equally, it seems certain that the transfer of resistant genes from the resistant to the sensitive organisms of the same or different species is involved in widening the range of organisms showing drug resistance (1).

Thus there is a selection pressure, of emergence of drug resistance over on successful therapy during infection. It would be useful to have reliable means for developing new strategies for circumventing the problem. Amongst the many practical approaches being suggested to tackle the problem of resistance, one that is most important is the avoidance of indiscriminate use of antibiotics. Selection of appropriate antibiotic is also very crucial for successful treatment especially in severe infections.

Selection of appropriate antibiotic for treatment of infection is normally done by in-vitro susceptibility testing of the isolated causative organisms. And reliable in-vitro testing method is necessary to guide in selection of appropriate antibiotic for treatment.

From clinical point of view a microorganisms may be considered "susceptible" to a particular antimicrobial agents if in-vitro studies

suggest that a patient infected by that organism is likely to respond favourably to the drug when it is given in dosages appropriate to the microorganism and type of infection (2). Term "resistant" implies that infection is not likely to respond to such a therapy (i.e. the minimum inhibitory concentration (MIC) exceeds the concentration of the drug that can be expected at the site of infection).

GENETIC MECHANISMS OF DRUG-RESISTANCE - SPONTANEOUS MUTATION

Gene mutations are said to be spontaneous when they occur unprovoked. Spontaneous mutations occur with low frequency i.e. one gene mutation per 10^5 to 10^7 cells per cell division (1). It can be easily and convincingly demonstrated by replica plate technique that, spontaneous mutation to drug resistance regularly occurs in sensitive population in absence of the drug against which resistance develops (1). In the mutational development of the usual chromosomal type of the drug resistance two distinct patterns are seen (3). :

1) The "multistep type" in which small increase in resistance occurs in step wise manner. Successive mutations occur at multiple loci e.g. Penicillin type (3).

2) Facultative "large step" e.g. Streptomycin type mutation occurs at a single locus converting sensitive culture to a highly resistant one (3).

"Large step" facultative type of spontaneous mutation is a greater threat to successful chemotherapy than the "multiple step" pattern, for when it occurs, an infectious disease may escape from drug control abruptly and completely at any time. For this reason combined therapy

is especially indicated with drugs and organisms showing this pattern.

ACQUIRED DRUG RESISTANCE

Bacterial cells are able to achieve resistance to many antimicrobial substances (particularly those devised by and made by man) by readjustment of their existing genetic information, so that, molecular target of the inhibitor is made less sensitive. In general antibiotics that are valuable therapeutically stand out against this background because the cell cannot readily achieve resistance by adjusting it's existing genetic information. A specific inactivating enzyme is often necessary and this needs presence of additional gene/s solely to code for this enzyme (1,4,5).

A remarkable mechanism mediates simultaneous transfer of resistance to several entirely unrelated antimicrobial agents whereas resistance arising through spontaneous mutation characteristically is specific for one particular class of agents (2,4). Transmissible multidrug resistance is found principally in enteric bacteria (1). Additional genes that are needed for resistance are more commonly carried as a plasmid and only sometimes they can be found on the chromosome. Staphylococcus aureus and enteric bacteria particularly have such plasmid borne drug resistance genes where inactivating enzyme often causes trouble in therapy.

Advantages to bacterial cell of plasmid location of resistance genes are - (1) easier transfer to other cells and maintenance of resistance genes as heritable entity; (2) higher level of resistance shown because of multiple copies of resistance genes (4).

Acquired resistance may result due to any one of the three

gene transfer mechanisms -

a) Transformation b) Transduction c) Conjugation.

a) TRANSFORMATION :

Property of drug resistance could be transferred to sensitive cells by treating them with DNA extracted from drug resistant mutants (1). Since transformation of morphological characteristics of pneumococcal cells from smooth to rough forms can occur in-vivo in infected animals, it is conceivable that transformation of sensitive cells to resistant could also occur in-vivo. However it should be remembered that transformation is a relatively inefficient way of spreading genetic information, since for each resistance marker acquired by the sensitive cell, a resistant cell must lyse in order to release the DNA. A somewhat more favourable situation may be envisaged in case of resistance determined by multiple genetic loci. In this situation, resistance is transferred into sensitive cells by unit steps each conferring partial resistance (1). Thus a number of recipient cells might acquire bits of DNA derived from a single cell each conferring partial drug resistance. Despite this consideration, genetic transformation probably does not contribute substantially to clinical problems of drug resistance (1).

b) TRANSDUCTION :

During the process of phage transduction which occurs both in gram positive and gram negative bacteria, genetic information is transferred by phage particles from one bacterial cell to a related phage susceptible cell. Transducing phage might pick up a stretch of DNA containing drug resistance determinant from resistant bacterium during the process of induction of lysogenic to lytic cycle. Lysis of cell releases multiple

copies of phage particles each carrying a copy of drug resistance marker. Subsequent infection of phage susceptible drug sensitive cells under conditions favourable for lysogeny results into newly infected cells becoming drug resistant. Transfer of plasmid determinants of drug resistance usually occurs with much greater facility than that of chromosomal determinants by transduction (1). Spread of drug resistance markers by transduction is of considerable significance in Staphylococcus aureus and is an important factor in emergence of drug resistant strains of Staphylococci. Phage transduction may also be significant to spread of drug resistance among the Streptococci.

c) CONJUGATION :

Conjugation is perhaps the most threatening means of drug transfer as far as clinical importance of emergence of drug resistance is concerned (1). The bacterial plasmid genes that are associated with transfer of drug resistance markers by cellular conjugation are called R factors (1). Existence of R factors carrying various combinations of drug resistance determinants and their ability to specify its own transfer to cells of distinct but related species because of self replication is crucial here.

In early work of R factors Japanese workers made a convincing case for believing that resistance to certain important antibiotics was transferred from R factor carrying line of Escherischia coli in a person suffering from shigellosis (4,6,7). In this case survival of pathogens depended on combination of gene transfer (to give them resistance genes that they lacked initially) and also on selection of those cells at the expense of their sensitive counterparts.

Gram negative bacteria can indeed transfer drug resistance not only

to cells of the same species but also to bacteria of different species or even different genera. And this is the reason why conjugation is a serious threat in emergence and spread of drug resistance. Such a conjugation process transferring drug resistance may occur within the body of animals and human beings harbouring them and also in the environment which is carrying (spreading) them.

ROLE OF TRANSPOSONS :

Transposons are discrete sequences of DNA that can move from one replicon to another, by a process of recombination called transposition or translocation (8). Composite transposons have a central region with markers (such as drug resistance markers) which are unconnected with transposition. Transposons function as portable regions of homology. Two copies of transposons at different locations (even on different chromosomes) provide sites for reciprocal recombination. Such exchanges could result, in deletions, insertions, inversions or translocations and thus playing an important role in evolution of some bacterial plasmids.

Until recently the process whereby several antibiotic resistance determinants came to be linked genetically on a single plasmid was a question but the discovery of transposons now provides information on how a multiple resistance plasmid arises (4).

The fact that such multiple resistance plasmids are common in practice, presumably speaks, therefore for the potency of pressure operating to select for survival of such plasmids which must be built up by stepwise accumulation of appropriate transposons.

METHODS OF IN-VITRO TESTING

Large number of isolates of bacteria from patients are examined in clinical laboratories in order to determine which antibiotics are likely to be effective in curing the infection. The tests are done therefore in such a way as to show whether an isolate is sensitive to the concentration of an antibiotic likely to be reached in infected tissues during treatment of the patient by methods and dosage rates employed by the physician (9).

Following methods are employed for testing in-vitro sensitivity of clinical isolates.

- 1) Broth dilution method
- 2) Agar dilution method
- 3) Agar diffusion method

BROTH DILUTION METHOD AND AGAR DILUTION METHOD :

The principle of these methods is similar. Different quantities of antibiotic stock solutions are delivered into a series of tubes or plates and increasing concentrations of antibiotic are achieved in the nutrient medium. These are then seeded with an isolate under test and incubated to see whether growth occurs or not. These tests will give information on minimum inhibitory concentration (MIC) required for treating infection by a particular isolate.

AGAR DIFFUSION METHOD :

Agar diffusion tests may be done by modifications such as i) cylinder cup test, ii) agar well method, iii) agar ditch method, iv) Baur and Kirby's disc diffusion method.

The principle underlying these modifications is same namely, concentration gradient varies continuously and zones of inhibitions for the seeded organism are observed. Baur and Kirby's paper disc diffusion technique is the one which is used by most of the clinical laboratories because of simplicity, convenience, reliability and practical usefulness (2).

The inverse relationship between size of zone of inhibition and minimum inhibitory concentration (MIC) is critically important to all agar diffusion techniques. When test conditions are held reasonably constant, there is a linear relationship between diameter of zone of inhibition as determined by disc diffusion method (method 3) and $\log_2$ of MIC as determined by standard dilution techniques (method 1 or 2).

For each antimicrobial agent a characteristic zone size : MIC relationship can be documented, thus permitting approximation of MIC from a given zone of inhibition determination. Also it is possible to establish MIC break points (2) above which an organism should be considered resistant and MIC below which an organism is classified as susceptible. With some drugs there will be an intermediate category between these two breakpoints.

Such MIC breakpoints are developed after considering 1) the pharmacokinetics of each antimicrobial agent 2) the behaviour of microorganism of known responsiveness or non-responsiveness and 3) overall distribution of MIC and diameter of zone of inhibition with relevant clinical isolates. The very same principles are used in Baur and Kirby's method in development of zone size interpretative criteria for disc diffusion method.

The concentration of drug in tissues varies considerably from patient to patient and from time to time in the same patient, so that for most clinical purposes it is pointless to employ laborious methods such as (1) and (2) to measure accurately the minimum (MIC) of the drug for the isolate. It is sufficient, to use a simple test such as (3) which distinguishes isolates as 'sensitive' or 'resistant' in fair concordance with probable outcome of the therapy.

Antimicrobial dilution tests in broth or agar (method 1 or 2) may be required : (a) if more quantitative measures of susceptibility are needed, (b) if test strains are relatively fastidious or slow growing, (c) if number of drugs to be tested at one time are very few, (d) when diffusion tests have been found to be unsatisfactory and (e) when potential for lethal activity is to be determined.

Agar diffusion tests (method 3) done for sensitivity are (a) more convenient for day to day utility, (b) used when simple qualitative information is sufficient, (c) when test strains are capable of growing at a fairly uniform rapid rate, and (d) when fairly large number of drugs are to be studied at the same time.

The only limitations is that the diffusion tests with agents and microorganisms being tested have to be standardised previously and shown to be satisfactory.

LIMITATIONS OF IN-VITRO SUSCEPTIBILITY TESTING :

A number of host factors can influence the in-vivo susceptibility of microorganisms to the drugs. These factors are not as a rule incorporated in the in-vitro tests currently employed but they must be

taken into account in designing strategies to tackle the problem of drug resistance.

The effects of host factors and other limitations can be exemplified as - (2) :

- 1) Pathophysiological state of the host
- 2) Severity of infection
- 3) Need of other supplementary measures
- 4) Concentration of the drug in the body fluid being not necessarily the same as used in testing.
- 5) Variations in the degree of susceptibility under different conditions within the body of the host.
- 6) Ingredients in the body and tissue fluids being different from those in the culture medium.
- 7) Agents present in the body and tissue fluids capable of biological/physicochemical influence on either the microbe or the drug.

1) Pathophysiological State of the Host :

Patients with acute leukemia, or uncontrollable diabetes mellitus may not respond to chemotherapy which is normally successful otherwise (2).

2) Severity of Infection :

The natural course of type of infection being treated should also be considered. Infections that normally carry high morbidity and mortality are often treated more intensely than infections that are less serious. Chemotherapy of less serious infections is often difficult to

evaluate since patients, may appear to respond to an anti-microbial agent given at the time of infection whereas causative agent would be eliminated naturally.

Apparent success in treating infections due to microorganisms properly classified as resistant may be quite common with such self limiting diseases. At the other extreme patients with serious underlying disease may fail to survive although their infection was appropriately treated with antimicrobial drugs to which etiological agent was susceptible in-vitro.

3) Need of Other Supplementary Measures :

The efficacy of antibiotic in treatment of bacterial infections depends upon interaction of drug, bacteria and phagocytes (10). Infections localised at sites protected from phagocytic system might also fail to respond completely. For example, when invading organism is lodged within an abscess or within vegetation of endocarditis, phagocytic system does not operate optimally although drug penetrates. Incision and drainage of abscesses are essential to the success of chemotherapy. Mechanisms responsible for drug failure when organism is within abscess are multifunctional and poorly understood. Drug inactivating enzymes are demonstrated in pus (11). Gentamycin inactivation is also demonstrated in purulent exudates (12) while chloramphenicol activity is not altered in pus (13).

4) Concentration of the Drug :

Microorganisms resistant to concentrations ordinarily obtained in blood might respond to therapy if infection is located in organ system

in which drug is normally concentrated e.g. urinary tract (2,14).

Similarly penetration of antibiotic in bile may be necessary for successful eradication of hepatobiliary infections (15). Therefore and concentrations reached in bile are important/in-vitro susceptibility testing may not be sufficient. Also poor penetration and subinhibitory concentrations reached in cerebrospinal fluid (CSF) are considered responsible, to some extent, for difficulty faced in bacterial killing in CSF (16). It is also observed that therapeutic concentrations of gentamycin and carbenicillin are not reached in broncheal secretions and lung tissues and organism though susceptible may not respond (17).

5) Variations in the Degree of Susceptibility under Different Conditions within the Body of the Host :

Several clinical reports have suggested that concentrations of gentamycin in CSF that far exceed the MIC, are needed in therapy of human gram negative bacillary meningitis. This necessitated administration of very large doses of gentamycin. In turn it resulted in levels of drug in serum that were 5 - 15 times more than those considered toxic to humans (16).

The concentration of gentamycin required to produce bacterial killing in CSF in-vivo was much higher than was predicted from in-vitro sensitivity testing.

Later on it was clear that poor penetration and (reduced) pH conditions in CSF are responsible for variation observed from in-vitro susceptibility results. Also duodeno pancreatic secretions enhance bactericidal activity of some antimicrobial drugs as is shown by modified in-vitro study (18) therefore usual in-vitro susceptibility tests are not enough.

In-vitro susceptibility tests of facultative organisms are routinely performed under aerobic conditions, despite the fact that many infections caused by these organisms occur in anerobic areas i.e. intra-abdominal abscesses. Hence it may not reflect the actual state of antibiotic-bacteria interaction under conditions of infection. Thus a laboratory report of an enteric organism being sensitive to an aminoglycoside may influence the clinician to treat the patient with a drug that is relatively ineffective in the millieu of the infection. Inhibitors of oxidative metabolism impair the transport of antibiotic and thus molecular oxygen may be necessary for optimal entrance of aminoglycoside into the bacterial cell (19).

The in-vitro activity of gentamycin judged by MIC determination was much reduced when normal aerobic conditions were replaced by either air plus 4 % CO₂ or by anaerobic conditions. The phenomenon was greatest on Staph. aureus where decrease in activity was twenty fold. For E.coli, Klebsiella, Enterobacter and Proteus it was between 2.5 to 15 fold. The influence that anaerobiosis and hypercapnic conditions have on activity of antibiotics must be borne in mind when choosing the antibiotics for therapy (20).

The increasing incidences of bacterial resistance to commonly prescribed antibiotics has markedly limited the usefulness of many and has necessiated the prescription of some of the more toxic agents when less toxic ones are ineffective (19). Hence in the use of aminoglycoside antibiotics, clinically relevant results in sensitivity testing are of great importance because of potential ototoxicity of these drugs (16).

Levels of antibiotics in urine rather than those in serum have been

shown to determine the outcome of urinary infections that are not complicated by sepsis (14).

The antibacterial effect of gentamycin against E.coli and Pseudomonas aeruginosa was studied (21,22,23) and it was shown that urine has inhibitory effect that is dependent on acidity and total osmolarity of urine. Upto 40 times gentamycin was needed to prevent E.coli or Pseudomonas growth in concentrated acidic human urine as is required in broth. This inhibitory effect may be particularly important when urinary concentration of gentamycin are reduced either because of reduction in dosage or because of decreased excretion due to renal inefficiency. KH_2PO_4 , Na_2SO_4 , inhibited effect of gentamycin. MgCl_2 showed less inhibitory effect. pH was not the only factor responsible for affecting gentamycin action in urine. This was shown in tests with urine of which pH was readjusted (21).

Electrolytes in concentrations normally found in urine have been shown to interfere with antibacterial action of streptomycin in-vitro as were Calcium and Magnesium ions in case of gentamycin. Ramirez et al proposed that this effect results from enhanced binding of gentamycin by bacteria in presence of Calcium and Magnesium ions (21).

Urinary inhibition of antibacterial effect of gentamycin may alter the outcome of therapy. Lower concentrations of gentamycin combined with inhibition by urinary constituents may result in failure to cure urinary tract infections (UTI) that are caused by seemingly susceptible organisms. Laboratory methods currently used to determine bacterial susceptibility may not thus reflect the ability of bacteria in urine of a patient to resist antibacterial action of gentamycin.

While some studies as described above report that pH as well as osmolarity and ionic antagonism have a combined effect on antibacterial action of certain antibiotics, earlier studies have shown that erythromycin and novobiocin were effective on gram negative organisms causing UTI provided that pH of the medium or urine was changed so as to maximise the activity (24).

The fact that appropriate adjustment of pH may markedly increase activity of some antibiotics suggests not only that some apparently inactive antibiotics may have potential activity thus disclosed but also that some highly active antibiotics such as kanamycin and gentamycin which have potentially serious toxicity when used in moderate large dosages might be used effectively in much smaller or less toxic doses for UTI. Further in the same study (24) the possibility of decreasing dose and potential toxicity of such drugs is suggested by using promising alkalising medication in conjunction (24).

In another study two mechanisms of potential biologic antagonism of gentamycin and other aminoglycosides in purulent sputum from patients with cystic fibrosis and bronchiectasis have been reported. Reduction in activity by ions and antibiotic binding is shown in which the latter is more important (25).

Necessity of Designing In-vitro Testing, Suitably Modified to Mimic Host Factors :

The rational use of antimicrobial agents thus depends to a large extent on reliability of in-vitro testing of susceptibility and solving the problem of discrepancy in in-vitro testing of susceptibility and in-vivo response to the drug.

For example medium with low concentrations of calcium and magnesium ions will report the organism to be sensitive to gentamycin as per Baur and Kirby's method whereas with high concentrations of calcium and magnesium ions high percentage of isolates will appear resistant (26,27) posing problem of withholding useful therapy or employing potentially toxic doses of the antibiotic. Therefore standard reference medium is the first prerequisite to have reliability of in-vitro susceptibility testing.

Because of the use of standard reference medium and in-vitro procedures, it is difficult to say whether the test would be able to reflect, different environments at different locations in body and in different body fluids (which vary greatly in acidity, anaerobic conditions, osmolarity, concentration of different ions) simultaneously and also overcome other earlier cited limitations.

Appreciation of variables operating in in-vitro and in-vivo situations permits more logical approach to chemotherapeutic use of drug and may explain discrepancies observed (28). Hence there is a necessity of designing in-vitro susceptibility testing suitably modified to mimic the host factors. Results of laboratory testing are often misunderstood and misapplied (29).

Towards fulfilling this necessity some studies have been done earlier to study the role of certain factors influencing effectiveness of drug in-vivo. These are -

Effect of pH- There are various reports about study of effect of pH of medium on activity of antibiotics (30,31). Strausbaugh and Sande (16) in their work on factors affecting therapy of experimental Proteus

mirabilis meningitis in rabbits report that reduced pH of CSF is one of the important factors responsible for failure of bacterial killing by gentamycin. Reduced pH of CSF could account for 8 fold or greater increase in concentration of gentamycin required for bacterial killing. Decreasing the pH of the test media from 7.8 to 5.0 has been shown to increase MIC of gentamycin for various gram negative bacilli 8 - 32 fold. And this effect is also demonstrated (24) to be important in therapy of urinary tract infections (as described earlier). Adjustment of urine pH in chemotherapy of urinary tract infections has been also discussed by Brumfitt (32).

EFFECT OF BILE ACIDS :

Effect of bile acids on activity of antibiotics and nitrofuran drugs is reported earlier (33,34,35,36) and even it was recommended that antibiotics with maximum activity associated with presence of patient's bile should be recommended for treatment of patients with inflammatory diseases of liver and urinary tract. This report (33) describes how bile enhances activity of some antibiotics.

The effect of bile acids on antibiotic resistant strains of S. aureus, E.coli, P.aerugenosa isolated from patients with different purulent, inflammatory processes is reported. It was shown that (33), glycocholic acid, deoxycholic acid lowered the in vitro resistance of the above organisms. These bile acids increased bacterial sensitivity to streptomycin, chloramphenicol and neomycin by 8 - 16, 10 - 20 and 40 - 80 times respectively. The effect of bile acids on resistant strains of E.coli and Proteus was less pronounced.

EFFECT OF ENZYMES :

In one of the studies (37) it has been shown that proteolytic enzymes like papain increased chemotherapeutic efficacy of antibiotics on average by 50 % . Study was carried out on resistant Staph aureus cultures causing septicemia in mice with antibiotics such as benzyl penicillin, streptomycin, chloramphenicol, tetracycline, erythromycin, novobiocin etc. It was reported that when used alone in dose of 0.1 mg per mouse, papain prevented death in 20 % animals. High chemotherapeutic efficacy of antibiotic combination with papain lead to recommending it for use in clinical practice for treatment of infections caused by antibiotic resistant Staphylococcus.

Use of enzyme dextranase is suggested to modify extracellular polysaccharide glycocalyx in treatment of experimental endocarditis due to viridans streptococci (38).

EFFECT OF EDTA :

Weiser et al reported (39) that bacterial resistance to antibiotics may be a consequence of development of permeability barrier which prevents antibiotics from reaching its site of action in the bacterial cell. Such permeability barriers are thought to be responsible for resistance E.coli and other gram negative organisms to chloramphenicol, actinomycin D, benzyl penicillin, vancomycin.

The permeability of several gram negative organisms to a variety of unrelated compounds including antibiotics has been shown to have increased by EDTA (39). Subinhibitory concentration of EDTA was used in the study. Maximum concentration of EDTA used was 2.8×10^{-2} M.

EDTA caused reduction in MIC for penicillin, ampicillin, chloramphenicol, tetracycline. Complete reversal of resistance was observed for tetracycline, penicillin and ampicillin for Pseudomonas aeruginosa. For E.coli, Proteus reversal of resistance for chloramphenicol, penicillin, ampicillin by EDTA was partial, MIC was not lowered to the sensitive range.

Action of EDTA was completely reversed by 0.1 M MgSO_4 (39). Weinberg (40) showed that addition of Mg^{++} and Fe^{++} reduced activity of tetracycline against gram negative bacteria. It is possible therefore that EDTA by chelating these ions enhances the activity of tetracycline.

EFFECT OF CALCIUM, MAGNESIUM AND FERROUS IONS :

Weinberg (40) in his study of effect of metallic ions reported that suppression of antimicrobial activity of drugs by such ions as Mg, Fe, occurred either by their combination with tetracycline to remove the drug from enzymatic sites or by replacement of essential cation taken from enzymes by antibiotics. In either case excess metallic ions over the drug was required for suppressing antibiotic action.

Tetracycline, oxytetracycline action was suppressed by Fe if Fe : drug was in (50 - 140 : 1) molar ratio. If metallic ions were in toxic concentration then tetracycline stimulated the growth instead of inhibiting it.

Action of streptomycin was suppressed by Ca, Mg, Ba because of water soluble chelates formed by streptomycin with these ions.

Chloramphenicol was not usually affected by metallic ions. Effect of cations on antibiotic action has been reported by many (41 - 47).

Work by Gilbert et al (41) and further by Zimilis and Jackson (42) reported about the effects of magnesium and calcium ions, on MIC of gentamycin for Pseudomonas and other organisms. It says that these cations interact with bacteria rather than with antibiotics.

Work by Medeiros et al shows that cations interact with bacteria rather than with antibiotic (43). Their results suggest that divalent cations in environment of bacterium can alter it's susceptibility to gentamycin in-vitro and presumably also in-vivo. High concentrations of divalent cations for example intracellularly might impair killing of Pseudomonas and other bacilli by gentamycin. On the other hand low concentrations of sodium chloride occurs in urine and can enhance it.

Now it has been shown (42) that calcium and magnesium ions exert influence on outer surface of bacterium rather than (on ribosomal sites) intracellularly. It is postulated that calcium and magnesium ions stiffen the lipoprotein membranes thus preventing conformational changes whereby the membrane is disrupted. Cations link polysaccharide subunits. Cations are important for structural integrity and weakening of this structural integrity reduces resistance.

Thus with increase in cations, cell-wall integrity will improve increasing the resistance of organism to the drugs that disrupt the cell wall.



AIMS AND OBJECTIVES

AIMS AND OBJECTIVES

In view of the above points following aims and objectives were set up.

- 1) To study antibiotic resistance levels of various resistant clinical isolates and to study stability of antibiotic resistance by serial transfer.
- 2) To study nature of antibiotic resistance i.e. whether it is plasmid borne or chromosomal.
- 3) To study in-vitro effects of some factors which can influence efficacy of the drug in-vivo. Here in-vitro tests are designed so as to mimic the in-vivo conditions.



MATERIALS AND METHODS

MATERIALS AND METHODS

Bacterial Strains :

Forty different clinical isolates from pathological samples such as stool, urine and pus of patients suffering from various infections were collected. These were obtained from Clinical Pathology Department of Haffkine Institute, Parel, and Pathology Department of Railway Hospital, Byculla, Bombay. Isolates were selected because of their in-vitro resistance to two or more antimicrobial agents.

Identification of Bacterial Isolate :

Pure cultures of bacteria isolated from clinical samples were identified using standard techniques as described in (48).

Antibiotics :

Pure dry powder of the following antibiotics were obtained from the source indicated in paranthesis.

1. Benzyl Penicillin (Alembic Chemicals)
2. Streptomycin Sulfate (Sarabhai Chemicals)
3. Ampicillin (Ranbaxy Laboratory)
4. Cloxacillin (Lyka Laboratory)
5. Chloramphenicol (Supplied by Hi-Media)
6. Tetracycline (Supplied by Hi-Media)
7. Neomycin Sulfate (Supplied by Hi-Media)
8. Gentamycin Sulfate (Supplied by Hi-Media)
9. Sulfamethoxazole (Supplied by Haffkine Institute)
10. Trimethoprim (Supplied by Haffkine Institute)

Antibiotic Sensitivity Testing :

Clinical isolates after identification were subjected to antibiotic sensitivity testing. Kirkby-Baur technique for disc sensitivity testing was used as described in (2,49)

Following antibiotics were used to determine antibiotic resistance pattern by disc sensitivity testing.

I) Antibiotics :

Choice of the drugs was done in such a way that antibiotics belonging to different groups were included. The antibiotics selected were also such that they are commonly employed in chemotherapy of infections caused by isolates under study here. Preparation of stock solutions and working stock solutions is given in Tables 1 and 2.

II) Preparations of Antibiotic Discs :

Whatman filter paper number 1 was used. Six mm diameter discs were punched. Filter paper discs were sterilized by placing them in empty glass plates in oven at 160 °C for 2 hours. Antibiotic solutions were prepared as described above. Hundred filter paper discs (sterile) were put in 1 ml of antibiotic solution of such concentration that final concentration of antibiotic received (absorbed) by each disc was as per concentration of the drug required for sensitivity testing.



TABLE 1
STOCK SOLUTIONS OF ANTIBIOTICS

Sr.No.	Antimicrobial Agent and Amount		Solvent and Amount in ml		Concentration per ml
1	Benzyl Penicillin	0.5 ml of 2 lac Unit per ml.	Water	9.5	10000 Units
2	Streptomycin	100 mg	Water	10.0	10 mg
3	Chloramphenicol	300 mg	Ethanol	10.0	30 mg
4	Tetracycline	300 mg	Water	10.0	30 mg
5	Ampicillin	100 mg	0.1 M PBS (pH 8.0)	10.0	10 mg
6	Cloxacilin	10 mg	Water	10.0	1 mg
7	Sulfonamide	300 mg	10% NaOH (Hot)	5.0	60 mg
8	Gentamycin	100 mg	0.1 M PBS (pH 8.0)	10.0	10 mg
9	Neomycin	300 mg	Water	10.0	30 mg
10	Trimoxazole (i.e. Trimethoprim plus Sulfamethoxazole	1.75 mg 23.25 mg	10% NaOH (Hot)	5.0	5 mg

TABLE 2
WORKING SOLUTIONS OF ANTIBIOTICS

Sr. No.	Antimicrobial Agent	Stock Solution		Diluent and	Amount in ml.	Final Concentration per ml.
		Conc./ml	Amount in ml			
1	Benzyl Penicillin	10000 Units	10.0	Water	90.0	1000 Units
2	Streptomycin	10 mg	10.0	Water	90.0	1 mg
3	Chloramphenicol	30 mg	10.0	Water	90.0	3 mg
4	Tetracycline	30 mg	10.0	Water	90.0	3 mg
5	Ampicillin	10 mg	10.0	0.1 M PBS (pH 6.0)	90.0	1 mg
6	Cloxacilin	1 mg	10.0	Water	90.0	100 μ g
7	Sulfonamide	60 mg	5.0	Water	5.0	30 mg
8	Gentamycin	10 mg	10.0	Water	90.0	1 mg
9	Neomycin	30 mg	10.0	Water	90.0	3 mg
10	Trimoxazole	5 mg	5.0	Water	5.0	2.5 mg

PBS - Phosphate Buffer Saline

<u>Antimicrobial Agent</u>	<u>Amount Per Disc</u>
1. Benzyl Penicillin	10 Units
2. Streptomycin	10 µg
3. Chloramphenicol	30 µg
4. Tetracycline	30 µg
5. Ampicillin	10 µg
6. Cloxacillin	1 µg
7. Sulfadimidine	300 µg
8. Neomycin	30 µg
9. Gentamycin	10 µg
10. Trimoxazole	25 µg

III. Nutrient Media for Sensitivity Testing :

Nutrient broth was used for bringing the organisms into log phase for sensitivity testing and Mueller and Hinton agar was used to carry out the actual sensitivity test. Composition of media is described in Appendix.

IV. Test Procedure :

Clinical isolate for which sensitivity testing was to be done, was first grown in nutrient broth. Loopful of 24 h old culture was transferred to 4 ml of nutrient broth and incubated at 37 °C for 4 h. The culture was diluted with sterile normal saline and matched with Brown's optical standards to get approximately 1.5×10^8 organisms per ml. Mueller and Hinton agar plates were first incubated at 37 °C for 48 h to check sterility. Properly dried sterile Mueller and Hinton agar plates were used for the test. Culture to be tested (of which density was adjusted) was streaked on dry surface of Mueller and Hinton agar.

Culture was allowed to dry. Antibiotic discs were placed on seeded agar plates. Standard drug sensitive strains of E.coli NCTC 10418 and Staphylococcus aureus (Oxford) 6571 were also employed to ensure the efficacy of the method. Plates were incubated at 37 °C overnight. Diameters of zones of inhibition were measured. Ten discs were placed in each plate.

V. Standardisation :

Employing the antibiotic discs prepared in our laboratory and the standard test culture E.coli NCTC 10418 and Staph aureus (oxford) 6571, a sensitivity chart was prepared. After sensitivity testing, isolates were designated as 'Sensitive', 'Intermediate', and 'Resistant' with reference to this chart, as shown in Table 3.

Agar Plate Dilution Technique for MIC Determination :

Level of antibiotic resistance of the clinical isolates to different antibiotics was determined by agar plate dilution technique as described in (2).

Media : Nutrient broth was used for bringing the bacterial strains into log phase for MIC testing. Mueller and Hinton agar was used for the actual MIC testing.

Procedure : Different amounts of working solutions of antibiotics were added to sterile petriplates(100 mm) so as to achieve final concentrations of 40, 80, 100, 200 and 300 µg/ml. Twenty-five ml of molten Mueller and Hinton agar was first cooled to 50 °C and then was poured in each plate. Plates were properly roated for thorough mixing of antibiotic with the molten agar. The antibiotic containing plates were

TABLE 3
SENSITIVITY REFERENCE CHART

Antibiotics	Diameter of Zone of Inhibition (mm)		
	Sensitive	Intermediate	Resistant
1 Benzyl Penicillin	≥ 15	11 - 15	≤ 10
2 Sulfadimidine (300 μ g/disc)	≥ 17	13 - 16	≤ 12
3 Streptomycin (10 μ g/disc)	≥ 15	12 - 14	≤ 11
4 Chloramphenicol (30 μ g/disc)	≥ 18	15 - 17	≤ 14
5 Ampicillin (10 μ g/disc)	≥ 14	12 - 13	≤ 11
6 Tetracycline (30 μ g/disc)	≥ 19	14 - 18	≤ 13
7 Cloxacilin (1 μ g/disc)	≥ 15	11 - 14	≤ 10
8 Neomycin (30 μ g/disc)	≥ 17	13 - 16	≤ 12
9 Gentamycin (10 μ g/disc)	≥ 13	11 - 12	≤ 10
10 Trimoxazole (25 μ g/disc)	≥ 17	13 - 16	≤ 12

cooled and preserved at 40 °C till use. Before use, the plates were dried at 37 °C and then used for MIC testing. Each plate was divided into 12 sectors so that 12 different cultures could be tested simultaneously. Loopful of 24 h old culture was transferred to 10 ml of nutrient broth and incubated at 37 °C for 24 h. Then 0.001 ml of 10^{-1} dilution of overnight broth culture was inoculated with a calibrated loop. Plates were incubated at 37 °C. After overnight (18 - 20 h) incubation, plates were examined for bacterial growth. Lowest concentration of antibiotic causing complete inhibition of cultures was taken as minimum inhibitory concentration (MIC). Plates of Mueller and Hinton agar without antibiotic were inoculated and incubated as control of the viability of the test organism.

Testing Stability of Antibiotic Resistance of Clinical Isolates :

Clinical isolates were transferred every three weeks on antibiotic free nutrient agar slants. These were preserved at 4 °C. MIC determination for various antibiotics of these cultures was done after 5, 10, 15, 20 serial transfers. MIC testing was done by agar plate dilution technique as described earlier.

Resistance Transfer Testing :

show

Bacterial strains which/ resistance to various antimicrobial agents may bear necessary genetic determinants either on chromosome or on plasmid. Simultaneous resistance to a number of antimicrobial agents is generally because of plasmid borne genetic determinants (3). Plasmid borne antibiotic resistance also has great significance in spread of antibiotic resistance to other sensitive organisms. Nature of resistance whether due to plasmid borne genes or R factors was

determined as per following resistance transfer testing procedure (50).

Procedure :

Culture Strains : All antibiotic resistant strains were tested for R factors. Resistant isolates which acted as donors in the conjugation experiment were first tested for their sensitivity to 50 µg of nalidixic acid per ml of medium. Only those isolates which were sensitive were used for Resistance transfer testing. Nalidixic acid resistant (to 50 µg/ml of medium) lactose negative E.coli K 12 strain was used as the recipient in the conjugation experiment. This culture was sensitive to Streptomycin, Chloramphenicol, Tetracycline, Neomycin, Gentamycin and Trimoxazole.

Media Nutrient broth was used to bring the donor and recipient strains into log phase. Mueller and Hinton agar containing 50 µg of nalidixic acid per ml was used for checking sensitivity or resistance after conjugation.

Donor multiresistant isolates and recipient E.coli K12 strain were first grown in nutrient broth at 37 °C. Overnight cultures of donor and recipient were mixed in the ratio of 1 : 2 . This mixture was then incubated for 24 h at 37 °C after addition of nutrient broth for further growth. 0.2 ml of 10⁻¹ dilution of overnight growth of the mixture was then used for flooding Mueller and Hinton agar (containing 50 µg nalidixic acid per ml of the medium). Seeded plates were allowed to dry and antibiotic discs were placed on agar surface in 10 sectors. Plates were incubated at 37 °C for 24 h. Antibiotic sensitivity pattern of the mixed culture after growth was checked. Simultaneously control

plates without nalidixic acid were inoculated separately for donor and recipient, to test antibiotic sensitivity pattern.

Evidence of resistance in recipient, E.coli K12 as shown on nalidixic acid containing plate as against its sensitivity as shown on control plate was interpreted to mean that resistance transfer had taken place.

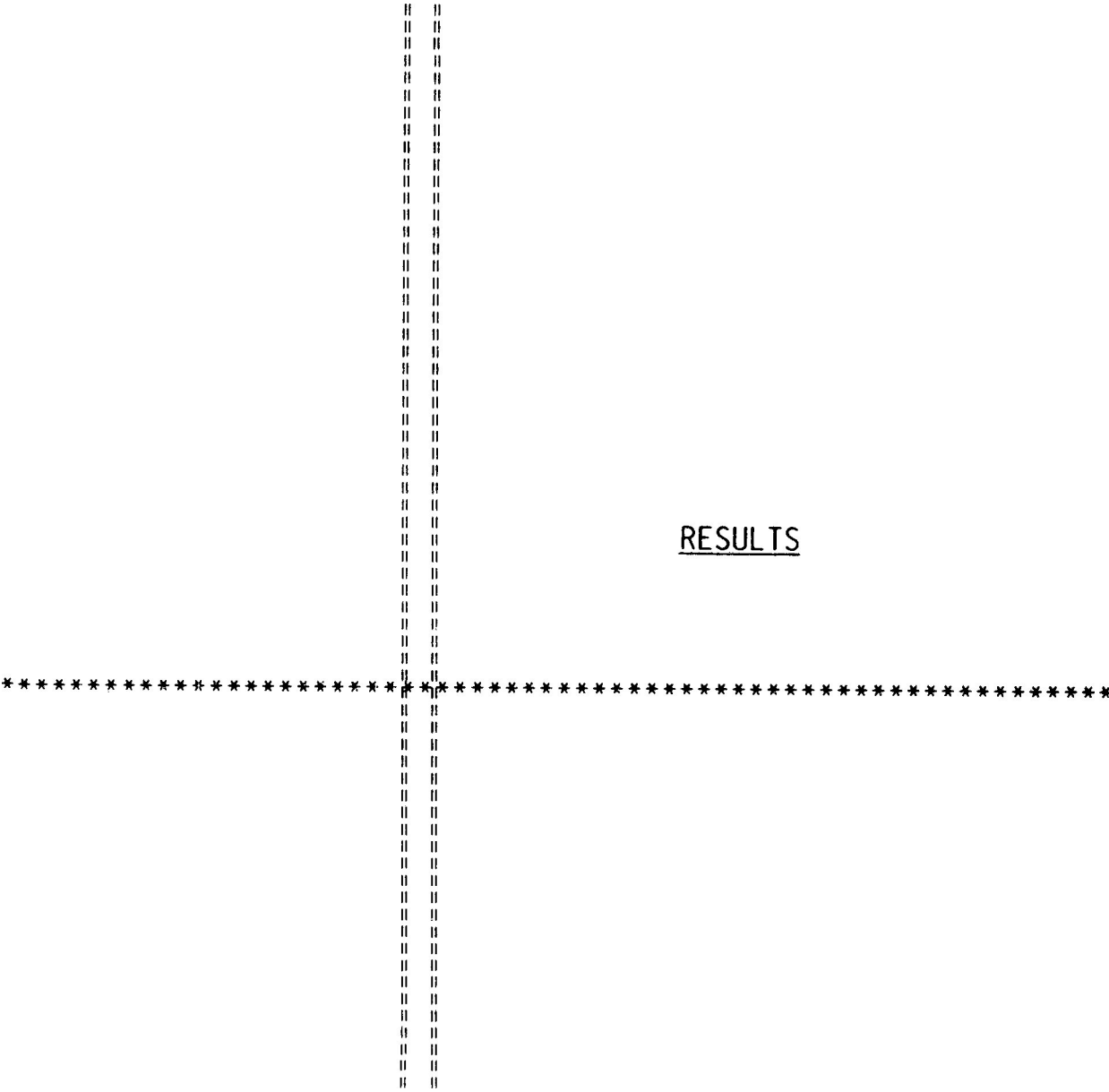
Study of Effect of Different Factors on MIC Levels of Antibiotics:

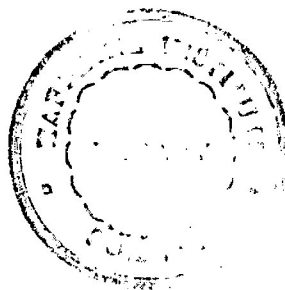
Clinical isolates were studied for their levels of resistance to different antibiotics in presence and in absence of various organic and inorganic chemicals.

Following factors were studied :

1. Effect of individual bile acids like Cholic acid, Sodium deoxycholate and Sodium taurocholate.
2. Effect of different combinations of two bile acids.
3. Effect of mixture of three bile acids.
4. Effect of magnesium ions.
5. Effect of phosphate ions.
6. Effect of potassium chloride.
7. Effect of EDTA
8. Effect of Fe^{++} ions.
9. Effect of pH.
10. Effect of proteolytic enzymes.

Details of the methods used for these studies are given in Section IV of the Results.





RESULTS

Bacterial Strains:

In all 40 bacterial strains were collected from clinical samples. Twenty one strains were obtained from Clinical Pathology Laboratory of Haffkine Institute, Parel, 18 strains were obtained from Clinical Pathology Laboratory of Railway Hospital, Byculla, Bombay and one strain from private clinical laboratory in New Bombay area. Bacteriological identification done at source was reconfirmed.

Thirty-one bacterial strains were isolated from urine, 2 strains from stool and 7 strains from subcutaneous abscesses.

Thirteen strains were Escherischia coli, 10 strains were Proteus spp., 5 strains were Klebsiella spp., 8 strains were Pseudomonas spp., 1 strain of Salmonella spp. and 3 strains of Staphylococcus aureus (Table 1).

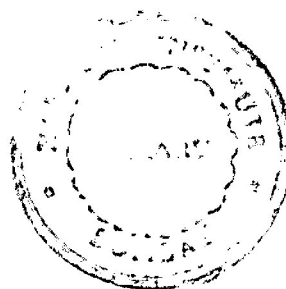
Antibiotic Sensitivity Testing :

All 40 strains had been tested for antibiotic sensitivity by the respective laboratories from where they were obtained and found to be multi drug resistant (i.e. resistant to more than 3 antibiotics). Confirmation of sensitivity and thereby antibiotic resistance pattern of these strains was done in our laboratory again using standard Kirby-Baur technique.

Out of 40 strains studied for sensitivity against 10 antibiotics - 28 bacterial strains were resistant to 10 antibiotics, 7 strains resistant to 9 antibiotics, 3 strains resistant to 8 antibiotics and 2 strains were

TABLE 1
DESCRIPTION OF ISOLATES

Sr. No.	Organism	Total No. of Strains	Source	No. of Strains
1	<u>Escherichia coli</u>	13	Urine	12
			Stool	1
2	<u>Proteus</u> spp.	10	Urine	8
			Shoulder Swab	1
			Stool	1
3	<u>Klebsiella</u> spp.	5	Urine	5
4	<u>Pseudomonas aeruginosa</u>	8	Urine	5
			Abscess Swab	3
5	<u>Salmonella</u> spp.	1	Urine	1
6	<u>Staphylococcus aureus</u>	3	Abscess Swab	3



resistant to 7 antibiotics.

Data of sensitivity testing is summarized in Tables (2A and 2B).

MIC of Antibiotics for Forty Clinical Isolates :

MIC of individual antibiotic against the 40 strains was also determined. The results are shown in Table (3) .

Antibiotic Resistance Stability Testing :

Antibiotic resistance level (MIC) of all 40 bacterial strains was checked after 5, 10, 15, 20 serial transfers on antibiotic-free media. It was observed that there was no change in MIC of various antibiotics tested for these bacterial strains. Thus antibiotic resistance level (MIC) was found to be stable for the antibiotics and cultures tested (Table 3).

Antibiotic Resistance Transfer Testing:

Nature of antibiotic resistance whether plasmid borne or chromosomal was decided by checking for presence of R factors. Conjugation experiment was carried on isolates as the donors and E.coli K 12 Nal^R as the recipient. Out of total 40 bacterial strains only 12 were nalidixic acid sensitive and therefore were suitable as donors for the method used here. Sensitivity testing on transconjugants obtained for each of the 12 donor strains after their conjugation with Nal^R E.coli K12 strain was carried out and results were as follows -

Three out of 12 cultures did not show presence of R factors. In remaining 9 cultures presence of R factors could be demonstrated by transfer of resistance that had occurred from donor (isolate) to recipient. Antibiotic pattern of transfer is shown in Table (4). It may be noted that 1 culture showed transfer of resistance for 6 antibiotics, 1 culture

TABLE 2 A
ANTIBIOTIC RESISTANCE PATTERN

Total Number of Strains Tested	Organism	Number of Strains Resistant to Antibiotics									
		Peni.	Str.	Chl.	Tetra.	Amp.	Clox.	Trimox.	Neo	Gent	Sulfa
13	<u>E.coli</u>	13	13	12	12	13	13	13	13	12	13
10	<u>Proteus</u> spp.	10	10	9	10	10	10	9	7	7	10
5	<u>Klebsiella</u> spp.	5	5	4	4	5	5	4	5	4	5
8	<u>Pseudomonas</u> spp.	8	8	8	8	8	8	8	8	6	8
1	<u>Salmonella</u> spp.	1	-	1	1	1	1	1	-	1	1
3	<u>Staphylococcus aureus</u>	3	3	3	3	3	3	3	3	3	3

Peni - Benzyl Penicillin

Str - Streptomycin

Chl - Chloramphenicol

Tetr - Tetracycline

Amp - Ampicillin

Clox - Cloxacilin

Trimox- Trimoxazole

Neo - Neomycin

Gent - Gentamycin

Sulfa - Sulfadimidine

TABLE 2 B
ANTIBIOTIC RESISTANCE PATTERN

Number of Antibiotics that Organisms were resistant to	Total Number of Strains	<u>E.coli</u>	<u>Proteus</u> spp.	<u>Klebsiella</u>	<u>Pseudomonas</u>	<u>Salmonella</u>	<u>Staph aureus</u>
0	--	--	--	--	--	--	---
1	--	--	--	--	--	--	--
2	--	--	--	--	--	--	--
3	--	--	--	--	--	--	--
4	--	--	--	--	--	--	--
5	--	--	--	--	--	--	--
6	--	--	--	--	--	--	--
7	2	--	2	--	--	--	--
8	3	2	--	1	--	--	--
9	7	2	2	1	2	--	--
10	28	9	6	3	6	1	3

STABILITY TESTING FOR ANTIBIOTIC RESISTANCE

[illegible]

Contd...

TABLE - 3 (Contd.)

STABILITY TESTING FOR ANTIBIOTIC RESISTANCE

Organism	Strain No.	Streptomycin MIC					Chloramphenicol MIC					Tetracycline MIC				
		Initial	No. of Passages				Initial	No. of Passages				Initial	No. of Passages			
			5	10	15	20		5	10	15	20		5	10	15	20
<u>Pseudomonas</u> spp.	1	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600
	2	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600
	3	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 200 < 300	> 200 < 300	> 200 < 300	> 200 < 300	> 200 < 300
	4	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600
	5	> 400 < 500	> 400 < 500	> 400 < 500	> 400 < 500	> 400 < 500	> 500 < 600	> 500 < 600	> 500 < 600	> 500 < 600	> 500 < 600	> 600	> 600	> 600	> 600	> 600
	6	> 600	> 600	> 600	> 600	> 600	> 500 < 600	> 500 < 600	> 500 < 600	> 500 < 600	> 500 < 600	> 200 < 300	> 200 < 300	> 200 < 300	> 200 < 300	> 200 < 300
	7	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600
	8	> 600	> 600	> 600	> 600	> 600	> 500 < 600	> 500 < 600	> 500 < 600	> 500 < 600	> 500 < 600	> 600	> 600	> 600	> 600	> 600
<u>Staph aureus</u>	1	> 600	> 600	> 600	> 600	> 600	> 500 < 600	> 500 < 600	> 500 < 600	> 500 < 600	> 500 < 600	> 200 < 300	> 200 < 300	> 200 < 300	> 200 < 300	> 200 < 300
	2	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600
	3	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600
<u>Salmonella</u> spp.	1	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 600	> 300 < 400	> 300 < 400	> 300 < 400	> 300 < 400	> 300 < 400

For testing stabilty of antibiotic resistance MIC was checked for 5 different antibiotics after 5, 10, 15, 20 serial passages of the test organisms on antibiotic free nutrient media.

[illegible]

Contd

TABLE - 3 (Contd.)

STABILITY TESTING FOR ANTIBIOTIC RESISTANCE

Organism	Strain No.	Neomycin MIC					Gentamycin MIC				
		Initial	No. of Passages				Initial	No. of Passages			
			5	10	15	20		5	10	15	20
<u>Pseudomonas</u> spp.	1	> 600 < 100	> 600 < 100	> 600 < 100	> 600 < 100	> 600 < 100	> 500 < 100	> 500 < 100	> 500 < 100	> 500 < 100	> 500 < 100
	2	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600	> 500 < 600	> 500 < 600	> 500 < 600	> 500 < 600	> 500 < 600
	3	> 500 < 100	> 500 < 100	> 500 < 100	> 500 < 100	> 500 < 100	< 50	< 50	< 50	< 50	< 50
	4	> 1000 < 200	> 1000 < 200	> 1000 < 200	> 1000 < 200	> 1000 < 200	< 50	< 50	< 50	< 50	< 50
	5	> 500 < 100	> 500 < 100	> 500 < 100	> 500 < 100	> 500 < 100	< 50	< 50	< 50	< 50	< 50
	6	> 1000 < 200	> 1000 < 200	> 1000 < 200	> 1000 < 200	> 1000 < 200	< 50	< 50	< 50	< 50	< 50
	7	> 2000 < 300	> 2000 < 300	> 2000 < 300	> 2000 < 300	> 2000 < 300	> 1000 < 200	> 1000 < 200	> 1000 < 200	> 1000 < 200	> 1000 < 200
	8	> 2000 < 300	> 2000 < 300	> 2000 < 300	> 2000 < 300	> 2000 < 300	> 1000 < 200	> 1000 < 200	> 1000 < 200	> 1000 < 200	> 1000 < 200
<u>Staph aureus</u>	1	> 2000 < 300	> 2000 < 300	> 2000 < 300	> 2000 < 300	> 2000 < 300	> 2000 < 300	> 2000 < 300	> 2000 < 300	> 2000 < 300	> 5000 < 600
	2	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600
	3	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600	> 600 < 600
<u>Salmonella</u> spp.	1	> 500 < 100	> 500 < 100	> 500 < 100	> 500 < 100	> 500 < 100	> 500 < 100	> 500 < 100	> 500 < 100	> 500 < 100	> 500 < 100

For testing stability of antibiotic resistance MIC was checked for 5 different antibiotics after 5, 10, 15, 20 serial passages of the test organisms on antibiotic free nutrient media.

TABLE 4

ANTIBIOTIC RESISTANCE TRANSFER TESTING

Isolate No	From Organism	Antibiotic Resistance Transfer Pattern to the Receptient
1	<u>E.coli</u>	No transfer
5	<u>Proteus</u> spp.	No transfer
9	<u>Pseudomonas</u> <u>aeruginosa</u>	No transfer
4	<u>Klebsiella</u> spp.	Str., Chl., Tetra., Neo.
8	<u>E.coli</u>	Chl., Neo, Gent.,
10	<u>Proteus</u> spp.	Str., Chl., Tetr., Neo., Gent., Trimox.
13	<u>Klebsiella</u> spp.	Str., Chl., Gent., Trimox.
16	<u>E.coli</u>	Chl., Tetr., Trimox.
23	<u>Klebsiella</u> spp.	Tetr., Neo., Gent., Trimox.
24	<u>Klebsiella</u> spp.	Chl., Tetr., Neo., Gent., Trimox.
25	<u>Klebsiella</u> spp.	Str., Chl., Neo., Gent.
26	<u>E.coli</u>	Chl., Tetr., Gent., Trimox.



showed transfer for 5 antibiotics, 5 cultures for 4 antibiotics and 2 cultures showed transfer of resistance for 3 antibiotics.

STUDY OF EFFECT OF INDIVIDUAL BILE ACID TREATMENT ON MIC OF ANTIBIOTICS :

Twenty four¹ out of forty bacterial strains were studied for effect of individual bile acid on MIC of different antibiotics. Cholic acid, sodium deoxycholate and sodium taurocholate were the three bile acids used in the study. First MIC determination was carried out for the bile acid using E.coli as the test organism. Broth dilution technique (2) was used here to prepare different dilutions of bile acid in nutrient broth. Maximum concentration of bile acid at which the growth of test organism could occur was 2.5 % (w/v) and this was employed for treatment of clinical isolate during it's study on MIC of antibiotics.

Bacterial strains to be tested were first grown in 10 ml nutrient broth by inoculating loopful of log-phase culture and incubating it at 37 °C for 18 - 20 h. This overnight growth of the culture was then centrifuged at 3000 g for 15 min. Bacterial pellet was suspended in normal saline and adjusted to original volume 4 ml of 10⁻¹ dilution of this was prepared and centrifuged at 3000 g for 15 min. Bacterial pellet obtained was then suspended in 4ml of 2.5 % bile acid in nutrient broth and incubated at 37 °C for 3 h. It was then centrifuged at 3000 g for 15 min to remove bile acid; one saline wash was given to the sediment to remove any remaining bile acid. Finally bacterial pellet was suspended in 4 ml of normal saline and this treated culture was used for MIC testing by agar plate dilution technique. Mueller and Hinton agar containing different concentration of antibiotics was used for this purpose.

Chloramphenicol, tetracycline, streptomycin and neomycin were used in the study. MIC determination was done in triplicate for all the samples. MICs of bile acid treated cultures and untreated control cultures were assayed for different antibiotics.

MICs of control and treated strains for each antibiotic were compared employing nonparametric statistical Mann-Whitney test (51). When each and every test result was less or greater than simultaneously assayed control result, it was concluded that the given bile acid treatment resulted in reduction or increase in MIC value respectively.

Tables (5, 6) give details of the effect of individual bile acids on MIC of antibiotics for various bacterial strains.

As can be seen from Table (7) treatment with bile acids resulted in reduction of MIC of the four antibiotics for E.coli, Proteus spp. and Klebsiella spp. in decreasing order as far as the number of strains affected. Pseudomonas aeruginosa, Staph aureus and Salmonella spp. did not show any effect. However number of these cultures tested was very small. Cholic acid was found to be more effective than sodium deoxycholate and sodium taurocholate in reduction of MIC of the four antibiotics tested.

Study of Effect of Treatment of Two Bile Acid Combination on MIC by Antibiotics :

Twenty four out of 40 bacterial strains were studied for the effect of treatment of combination of two bile acids on MIC of antibiotics. Subinhibitory concentrations were prepared using 1.25 % (w/v) of each bile acid resulting into a combined bile acid strength of 2.5 % in nutrient broth. Combinations used were (i) cholic acid and sodium deoxycholate

TABLE 5

EFFECT OF INDIVIDUAL BILE ACID ON MIC OF ANTIBIOTICS

Organism	Strain No.	Chloramphenicol MIC				Strain No.	Tetracycline MIC			
		Control	Ca	Sd	Sta		Control	Ca	Sd	Sta.
<u>E.coli</u>	1	> 500 & < 600	> 200 & < 300	> 200 & < 300	> 200 & < 300	1	> 600	> 600	> 100 & < 200	> 600
	2	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 500	2	> 600	> 200 & < 300	> 200 & < 300	> 600
	3	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	3	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
	4	> 500 & < 600	> 100 & < 200	> 200 & < 300	> 200 & < 300	4	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400
	5	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	5	> 600	> 600	> 600	> 600
	6	> 500 & < 600	> 200 & < 300	> 500 & < 600	> 500 & < 600	6	> 600	> 200 & < 300	> 200 & < 300	> 600
	7	> 500 < 600	> 200 & < 300	> 200 & < 300	> 200 & < 300	7	> 600	> 600	> 600	> 600
<u>Proteus spp.</u>	1	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	1	> 300 & < 400	> 200 & < 300	> 200 & < 300	> 200 & < 300
	2	> 600	> 600	> 600	> 600	2	> 600	> 600	> 600	> 600
	3	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	3	> 600	> 600	> 600	> 600
	4	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	4	> 600	> 600	> 600	> 600
	5	> 200 & < 300	> 50 & < 100	> 50 & < 100	> 50 & < 100	5	> 200 & < 300	> 100 & < 200	> 50 & < 100	> 200 & < 300
	6	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400	6	> 500 & < 600	> 300 & < 400	> 300 & < 400	> 300 & < 400
	7	> 500 < 600	> 200 < 300	> 200 < 300	> 500 < 600	7	> 300 & < 400	> 100 & < 200	> 100 & < 200	> 300 & < 400
	8	> 600	> 600	> 600	> 600	8	> 600	> 600	> 600	> 600
<u>Klebsiella spp.</u>	1	> 600	> 600	> 600	> 600	1	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400
	2	> 600	> 600	> 600	> 600	2	> 600	> 600	> 600	> 600
	3	> 600	> 600	> 600	> 600	3	> 600	> 600	> 600	> 600
	4	> 200 & < 300	> 100 & < 200	> 200 & < 300	> 200 & < 300	4	> 500 & < 600	> 200 & < 300	> 200 & < 300	> 500 & < 600
<u>Staph aureus</u>	1	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	1	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
	2	> 600	> 600	> 600	> 600	2	> 600	> 600	> 600	> 600
<u>Pseudomonas spp.</u>	1	> 600	> 600	> 600	> 600	1	> 600	> 600	> 600	> 600
	2	> 600	> 600	> 600	> 600	2	> 600	> 600	> 600	> 600
<u>Salmonella spp.</u>	1	> 600	> 600	> 600	> 600	1	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400

MIC values determined in triplicate in each case.

Ca - Cholic Acid, Sd - Sodium Deoxycholate, Sta - Sodium Taurocholate

TABLE 6
EFFECT OF INDIVIDUAL BILE ACID ON MIC OF ANTIBIOTICS

Organism	Strain No.	Streptomycin MIC				Strain No.	Neomycin MIC			
		Control	Ca	Sd	Sta		Control	Ca	Sd	Sta.
<u>E.coli</u>	1	> 600	> 200 & < 300	> 200 & < 300	> 600	1	> 200 & < 300	> 100 & < 200	> 100 & < 200	> 50 & < 100
	2	> 600	> 600	> 200 & < 300	> 600	2	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400
	3	> 600	> 200 & < 300	> 200 & < 300	> 200 & < 300	3	> 200 & < 300	> 100 & < 200	> 100 & < 200	> 200 & < 300
	4	> 600	> 600	> 600	> 600	4	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	5	> 600	> 200 & < 300	> 300 & < 400	> 300 & < 400	5	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
	6	> 600	> 100 & < 200	> 100 & < 200	> 100 & < 200	6	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	7	> 600	> 300 & < 400	> 600	> 600	7	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
<u>Proteus spp.</u>	1	> 600	> 200 & < 300	> 200 & < 300	> 200 & < 300	1	> 200 & < 300	> 50 & < 100	> 50 & < 100	> 50 & < 100
	2	> 600	> 600	> 600	> 600	2	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	3	> 600	> 600	> 600	> 600	3	> 100 & < 200	> 100 & < 200	> 100 & < 200	> 100 & < 200
	4	> 600	> 600	> 600	> 600	4	> 100 & < 200	> 100 & < 200	> 100 & < 200	> 100 & < 200
	5	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300	5	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	6	> 600	> 600	> 600	> 600	6	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600
	7	> 500 < 600	> 200 & < 300	> 200 & < 300	> 200 & < 300	7	> 200 & < 300	< 50	< 50	< 50
	8	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	8	> 300 & < 400	> 300 & < 400	> 50 & < 100	> 300 & < 400
<u>Klebsiella spp.</u>	1	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400	1	> 500 < 600	> 500 < 600	> 500 < 600	> 500 < 600
	2	> 600	> 600	> 600	> 600	2	> 200 < 300	> 50 & < 100	> 50 & < 100	> 200 < 300
	3	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	3	> 200 & < 300	> 100 & < 200	> 100 & < 200	> 200 & < 300
	4	> 600	> 600	> 300 & < 400	> 600	4	> 300 & < 400	> 200 & < 300	> 200 & < 300	> 300 & < 400
<u>Staph aureus</u>	1	> 600	> 600	> 600	> 600	1	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
	2	> 600	> 600	> 600	> 600	2	> 600	> 600	> 600	> 600
<u>Pseudomonas spp.</u>	1	> 600	> 600	> 600	> 600	1	> 600	> 600	> 600	> 600
	2	> 600	> 600	> 600	> 600	2	> 600	> 600	> 600	> 600
<u>Salmonella spp.</u>	1	> 600	> 600	> 600	> 600	1	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100

MIC values determined in triplicate in each case.

Ca - Cholic Acid, Sd - Sodium Deoxycholate, Sta - Sodium Taurcholate

TABLE 7
EFFECT OF INDIVIDUAL BILE ACID TREATMENT ON MIC OF 4 ANTIBIOTICS
FOR DIFFERENT ORGANISMS

Organism	Strains Tested	Effect of	Number of Strains Showing Reduction in MIC of			
			Chl.	Tetr.	Str.	Neo.
<u>E.coli</u>	7	Cholic acid	4	2	5	2
		Sod. Deoxy.	3	3	5	2
		Sod. Taurch.	3	0	3	1
<u>Proteus</u> spp.	8	Cholic acid	2	4	2	2
		Sod. Deoxy.	2	4	2	3
		Sod. Taurch.	1	2	2	2
<u>Klebsiella</u> spp.	4	Cholic acid	1	1	0	3
		Sod. Deoxy	0	1	1	3
		Sod. Taurch.	0	0	0	0
<u>Staph aureus</u>	2	Cholic acid	0	0	0	0
		Sod. Deoxy.	0	0	0	0
		Sod. Taurch.	0	0	0	0
<u>Pseudomonas</u> spp.	2	Cholic acid	0	0	0	0
		Sod. Deoxy.	0	0	0	0
		Sod. Taurch.	0	0	0	0
<u>Salmonella</u> spp	1	Cholic acid	0	0	0	0
		Sod. Deoxy.	0	0	0	0
		Sod. Taurch.	0	0	0	0

Chl. - Chloramphenicol
Tetr. - Tetracycline
Str. - Streptomycin
Neo. - Neomycin

Sod. Deoxy - Sodium Deoxycholate Sod. Taurch - Sodium Taurcholate

(ii) cholic acid and sodium taurocholate (iii) sodium deoxycholate and sodium taurocholate.

The bacterial cells were treated with the above combination of bile acid and effect of such treatment on MIC of different antibiotics was evaluated as described earlier. Chloramphenicol, tetracycline, streptomycin and neomycin were used in the study. Tables (8, 9) give details of effect of treatment of combination of two bile acids on MIC of antibiotics.

As can be seen from Table (10) treatment of combination of two bile acids resulted in reduction of MIC of antibiotics for E.coli, Proteus spp. and Klebsiella spp. in decreasing order as far as the number of strains affected. For Staph aureus the MIC of tetracycline only was reduced. Pseudomonas aeruginosa and Salmonella spp. did not show any effect. However the number of these cultures tested was very small. Combination of cholic acid and sodium deoxycholate was found to be more effective in reduction of MIC of antibiotics than the other two combination as can be seen from the Table.

Study of Effect of Mixture of 3 Bile Acids on MIC of Antibiotics :

Twenty four out of total forty bacterial strains were selected for the study of effect of treatment of mixture of 3 bile acids on MIC of antibiotics. Equal amounts of cholic acid, sodium deoxycholate and sodium taurocholate were used to prepare 2.5 % bile acid containing broth.

The bacterial cells were treated with above said mixture of bile acids in broth and effect of such treatment on MIC of different antibiotics was evaluated as described earlier. Chloramphenicol, tetracycline, streptomycin and gentamycin were used in the study. MIC of treated test

EFFECT OF COMBINATION OF TWO BILE ACIDS ON MIC OF ANTIBIOTICS

Organism	Strain No	Chloramphenicol MIC				Strain No	Tetracycline MIC			
		Control	Ca + Sd	Ca + Sta	Sd + Sta		Control	Ca + Sd	Ca + Sta	Sd + Sta
<u>E.coli</u>	1	> 500 & < 600	> 200 & < 300	> 200 & < 300	> 200 & < 300	1	> 600	> 200 & < 300	> 600	> 200 & < 300
	2	> 500 & < 600	> 200 & < 300	> 500 & < 600	> 500 & < 600	2	> 600	> 600	> 600	> 600
	3	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	3	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
	4	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	4	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400
	5	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	5	> 600	> 600	> 600	> 600
	6	> 500 & < 600	> 200 & < 300	> 500 & < 600	> 500 & < 600	6	> 600	> 200 & < 300	> 200 & < 300	> 600
	7	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	7	> 600	> 600	> 600	> 600
<u>Proteus spp.</u>	1	> 500 & < 600	> 300 & < 400	> 300 & < 400	> 300 & < 400	1	> 300 & < 400	> 200 & < 300	> 300 & < 400	> 200 & < 300
	2	> 600	> 600	> 600	> 600	2	> 600	> 600	> 600	> 600
	3	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	3	> 600	> 600	> 600	> 600
	4	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	4	> 600	> 600	> 600	> 600
	5	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300	5	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
	6	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400	6	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600
	7	> 500 & < 600	> 200 & < 300	> 500 & < 600	> 500 & < 600	7	> 300 & < 400	> 200 & < 300	> 300 & < 400	> 200 & < 300
	8	> 600	> 600	> 600	> 600	8	> 600	> 600	> 600	> 600
<u>Klebsiella spp.</u>	1	> 600	> 600	> 600	> 600	1	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400
	2	> 600	> 600	> 600	> 600	2	> 600	> 600	> 600	> 600
	3	> 600	> 600	> 600	> 600	3	> 600	> 600	> 600	> 600
	4	> 200 & < 300	> 100 & < 200	> 200 & < 300	> 200 & < 300	4	> 500 & < 600	> 300 & < 400	> 300 & < 400	> 300 & < 400
<u>Staph aureus</u>	1	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	1	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
	2	> 600	> 600	> 600	> 600	2	> 600	> 600	> 600	> 600
<u>Pseudomonas spp.</u>	1	> 600	> 600	> 600	> 600	1	> 600	> 600	> 600	> 600
	2	> 600	> 600	> 600	> 600	2	> 600	> 600	> 600	> 600
<u>Salmonella spp.</u>	1	> 600	> 600	> 600	> 600	1	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400

MIC values determined in triplicate in each case.

Ca - Cholic Acid, Sd - Sodium Deoxycholate, Sta - Sodium Taurcholate

TABLE 9

EFFECT OF COMBINATION OF TWO BILE ACIDS ON MIC ANTIBIOTICS

Organism	Strain No.	Streptomycin MIC				Strain No.	Neomycin MIC			
		Control	Ca + Sd	Ca + Sta	Sd + Sta		Control	Ca + Sd	Ca + Sta	Sd + Sta
<u>E.coli</u>	1	> 600	> 600	> 600	> 600	1	> 200 & < 300	> 100 & < 200	> 100 & < 200	> 100 & < 200
	2	> 600	> 600	> 200 & < 300	> 600	2	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400
	3	> 600	> 200 & < 300	> 100 & < 200	> 100 & < 200	3	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
	4	> 600	> 600	> 600	> 600	4	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	5	> 600	> 300 & < 400	> 300 & < 400	> 300 & < 400	5	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
	6	> 600	> 200 & < 300	> 200 & < 300	> 200 & < 300	6	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	7	> 600	> 600	> 600	> 600	7	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
<u>Proteus spp.</u>	1	> 600	> 600	> 600	> 600	1	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
	2	> 600	> 600	> 600	> 600	2	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	3	> 600	> 600	> 600	> 600	3	> 100 & < 200	> 100 & < 200	> 100 & < 200	> 100 & < 200
	4	> 600	> 600	> 600	> 600	4	> 100 & < 200	> 100 & < 200	> 100 & < 200	> 100 & < 200
	5	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300	5	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	6	> 600	> 600	> 600	> 600	6	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600
	7	> 500 & < 600	> 300 & < 400	> 500 & < 600	> 500 & < 600	7	> 200 & < 300	> 10 & < 20	> 10 & < 20	> 10 & < 20
	8	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	8	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400
<u>Klebsiella spp</u>	1	> 300 & < 400	> 200 & < 300	> 200 & < 300	> 200 & < 300	1	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600
	2	> 600	> 600	> 600	> 600	2	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
	3	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	3	> 200 & < 300	> 100 & < 200	> 200 & < 300	> 200 & < 300
	4	> 600	> 600	> 200 & < 300	> 600	4	> 300 & < 400	> 200 & < 300	> 300 & < 400	> 300 & < 400
<u>Staph aureus</u>	1	> 600	> 600	> 600	> 600	1	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
	2	> 600	> 600	> 600	> 600	2	> 600	> 600	> 600	> 600
<u>Pseudomonas spp.</u>	1	> 600	> 600	> 600	> 600	1	> 600	> 600	> 600	> 600
	2	> 600	> 600	> 600	> 600	1	> 600	> 600	> 600	> 600
<u>Salmonella spp.</u>	1	> 600	> 600	> 600	> 600	1	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100

MIC values determined in triplicate in each case.

Ca - Cholic Acid, Sd - Sodium Deoxycholate, Sta - Sodium Taurcholate

TABLE 10
EFFECT OF COMBINATION OF 2 BILE ACIDS COMBINATION
TREATMENT ON MIC OF 4 ANTIBIOTICS FOR DIFFERENT ORGANISMS

Organism	Strains Tested	Effect of	Number of Strains Showing Reduction in MIC of			
			Chl.	Tetr.	Str.	Neo.
<u>E.coli</u>	7	Ca + Sd	3	2	3	1
		Ca + Sta	1	1	4	1
		Sd + Sta	1	1	3	1
<u>Proteus spp.</u>	8	Ca + Sd	2	2	1	1
		Ca + Sta	1	0	0	1
		Sd + Sta	1	2	0	1
<u>Klebsiella spp.</u>	4	Ca + Sd	1	1	1	2
		Ca + Sta	0	1	2	0
		Sd + Sta	0	1	1	0
<u>Staph aureus</u>	2	Ca + Sd	0	0	0	0
		Ca + Sta	0	0	0	0
		Sd + Sta	0	0	0	0
<u>Pseudomonas spp.</u>	2	Ca + Sd	0	0	0	0
		Ca + Sta	0	0	0	0
		Sd + Sta	0	0	0	0
<u>Salmonella spp.</u>	1	Ca + Sd	0	0	0	0
		Ca + Sta	0	0	0	0
		Sd + Sta	0	0	0	0

Chl. - Chloramphenicol
Tetr. - Tetracycline
Str. - Streptomycin
Neo. - Neomycin

Ca - Cholic Acid, Sd - Sodium Deoxycholate, Sta - Sodium Tarucholate

and untreated control cultures was determined each in triplicate for all the 4 antibiotics. As before MIC values of test and control were compared by Mann-Whitney test.

Table (11) gives the details of effect of mixture of bile acids on MIC of 4 antibiotics for the cultures affected.

As can be seen from Table (12) treatment with mixture of 3 bile acids resulted in reduction of MIC of antibiotics for E.coli, Proteus spp., Klebsiella spp. and Staph aureus in decreasing order as far as the number of strains affected. Pseudomonas aeruginosa did not show any effect, 3 bile acid combination appeared to be more effective in reducing MIC of streptomycin than that of other antibiotics. MIC of tetracycline was reduced only in one strain of E.coli.

STUDY OF EFFECT OF MAGNESIUM IONS ON MIC OF ANTIBIOTICS:

Twenty four out of total forty bacterial strains were studied for effect of magnesium ions on MIC of antibiotics.

Bacterial cells were treated with 1 mg/ml $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in broth at 37°C for 3 h and effect of such treatment on MIC of four antibiotics was evaluated as described earlier. MIC of treated test and untreated control cultures was determined each in triplicate for all the antibiotics. As before MIC values of test and control were compared by Mann-Whitney test. Chloramphenicol, tetracycline, streptomycin and neomycin were the antibiotics used in study.

Table (13) gives details of effect of magnesium ions on MIC of four antibiotics for the cultures affected.

Table (14) shows that treatment with magnesium ions resulted

TABLE 11

EFFECT OF MIXTURE OF 3 BILE ACIDS (Cd + Sd + Sta) ON MIC OF ANTIBIOTICS

Organism	Strain No.	Chloramphenicol MIC		Strain No.	Tetracycline MIC		Strain No.	Streptomycin MIC		Strain No.	Gentamycin MIC	
		Control	Test		Control	Test		Control	Test		Control	Test
<u>E.coli</u>	1	> 500 & < 600	> 500 & < 600	1	> 600	> 200 & < 300	1	> 600	> 200 & < 300	1	> 50 & < 100	> 50 & < 100
	2	> 500 & < 600	> 500 & < 600	2	> 600	> 600	2	> 600	> 600	2	> 50 & < 100	> 50 & < 100
	3	> 500 & < 600	> 400 & < 500 &	3	> 200 & < 300	> 200 & < 300	3	> 600	> 200 & < 300	3	> 50 & < 100	> 50 & < 100
	4	> 500 & < 600	> 200 & < 300	4	> 300 & < 400	> 300 & < 400	4	> 600	> 600	4	> 50 & < 100	> 50 & < 100
	5	> 500 & < 600	> 500 & < 600	5	> 600	> 600	5	> 600	> 200 & < 300	5	> 200 & < 300	> 200 & < 300
	6	> 500 & < 600	> 500 & < 600	6	> 600	> 600	6	> 600	> 300 & < 400	6	> 50 & < 100	> 50 & < 100
	7	> 500 & < 600	> 500 & < 600	7	> 600	> 600	7	> 600	> 600	7	> 600	> 600
<u>Proteus spp.</u>	1	> 500 & < 600	> 300 & < 400	1	> 300 & < 400	> 300 & < 400	1	> 600	> 500 & < 600	1	> 100 & < 200	> 100 & < 200
	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 100 & < 200	> 100 & < 200
	3	> 500 & < 600	> 200 & < 300	3	> 600	> 600	3	> 600	> 600	3	> 200 & < 300	> 200 & < 300
	4	> 500 & < 600	> 500 & < 600	4	> 600	> 600	4	> 600	> 600	4	> 500 & < 600	> 200 & < 300
	5	> 200 & < 300	> 200 & < 300	5	> 200 & < 300	> 200 & < 300	5	> 200 & < 300	> 200 & < 300	5	> 50 & < 100	> 50 & < 100
	6	> 300 & < 400	> 300 & < 400	6	> 500 & < 600	> 500 & < 600	6	> 600	> 600	6	> 200 & < 300	> 200 & < 300
	7	> 500 & < 600	> 500 & < 600	7	> 300 & < 400	> 300 & < 400	7	> 500 & < 600	> 200 & < 300	7	> 300 & < 400	> 50 & < 100
	8	> 600	> 600	8	> 600	> 600	8	> 500 & < 600	> 500 & < 600	8	> 100 & < 200	> 100 & < 200
	9	> 600	> 600	9	> 600	> 600	9	> 300 & < 400	> 300 & < 300	9	> 100 & < 200	> 50 & < 100
<u>Klebsiella spp.</u>	1	> 600	> 600	1	> 300 & < 400	> 300 & < 400	1	> 300 & < 400	> 300 & < 400	1	> 50 & < 100	> 50 & < 100
	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 200 & < 300	> 50 & < 100
	3	> 600	> 600	3	> 600	> 600	3	> 500 & < 600	> 500 & < 600	3	> 500 & < 600	> 400 & < 500
	4	> 200 & < 300	> 200 & < 300	4	> 500 & < 600	> 500 & < 600	4	> 600	> 200 < 300	4	> 400 & < 500	> 200 & < 300
<u>Staph aureus</u>	1	> 500 & < 600	> 500 & < 600	1	> 200 & < 300	> 200 & < 300	1	> 600	> 500 & < 600	1	> 200 & < 300	> 50 & < 100
	2	> 600	> 300 & < 400	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600
<u>Pseudomonas spp.</u>	1	> 600	> 600	1	> 600	> 600	1	> 600	> 600	1	> 50 & < 100	> 50 & < 100
	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 500 & < 600	> 500 & < 600

MIC values determined in triplicate in each case.

Ca - Cholic Acid, Sd - Sodium Deoxycholate, Sta - Sodium Taurcholate.

TABLE 12

EFFECT OF MIXTURE OF 3 BILE ACIDS (Ca + Sd + Sta) ON MIC OF 4
ANTIBIOTICS FOR DIFFERENT ORGANISMS

Sr. No.	Organism	Strains Tested	Number of Strains Showing Reduction In MIC of			
			Chl.	Tetr.	Str.	Gentamycin
1	<u>E.coli</u>	7	2	1	4	0
2	<u>Proteus</u> spp.	9	2	0	3	3
3	<u>Klebsiella</u> spp.	4	0	0	1	3
4	<u>Staph aureus</u>	2	1	0	1	1
5	<u>Pseudomonas</u> spp.	2	0	0	0	0

Chl. - Chloramphenicol

Tetr. - Tetracycline

Str. - Streptomycin



TABLE 13

EFFECT OF MAGNESIUM IONS ON MIC OF ANTIBIOTICS

Organism	Strain No.	Chloramphenicol MIC		Strain No.	Tetracycline MIC		Strain No.	Streptomycin MIC		Strain No.	Neomycin MIC	
		Control	Test		Control	Test		Control	Test		Control	Test
<u>E. coli</u>	1	> 200 & < 300	> 500 & < 600	1	> 600	> 600	1	> 600	> 600	1	> 200 & < 300	> 100 && < 200
	2	> 500 & < 600	> 500 & < 600	2	> 600	> 600	2	> 600	> 600	2	> 300 & < 400	> 300 & < 400
	3	> 500 & < 600	> 500 & < 600	3	> 200 & < 300	> 200 & < 300	3	> 500 & < 600	> 600	3	> 200 & < 300	> 600
	4	> 500 & < 600	> 500 & < 600	4	> 300 & < 400	> 300 & < 400	4	> 600	> 600	4	> 50 & < 100	> 50 & < 100
	5	> 500 & < 600	> 600	5	> 600	> 600	5	> 600	> 600	5	> 200 & < 300	> 200 & < 300
	6	> 500 & < 600	> 600	6	> 600	> 600	6	> 600	> 600	6	> 50 & < 100	> 50 & < 100
<u>Proteus spp.</u>	1	> 500 & < 600	> 500 & < 600	1	> 300 & < 400	> 200 & < 300	1	> 600	> 600	1	> 200 & < 300	> 200 & < 300
	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 50 & < 100	> 50 & < 100
	3	> 500 & < 600	> 600	3	> 600	> 600	3	> 600	> 600	3	> 100 & < 200	> 100 & < 200
	4	> 500 & < 600	> 500 & < 600	4	> 600	> 600	4	> 600	> 600	4	> 100 & < 200	> 100 & < 200
	5	> 200 & < 300	> 200 & < 300	5	> 200 & < 300	> 200 & < 300	5	> 200 & < 300	> 200 & < 300	5	> 50 & < 100	> 50 & < 100
	6	> 300 & < 400	> 300 & < 400	6	> 500 & < 600	> 500 & < 600	6	> 600	> 600	6	> 500 & < 600	> 200 & < 300
	7	> 500 & < 600	> 500 & < 600	7	> 300 & < 400	> 300 & < 400	7	> 500 & < 600	> 500 & < 600	7	> 200 & < 300	> 200 & < 300
	8	> 600	> 500 & < 600	8	> 600	> 500 & < 600	8	> 500 & < 600	> 500 & < 600	8	> 300 & < 400	> 100 & < 200
	9	> 600	> 200 & < 300	9	> 600	> 400 & < 500	9	> 300 & < 400	> 300 & < 400	9	> 300 & < 400	> 300 & < 400
<u>Klebsiella spp.</u>	1	> 600	> 600	1	> 300 & < 400	> 500 & < 600	1	> 300 & < 400	> 300 & < 400	1	> 500 & < 600	> 300 & < 400
	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 200 & < 300	> 200 & < 300
	3	> 600	> 600	3	> 600	> 600	3	> 500 & < 600	> 500 & < 600	3	> 200 & < 300	> 200 & < 300
	4	> 200 & < 300	> 200 & < 300	4	> 500 & < 600	> 500 & < 600	4	> 600	> 600	4	> 300 & < 400	> 300 & < 400
<u>Pseudomonas spp.</u>	1	> 600	> 600	1	> 600	> 600	1	> 600	> 600	1	> 600	> 600
	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600
	3	> 600	> 600	3	> 600	> 600	3	> 600	> 600	3	> 50 & < 100	> 50 & < 100
<u>Staph aureus</u>	1	> 500 & < 600	> 300 & < 400	1	> 200 & < 300	> 100 & < 200	1	> 600	> 500 & < 600	1	> 200 & < 300	> 200 & < 300
	2	> 600	> 200 & < 300	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600

MIC values determined in triplicate in each case.

TABLE 14
EFFECT OF MAGNESIUM IONS ON MIC OF 4 ANTIBIOTICS
OF DIFFERENT ORGANISMS

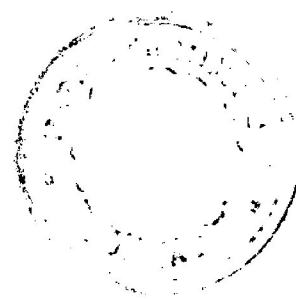
Sr. No.	Organism	Strains Tested	Number of Strains Showing Effect On MIC of			
			Chl.	Tetr.	Str.	Neo.
1	<u>E.coli</u>	6	3	0	1	2
2	<u>Proteus</u> spp.	9	3	3	0	2
3	<u>Klebsiella</u> spp.	4	0	1	0	1
4	<u>Pseudomonas</u> spp.	3	0	0	0	0
5	<u>Staph. aureus</u>	2	2	1	1	0

Chl. - Chloramphenicol

Tetr. - Tetracycline

Str. - Streptomycin

Neo. - Neomycin



in change of MIC of 4 antibiotics for Proteus spp., E.coli, Staph aureus and Kiebsiella spp. in decreasing order as far as the number of strains affected. Pseudomonas aerugenosa did not show any effect for any antibiotic .

As can be seen from Table (13) effect of magnesium ions was quite variable. Three strains of E.coli and one strain of Proteus spp. showed increase in MIC of chloramphenicol whereas 2 strains of Proteus spp. and 2 of Staph aureus showed decrease in MIC of chloramphenicol. Three strains of Proteus spp. and, one Staph aureus showed decrease in MIC of tetracycline whereas Klebsiella spp. shows increase in MIC of tetracycline. In case of streptomycin, one E.coli strain showed increase in MIC whereas one Staph aureus showed decrease in MIC.

As far as MIC of neomycin is concerned Proteus spp. Klebisella spp. and one strain of E.coli showed decrease and only 1 E.coli strain showed increase in MIC with magnesium ions.

STUDY OF EFFECT OF PHOSPHATE IONS ON MIC OF ANTIBIOTICS :

Twenty four out of total forty bacterial strains were studied for effect of phosphate ions on MIC of 4 antibiotics. Chloramphenicol, tetracycline, streptomycin and neomycin were the antibiotics studied.

Bacterial cells were treated with 1 mg/ml of K_2HPO_4 in broth at $37^{\circ}C$ for 3 h and effect of such treatment on MIC of antibiotics was evaluated as described earlier. MIC of treated test and untreated control cultures was determined each in triplicate for all the 4 antibiotics. As before MIC values of test and control were compared by Mann-Whitney test.

Table (15) gives details of effect of phosphate ions on MIC of 4 antibiotics for the cultures affected.

Table (16) shows that overall the effect of phosphate ions on MIC of antibiotics was quite of low order. And amongst them Proteus spp. E.coli, Klebsiella spp. and Staph aureus responded in decreasing order. Pseudomonas aeruginosa did not show any effect.

As can be seen from Table (15) there was no effect of phosphate ions on MIC of streptomycin in any case. MIC of neomycin increased in all the cultures responding to phosphate ions treatment. MIC of tetracycline decreased in all the cases responding to phosphate ions treatment. MIC of chloramphenicol decreased in all the responding cultures except one strain of Proteus spp. where increase was observed.

STUDY OF EFFECT OF PROTEOLYTIC ENZYMES ON MIC OF ANTIBIOTICS

Twenty four out of total forty bacterial strains were studied for the effect of proteolytic enzymes on MIC of antibiotics. Commercial preparations of (i) Pancreatin from pig pancreas activity equivalent to NF supplied by Loba-Cheme Indoaustral Co. and contains amylase as well as proteases. (ii) Trypsin (supplied by Hi-Media and contains bovine 0.2 Anson U/gm or about, 20000 full gross units/gm). (iii) Papain (supplied by Research Laboratory, Bombay) were used in the study. The concentration of these enzymes employed for treatment was 1 mg/ml prepared in buffered saline. Chloramphenicol, tetracycline, streptomycin and gentamycin were used in the study.

Bacterial cells were treated with above said enzyme solutions for 1 h at 37 °C after which the bacterial cells were washed and effect

TABLE 15

EFFECCT OF PHOSPHATE IONS ON MIC OF ANTIBIOTICS

Organism	Strain No.	Chloramphenicol MIC		Strain No.	Tetracycline MIC		Strain No.	Streptomycin MIC		Strain No.	Neomycin MIC	
		Control	Test		Control	Test		Control	Test		Control	Test
<u>E.coli</u>	1	> 500 & < 600	> 300 & < 400	1	> 600	> 300 & < 400	1	> 600	> 600	1	> 200 & < 300	> 500 & < 600
	2	> 500 & < 600	> 500 & < 600	2	> 600	> 600	2	> 600	> 600	2	> 300 & < 400	> 500 & < 600
	3	> 500 & < 600	> 500 & < 600	3	> 300 & < 400	> 100 & < 200	3	> 500 & < 600	> 500 & < 600	3	> 200 & < 300	> 600
	4	> 500 & < 600	> 500 & < 600	4	> 300 & < 400	> 300 & < 400	4	> 600	> 600	4	> 50 & < 100	> 50 & < 100
	5	> 500 & < 600	> 500 & < 600	5	> 600	> 600	5	> 600	> 600	5	> 200 & < 300	> 200 & < 300
	6	> 500 & < 600	> 500 & < 600	6	> 600	> 600	6	> 600	> 600	6	> 50 & < 100	> 300 & < 400
<u>Proteus spp.</u>	1	> 500 & < 600	> 300 & < 400	1	> 300 & < 400	> 200 & < 300	1	> 600	> 600	1	> 200 & < 300	> 200 & < 300
	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 50 & < 100	> 50 & < 100
	3	> 500 & < 600	> 500 & < 600	3	> 600	> 600	3	> 600	> 600	3	> 100 & < 200	> 300 & < 400
	4	> 500 & < 600	> 500 & < 600	4	> 600	> 600	4	> 600	> 600	4	> 100 & < 200	> 100 & < 200
	5	> 200 & < 300	> 200 & < 300	5	> 200 & < 300	> 200 & < 300	5	> 200 & < 300	> 200 & < 300	5	> 50 & < 100	> 50 & < 100
	6	> 300 & < 400	> 300 & < 400	6	> 500 & < 600	> 500 & < 600	6	> 600	> 600	6	> 500 & < 600	> 500 & < 600
	7	> 500 & < 600	> 600	7	> 300 & < 400	> 200 & < 300	7	> 500 & < 600	> 500 & < 600	7	> 300 & < 400	> 500 & < 600
	8	> 600	> 600	8	> 600	> 600	8	> 500 & < 600	> 500 & < 600	8	> 300 & < 400	> 500 & < 600
	9	> 600	> 600	9	> 600	> 600	9	> 300 & < 400	> 300 & < 400	9	> 300 & < 400	> 300 & < 400
<u>Klebsiella spp.</u>	1	> 600	> 600	1	> 300 & < 400	> 300 & < 400	1	> 300 & < 400	> 300 & < 400	1	> 300 & < 400	> 500 & < 600
	2	> 600	> 600	2	> 600	> 500 & < 600	2	> 600	> 600	2	> 500 & < 600	> 600
	3	> 600	> 600	3	> 600	> 600	3	> 500 & < 600	> 500 & < 600	3	> 200 & < 300	> 200 & < 300
	4	> 200 & < 300	> 200 & < 300	4	> 500 & < 600	> 500 & < 600	4	> 600	> 600	4	> 300 & < 400	> 300 & < 400
<u>Pseudomonas spp.</u>	1	> 600	> 600	1	> 600	> 600	1	> 600	> 600	1	> 600	> 600
	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600
	3	> 600	> 600	3	> 600	> 600	3	> 600	> 600	3	> 50 & 100	> 50 & 100
<u>Staph aureus</u>	1	> 500 & < 600	> 500 & < 600	1	> 200 & < 300	> 100 & < 200	1	> 600	> 600	1	> 200 & < 300	> 200 & < 300
	2	> 600	> 500 & < 600	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600

MIC values determined in triplicate in each case.

TABLE 16
EFFECT OF PHOSPHATE IONS ON MIC OF 4 ANTIBIOTICS
OF DIFFERENT ORGANISMS

Sr. No.	Organism	Strains Tested	Number of Strains Showing Effect on MIC of			
			Chl.	Tetr.	Str.	Neo.
1	<u>E.coli</u>	6	1	2	0	4
2	<u>Proteus</u> spp.	9	2	2	0	3
3	<u>Klebsiella</u> spp.	4	0	1	0	2
4	<u>Pseudomonas</u> spp.	3	0	0	0	0
5	<u>Staph aureus</u>	2	1	1	0	0

Chl. - Chloramphenicol

Tetr. - Tetracycline

Str. - Streptomycin

Neo. - Neomycin

of such treatment, on MIC of 4 antibiotics was evaluated separately for each of the enzymes. Procedure followed for this evaluation of MIC was as described earlier. Untreated cultures were simultaneously studied as control and received similar steps of treatment except the use of enzyme solution. MIC values of control and test were compared by Mann-Whitney test.

Tables (17,18) give details of the effect of the 3 enzymes on MIC of 4 antibiotics.

As can be seen from Table (19) treatment with proteolytic enzymes resulted in reduction of MIC of 4 antibiotics for Proteus spp Klebsiella spp., Pseudomonas spp., Staph aureus and E.coli in decreasing order as far as the number of strains affected.

Tables (17,18) show that papain, pancreatin and trypsin were effective in reduction of MIC of 4 antibiotics in the order mentioned as far as number of strains affected is concerned. Effect of the enzyme treatment on the MIC of tetracycline and chloramphenicol was greater than that in MIC of streptomycin and gentamycin.

STUDY OF EFFECT OF POTASSIUM CHLORIDE (KCl) ON MIC OF ANTIBIOTICS

Twenty four out of forty bacterial strains were studied for effect of KCl on MIC of 4 antibiotics. Chloramphenicol, tetracycline, streptomycin and gentamycin were the 4 antibiotics studied.

Bacterial cells were treated with 1 mg/ml of KCl in broth by incubating at 37 °C for 3 h. On such treated cultures MIC was determined to evaluate effect of treatment as described earlier MIC for treated test and untreated control was found out each in triplicate for

TABLE - 17
EFFECT OF PROTEOLYTIC ENZYMES ON MIC OF ANTIBIOTICS

Strain No.	Chloramphenicol MIC				Strain No.	Tetracycline MIC			
	Control	Pancreatin	Papain	Trypsin		Control	Pancreatin	Papain	Trypsin
1	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	1	> 600	> 600	> 600	> 600
2	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	2	> 600	> 600	> 600	> 600
3	> 500 & < 600	> 300 & < 400	> 200 & < 300	> 500 & < 600	3	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
4	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	4	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400
5	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	5	> 600	> 600	> 600	> 600
6	> 500 & < 600	> 500 & < 600	> 300 & < 400	> 500 & < 600	6	> 600	> 600	> 600	> 600
1	> 500 & < 600	> 200 & < 300	> 100 & < 200	> 100 & < 200	1	> 300 & < 400	> 200 & < 300	> 200 & < 300	> 200 & < 300
2	> 600	> 600	> 600	> 600	2	> 600	> 500 & < 600	> 400 & < 500	> 500 & < 600
3	> 300 & < 400	> 100 & < 200	> 100 & < 200	> 300 & < 400	3	> 600	> 600	> 500 & < 600	> 600
4	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	4	> 600	> 500 & < 600	> 500 & < 600	> 500 & < 600
5	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300	5	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
6	> 300 & < 400	> 200 & < 300	> 200 & < 300	> 200 & < 300	6	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600
7	> 600	> 200 & < 300	> 500 & < 600	> 500 & < 600	7	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400
8	> 600	> 600	> 600	> 600	8	> 600	> 600	> 600	> 600
9	> 600	> 600	> 600	> 600	9	> 600	> 600	> 600	> 600
la 1	> 600	> 300 & < 400	> 100 & < 200	> 600	1	> 300 & < 400	> 200 & < 300	> 200 & < 300	> 200 & < 300
2	> 600	> 600	> 600	> 600	2	> 600	> 600	> 600	> 600
3	> 600	> 600	> 600	> 600	3	> 600	> 600	> 600	> 600
4	> 200 & < 300	> 100 & < 200	> 100 & < 200	> 200 & < 300	4	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600
nas 1	> 600	> 600	> 600	> 600	1	> 600	> 500 & < 600	> 500 & < 600	> 500 & < 600
2	> 600	> 600	> 600	> 600	2	> 600	> 500 & < 600	> 500 & < 600	> 600
4	> 600	> 600	> 600	> 600	4	> 600	> 200 & < 300	> 200 & < 300	> 200 & < 300
reus 1	> 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	1	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
2	> 600	> 500 & < 600	> 500 & < 600	> 600	2	> 600	> 600	> 600	> 600

MIC values determined in triplicate in each case.

TABLE - 18

EFFECT OF PROTEOLYTIC ENZYMES ON MIC OF ANTIBIOTICS

Organism	Strain No.	Streptomycin MIC				Strain No.	Gentamycin MIC			
		Control	Pancreatin	Papain	Trypsin		Control	Pancreatin	Papain	Trypsin
<u>E.coli</u>	1	> 600	> 600	> 600	> 600	1	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	2	> 600	> 600	> 600	> 600	2	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	3	> 600	> 600	> 600	> 600	3	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	4	> 600	> 600	> 600	> 600	4	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	5	> 600	> 600	> 600	> 600	5	> 200 & < 300	> 100 & < 200	> 200 & < 300	> 100 & < 200
	6	> 600	> 600	> 600	> 600	6	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
<u>Proteus spp.</u>	1	> 600	> 600	> 600	> 600	1	> 100 & < 200	> 100 & < 200	> 100 & < 200	> 100 & < 200
	2	> 600	> 600	> 600	> 600	2	> 100 & < 200	> 100 & < 200	> 100 & < 200	> 100 & < 200
	3	> 600	> 600	> 600	> 600	3	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
	4	> 600	> 600	> 600	> 600	4	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600
	5	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300	5	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	6	> 600	> 600	> 600	> 600	6	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 100 & < 200
	7	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	7	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400
	8	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	8	> 100 & < 200	> 100 & < 200	> 100 & < 200	> 100 & < 200
	9	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400	9	> 500 & < 600	> 200 & < 300	> 200 & < 300	> 200 & < 300
<u>Kiebsiella spp.</u>	1	> 300 & < 400	> 200 & < 300	> 200 & < 300	> 300 & < 400	1	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	2	> 600	> 600	> 600	> 600	2	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 7 < 300
	3	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	3	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600
	4	> 600	> 600	> 600	> 600	4	> 400 & < 500	> 400 & < 500	> 400 & < 500	> 400 & < 500
<u>Pseudomonas spp.</u>	1	> 600	> 600	> 600	> 600	1	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	2	> 600	> 600	> 600	> 600	2	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600
	4	> 600	> 600	> 600	> 600	3	< 50	< 50	< 50	< 50
<u>Staph</u>	1	> 600	> 600	> 600	> 600	1	> 500 & < 600	> 300 & < 400	> 300 & < 400	> 300 & < 400
	2	> 600	> 600	> 600	> 600	2	> 600	> 600	> 600	> 600

MIC values determined in triplicate in each case.

TABLE 19
EFFECT OF TREATMENT WITH PROTEOLYTIC ENZYMES ON MIC OF 4
ANTIBIOTICS FOR DIFFERENT ORGANISMS

Organism	Strains	Effect	Number of Strains Showing Reduction in MIC of			
			Chl.	Tetr.	Str.	Gen.
<u>E.coli</u>	6	Pancreatin	1	0	0	1
		Papain	2	0	0	0
		Trypsin	0	0	0	1
<u>Proteus</u> spp.	9	Pancreatin	4	3	0	1
		Papain	4	4	0	1
		Trypsin	3	3	0	2
<u>Klebsiella</u> spp.	4	Pancreatin	2	1	1	0
		Papain	2	1	1	0
		Trypsin	0	1	0	0
<u>Pseudomonas</u> spp.	4	Pancreatin	0	3	0	0
		Papain	0	3	0	0
		Trypsin	0	2	0	0
<u>Staph aureus</u>	2	Pancreatin	2	0	0	1
		Papain	2	0	0	1
		Trypsin	1	0	0	1

Chl. - Chloramphenicol
Tetr - Tetracycline
Str. - Streptomycin
Gen. - Gentamycin



all the 4 antibiotics. As before MIC values of test and control were compared by Mann-Whitney test.

Table (20) gives details of effect of KCl on MIC of 4 antibiotics.

Table (21) shows that, overall there were less incidences of effect of KCl on MIC of antibiotics. And amongst them Klebsiella spp., E.coli, Proteus spp., Staph aureus and Pseudomonas spp. responded in decreasing order as far as number of strains affected.

As can be seen from Table (20) MIC of chloramphenicol increased for Klebsiella spp. and Staph aureus whereas that for E.coli strain decreased with KCl treatment. MIC of tetracycline increased for Staph aureus and Pseudomonas aeruginosa with KCl treatment, whereas no effect, on other cultures. MIC of streptomycin increased for Klebsiella spp. MIC of gentamycin increased for Proteus spp., Klebsiella spp. and E.coli strains. One E.coli strain however showed decreased in MIC of gentamycin.

STUDY OF EFFECT OF EDTA (Ethylene Diamine Tetra-acetate) ON MIC OF ANTIBIOTICS :

Twenty four out of total forty bacterial strains were studied for effect of EDTA on MIC of antibiotics.

Bacterial cells were treated with 2 mg/ml of EDTA in broth at 37 °C for 3 h and effect of such treatment on MIC of 4 antibiotics was evaluated as described earlier. MIC of treated test and untreated control cultures was determined each in triplicate for all the antibiotics. As before MIC values of test and control were compared by Mann-Whitney test. Chloramphenicol, tetracycline, streptomycin and gentamycin were

TABLE 20

EFFECT OF KCl ON MIC OF ANTIBIOTICS

Strain No.	Chloramphenicol MIC		Strain No.	Tetracycline MIC		Strain No.	Streptomycin MIC		Strain No.	Gentamycin MIC	
	Control	Test		Control	Test		Control	Test		Control	Test
1	> 500 & < 600	> 200 & < 300	1	> 600	> 600	1	> 600	> 600	1	> 50 & < 100	> 200 & < 300
2	> 500 & < 600	> 500 & < 600	2	> 600	> 600	2	> 600	> 600	2	> 50 & < 100	> 50 & < 100
3	> 500 & < 600	> 500 & < 600	3	> 200 & < 300	> 200 & < 300	3	> 600	> 600	3	> 50 & < 100	> 50 & < 100
4	> 500 & < 600	> 500 & < 600	4	> 300 & < 400	> 300 & < 400	4	> 600	> 600	4	> 50 & < 100	> 300 & < 400
5	> 500 & < 600	> 500 & < 600	5	> 600	> 600	5	> 600	> 600	5	> 50 & < 100	> 100 & < 200
6	> 500 & < 600	> 500 & < 600	6	> 600	> 600	6	> 600	> 600	6	> 50 & < 100	> 50 & < 100
7	> 500 & < 600	> 500 & < 600	7	> 600	> 600	7	> 600	> 600	7	> 600	> 600
1	> 500 & < 600	> 500 & < 600	1	> 300 & < 400	> 300 & < 400	1	> 600	> 600	1	> 50 & < 100	> 200 & < 300
2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 100 & < 200	> 100 & < 200
3	> 500 & < 600	> 500 & < 600	3	> 600	> 600	3	> 600	> 600	3	> 200 & < 300	> 200 & < 300
4	> 500 & < 600	> 500 & < 600	4	> 600	> 600	4	> 600	> 600	4	> 500 & < 600	> 500 & < 600
5	> 200 & < 300	> 200 & < 300	5	> 200 & < 300	> 200 & < 300	5	> 200 & < 300	> 200 & < 300	5	> 50 & < 100	> 50 & < 100
6	> 300 & < 400	> 300 & < 400	6	> 500 & < 600	> 500 & < 600	6	> 600	> 600	6	> 200 & < 300	> 200 & < 300
7	> 500 & < 600	> 500 & < 600	7	> 300 & < 400	> 300 & < 400	7	> 500 & < 600	> 500 & < 600	7	> 300 & < 400	> 300 & < 400
8	> 600	> 600	8	> 600	> 600	8	> 500 & < 600	> 500 & < 600	8	> 100 & < 200	> 100 & < 200
9	> 600	> 600	9	> 600	> 600	9	> 300 & < 400	> 300 & < 400	9	> 500 & < 600	> 50 & < 100
1a	> 600	> 600	1	> 300 & < 400	> 300 & < 400	1	> 300 & < 400	> 300 & < 400	1	> 50 & < 100	> 50 & < 100
2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 200 & < 300	> 200 & < 300
3	> 600	> 600	3	> 600	> 600	3	> 500 & < 600	> 500 & < 600	3	> 500 & < 600	> 500 & < 600
4	> 200 & > 300	> 600	4	> 500 & < 600	> 500 & < 600	4	> 600	> 600	4	> 200 & < 300	> 300 & < 400
5	> 200 & < 200 &	> 600	5	> 50 & < 100	> 50 & < 100	5	> 100 & < 200	> 600	5	> 50 & < 100	> 300 & < 400
1	> 400 & < 600	> 600	1	> 200 & < 300	> 500 & < 600	1	> 600	> 600	1	> 200 & < 300	> 200 & < 300
2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600
onas 1	> 600	> 600	1	> 400 < 500	> 500 < 600	1	> 600	> 600	1	> 500 & < 600	> 500 & < 600

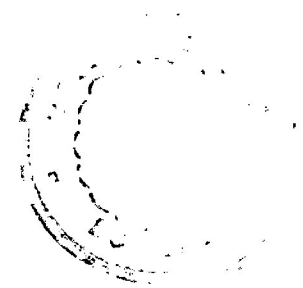
MIC values determined in triplicate in each case

TABLE 21

EFFECT OF KCl TREATMENT ON MIC OF 4 ANTIBIOTICS FOR
DIFFERENT ORGANISMS

Sr. No.	Organism	Strains Tested	Number of Strains Showing Effect on MIC of			
			Chl.	Tetr.	Str.	Gentamycin
1	<u>E.coli</u>	7	1	0	0	3
2	<u>Proteus</u> spp.	9	0	0	0	2
3	<u>Klebsiella</u> spp	5	2	0	1	2
4	<u>Staph aureus</u>	2	1	1	0	0
5	<u>Pseudomonas</u> spp.	1	0	1	0	0

Chl. - Chloramphenicol
Tetr - Tetracycline
Str. - Streptomycin



used in the study.

Table (22) gives details of effect of EDTA on MIC of 4 antibiotics for the cultures affected.

Table (23) shows that treatment with EDTA resulted in change of MIC of 4 antibiotics for Proteus spp., Klebsiella spp., E.coli, Staph aureus and Pseudomonas aeruginosa in decreasing order as far as number of strains affected. However overall number of incidences were few.

Table (22) shows that effect of EDTA treatment was of mixed nature and either increase or decrease was observed. E.coli and Klebsiella spp. showed decrease in MIC for chloramphenicol and tetracycline. Staph aureus showed increase in MIC for chloramphenicol and tetracycline. Also Proteus spp. shows increase in MIC for tetracycline, streptomycin and gentamycin (with one exception). Pseudomonas aeruginosa shows increase in MIC for tetracycline while E.coli shows increase in MIC for gentamycin.

STUDY OF EFFECT OF Fe^{++} IONS ON MIC OF ANTIBIOTICS :

Twenty four out of total forty bacterial strains were studied for effect of Fe^{++} ions on MIC of 4 antibiotics.

Bacterial cells were treated with 2 mg/ml of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in broth at 37°C for 3 h and effect of such treatment on MIC of 4 antibiotics was evaluated as described earlier. MIC of treated test and untreated control cultures was determined each in triplicate for all the 4 antibiotics. As before MIC values of test and control were compared by Mann-Whitney test. Chloramphenicol, tetracycline, streptomycin and gentamycin were used in the study.

- 71 -
TABLE 22

EFFECT OF EDTA ON MIC OF ANTIBIOTICS

Organism	Strain No.	Chloramphenicol MIC		Strain No.	Tetracycline MIC		Strain No.	Streptomycin MIC		Strain No.	Gentamycin MIC	
		Control	Test		Control	Test		Control	Test		Control	Test
<u>E.coli</u>	1	> 500 & < 600	> 200 & < 300	1	> 600	> 600	1	> 600	> 600	1	> 50 & < 100	> 100 & < 200
	2	> 500 & < 600	> 500 & < 600	2	> 600	> 600	2	> 600	> 600	2	> 50 & < 100	> 50 & < 100
	3	> 500 & < 600	> 500 & < 600	3	> 200 & < 300	> 200 & < 300	3	> 600	> 600	3	> 50 & < 100	> 50 & < 100
	4	> 500 & < 600	> 500 & < 600	4	> 300 & < 400	> 300 & < 400	4	> 600	> 600	4	> 50 & < 100	> 50 & < 100
	5	> 500 & < 600	> 500 & < 600	5	> 600	> 600	5	> 600	> 600	5	> 200 & < 300	> 200 & < 300
	6	> 500 & < 600	> 500 & < 600	6	> 600	> 600	6	> 600	> 600	6	> 50 & < 100	> 50 & < 100
	7	> 500 & < 600	> 500 & < 600	7	> 600	> 600	7	> 600	> 600	7	> 600	> 600
<u>Proteus spp.</u>	1	> 500 & < 600	> 500 & < 600	1	> 300 & < 400	> 300 & < 400	1	> 600	> 600	1	> 50 & < 100	> 200 & < 300
	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 100 & < 200	> 500 & < 600
	3	> 300 & < 400	> 300 & < 400	3	> 600	> 600	3	> 600	> 600	3	> 200 & < 300	> 200 & < 300
	4	> 500 & < 600	> 500 & < 600	4	> 600	> 600	4	> 600	> 600	4	> 600	> 500 & < 600
	5	> 200 & < 300	> 200 & < 300	5	> 200 & < 300	> 200 & < 300	5	> 200 & < 300	> 200 & < 300	5	> 50 & < 100	> 50 & < 100
	6	> 300 & < 400	> 300 & < 400	6	> 500 & < 600	> 500 & < 600	6	> 600	> 600	6	> 200 & < 300	> 200 & < 300
	7	> 600	> 600	7	> 300 & < 400	> 600	7	> 500 & < 600	> 600	7	> 300 & < 400	> 300 & < 400
	8	> 600	> 600	8	> 600	> 600	8	> 500 & < 600	> 500 & < 600	8	> 100 & < 200	> 100 & < 200
	9	> 600	> 600	9	> 600	> 600	9	> 300 & < 400	> 300 & < 400	9	> 500 & 600	> 500 & < 600
<u>Klebsiella spp.</u>	1	> 600	> 600	1	> 300 & < 400	> 300 & < 400	1	> 300 & < 400	> 300 & < 400	1	> 50 & < 100	> 50 & < 100
	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 200 & < 300	> 200 & < 300
	3	> 600	> 600	3	> 600	> 600	3	> 500 & < 600	> 500 & < 600	3	> 500 & < 600	> 500 & < 600
	4	> 200 & < 300	> 50 & < 100	4	> 500 & < 600	> 300 & < 400	4	> 600	> 600	4	> 400 & < 500	> 400 & < 500
	5	> 200 & < 300	> 50 & < 100	5	> 100 & < 200	> 50 < 100	5	> 100 & < 200	> 100 & < 200	5	> 50 & < 100	> 50 & < 100
<u>Staph aureus</u>	1	> 200 & < 600	> 600	1	> 200 & < 300	> 500 & < 600	1	> 600 &	> 600 &	1	> 200 & < 300	> 200 & < 300
	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600
<u>Pseudomonas s.p.</u>	1	> 600	> 600	1	> 400 & < 500	> 500 & < 600	1	> 600	> 600	1	> 500 & < 600	> 500 & < 600

MIC values determined in triplicate in each case.

TABLE 23

EFFECT OF EDTA TREATMENT ON MIC OF 4 ANTIBIOTICS FOR DIFFERENT
ORGANISMS

Sr. No.	Organism	Strains Tested	Number of Strains Showing Effect on MIC of			
			Chl.	Tetr.	Str.	Gent.
1	<u>E.coli</u>	7	1	0	0	1
2	<u>Proteus spp.</u>	9	0	1	1	3
3	<u>Klebsiella</u> <u>spp.</u>	5	2	2	0	0
4	<u>Staph aureus</u>	2	1	1	0	0
5	<u>Pseudomonas</u> <u>aeruginosa</u>	1	0	1	0	0

Chl. - Chloramphenicol

Tetr - Tetracycline

Str. - Streptomycin

Gent - Gentamycin

Table (24) gives details of effect of Fe^{++} ions on MIC of 4 antibiotics for the cultures affected by treatment.

Table (25) shows that treatment with Fe^{++} ions resulted in change of MIC of antibiotics for Proteus spp., E.coli, Klebsiella spp., Staph aureus and Pseudomonas aerugenosa in decreasing order as far as the number of strains affected.

As can be seen from Table (24) E.coli showed reduction in MIC of chloramphenicol and streptomycin. Proteus spp., showed reduction in MIC for 4 antibiotics whenever change occurred with Fe^{++} ions treatment. Klebsiella spp. showed reduction in MIC for chloramphenicol and tetracycline. Pseudomonas aerugenosa showed decrease in MIC of chloramphenicol, Staph aureus showed decrease in MIC for gentamycin but increase in MIC for tetracycline.

STUDY OF EFFECT OF pH ON MIC OF ANTIBIOTICS :

Twenty four out of total forty bacterial strains were studied for effect of pH on MIC of 4 antibiotics. Mueller and Hinton agar media having pH 6.5, 7.0, 7.5 and 8.0 were prepared. Using these media four sets of Mueller and Hinton plates containing different concentrations of four antibiotics were prepared. Chloramphenicol, tetracycline, streptomycin and gentamycin were used in the study.

Bacterial strains were prepared and inoculated on these plates as described earlier. MIC determination for different antibiotics at different pH was done each in triplicate. Results of assays at pH 7.5 were considered as control MIC values and results of MIC at pH 6.5, 7.0 and 8.0 were considered as test values. As before MIC values of test and control were compared by Mann-Whitney test.

EFFECT OF Fe⁺⁺ IONS ON MIC OF ANTIBIOTICS

Organism	Strain No.	Chloramphenicol MIC		Strain No.	Tetracycline MIC		Strain No.	Streptomycin MIC		Strain No.	Gentamycin MIC	
		Control	Test		Control	Test		Control	Test		Control	Test
<u>E.coli</u>	1	> 500 & < 600	> 300 & < 400	1	> 600	> 600	1	> 600	> 500 & < 600	1	> 50 & < 100	> 50 & < 100
	2	> 500 & < 600	> 500 & < 600	2	> 600	> 600	2	> 600	> 600	2	> 50 & < 100	> 50 & < 100
	3	> 500 & < 600	> 500 & < 600	3	> 200 & < 300	> 200 & < 300	3	> 600	> 600	3	> 50 & < 100	> 50 & < 100
	4	> 500 & < 600	> 500 & < 600	4	> 300 & < 400	> 300 & < 400	4	> 600	> 600	4	> 50 & < 100	> 50 & < 100
	5	> 500 & < 600	> 200 & < 300	5	> 600	> 600	5	> 600	> 100 & < 200	5	> 200 & < 300	> 200 & < 300
	6	> 500 & < 600	> 500 & < 600	6	> 600	> 600	6	> 600	> 600	6	> 50 & < 100	> 50 & < 100
	7	> 500 & < 600	> 500 & < 600	7	> 600	> 600	7	> 600	> 600	7	> 600	> 600
<u>Proteus spp.</u>	1	> 500 & < 600	> 500 & < 600	1	> 300 & < 400	> 300 & < 400	1	> 600	> 600	1	> 100 & < 200	> 100 & < 200
	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 100 & < 200	> 100 & < 200
	3	> 500 & < 600	> 500 & < m600	3	> 600	> 300 & < 400	3	> 600	> 500 & < 600	3	> 200 & < 300	> 200 & < 300
	4	> 500 & < 600	> 500 & < 600	4	> 600	> 600	4	> 600	> 600	4	> 500 & < 600	> 100 & < 200
	5	> 200 & < 300	> 200 & < 300	5	> 200 & < 300	> 200 & < 300	5	> 200 & < 300	> 200 & < 300	5	> 50 & < 100	> 50 & < 100
	6	> 500 & < 600	> 400 & < 500	6	> 500 & < 600	> 500 & < 600	6	> 600	> 600	6	> 200 & < 300	> 50 & < 100
	7	> 500 & < 600	> 500 & < 600	7	> 300 & < 400	> 200 & < 300	7	> 500 & < 600	> 50 & < 100	7	> 300 & < 400	> 300 & < 400
	8	> 600	> 600	8	> 600	> 600	8	> 500 & < 600	> 400 & < 500	8	> 100 & < 200	> 100 & < 200
	9	> 600	> 600	9	> 600	> 600	9	> 300 & < 400	> 200 & < 300	9	> 400 & < 500	> 50 & < 100
<u>Klebsiella spp.</u>	1	> 600	> 600	1	> 300 & < 400	> 300 & < 400	1	> 300 & < 400	> 300 & < 400	1	> 50 & < 100	> 50 & < 100
	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 200 & < 300	> 200 & < 300
	3	> 600	> 600	3	> 600	> 600	3	> 500 & < 600	> 500 & < 600	3	> 500 & < 600	> 500 & < 600
	4	> 200 & < 300	> 50 & < 100	4	> 500 & > 600	> 300 & > 400	4	> 600	> 600	4	> 400 & > 500	> 400 & > 500
	5	> 200 & < 300	> 50 & < 100	5	> 100 & < 200	> 50 & < 100	5	> 100 & < 200	> 100 & < 200	5	> 50 & < 100	> 50 & < 100
<u>Staph aureus</u>	1	> 500 & < 600	> 500 & < 600	1	> 200 & < 300	> 500 & < 600	1	> 600	> 600	1	> 200 & < 300	> 200 & < 300
	2	> 600	> 600	2	> 600	> 600	2	> 600	> 600	2	> 600	> 200 & < 300
<u>Pseudomonas spp.</u>	1	> 600	> 400 & < 500	1	> 600	> 600	1	> 600	> 600	1	> 50 & < 100	> 50 & < 100

MIC values determined in triplicate in each case.

TABLE 25

EFFECT OF Fe⁺⁺ IONS ON MIC OF 4 ANTIBIOTICS FOR
DIFFERENT ORGANISMS

Sr. No.	Organism	Strains Tested	Number of Strains Showing Effect on MIC of			
			Chl.	Tetr.	Str.	Gent.
1	<u>E.coli</u>	7	2	0	2	0
2	<u>Proteus</u> spp.	9	1	2	4	3
3	<u>Klebsiella</u> spp.	5	2	2	0	0
4	<u>Staph aureus</u>	2	0	1	0	1
5	<u>Pseudomonas</u> <u>aeruginosa</u>	1	1	0	0	0

Chl. - Chloramphenicol

Tetr. - Tetracycline

Str. - Streptomycin

Gent - Gentamycin



Details of effect of pH on MIC of 4 antibiotics for the cultures showing response are given in Tables (26,27).

As can be seen from Table (28) effect of pH on MIC of antibiotics for Proteus spp., E.coli, Klebsiella spp., Pseudomonas aeruginosa and Staph aureus was in decreasing order as far as the number of strains affected is concerned.

Tables (26,27) shows that, 3 strains of E.coli showed decrease in MIC of chloramphenicol for pH > 7.5 and 1 strain showed increase while other two showed decrease for pH < 7.5 . Pseudomonas aeruginosa showed increase in MIC of chloramphenicol for pH < 7.5 and no change for pH > 7.5 . One Proteus strain showed decrease in MIC of chloramphenicol for pH < 7.5 and no change for pH > 7.5 .

Strains of E.coli, Proteus spp., Klebsiella spp., Pseudomonas and Staph aureus which responded to change in pH/ decrease in MIC of tetracycline for pH < 7.5 and no change for pH > 7.5 with exception of 2 strains of Proteus spp., where decrease in MIC was observed also for pH > 7.5. One strain of Proteus and 1 strain of Klebsiella showed increase in MIC for pH > 7.5 .

Two strains of E.coli and 2 of Proteus showed decrease in MIC of streptomycin for pH < 7.5 and no change for pH > 7.5 . Two strains of Proteus and 1 strain of Pseudomonas aeruginosa showed increase in MIC of streptomycin for pH < 7.5 and no change for pH > 7.5 .

Proteus spp., Klebsiella spp. Pseudomonas aeruginosa and 2 strains out of 3 of E.coli showed increase in MIC of gentamycin for pH < 7.5. One strain of Proteus, E.coli and Staph aureus show decrease in MIC of gentamycin for pH > 7.5 while other strains show no change.

TABLE - 26

EFFECT OF pH ON MIC OF ANTIBIOTICS

Organism	Strain No.	Chloramphenicol MIC at pH				Strain No.	Tetracycline MIC at pH			
		Control (7.5)	6.5	7.0	8.0		Control (7.5)	6.5	7.0	8.0
<u>E.coli</u>	1	> 300 & < 400	> 500 & < 600	> 500 & < 600	> 300 & < 400	1	> 600	> 200 & < 300	> 500 & < 600	> 600
	2	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 300 & < 400	2	> 600	> 300 & < 400	> 200 & < 300	> 600
	3	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	3	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
	4	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	4	> 300 & < 300	> 300 & < 400	> 300 & < 400	> 300 & < 400
	5	> 500 & < 600	> 300 & < 400	> 500 & < 600	> 300 & < 400	5	> 600	> 200 & < 300	> 300 & < 400	> 600
	7	> 600	> 600	> 600	> 500 & < 600	7	> 600	> 300 & < 400	> 300 & < 400	> 500 & < 600
<u>Proteus spp.</u>	1	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	1	> 300 & < 400	> 200 & < 300	> 200 & < 300	> 300 & < 400
	2	> 600	> 500 & < 600	> 600	> 600	2	> 600	> 200 & < 300	> 200 & < 300	> 500 & < 600
	3	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	3	> 600	> 300 & < 400	> 300 & < 400	> 500 & < 600
	4	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	4	> 500 & < 600	> 200 & < 300	> 500 & < 600	> 500 & < 600
	5	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300	5	> 200 & < 300	> 100 & < 200	> 100 & < 200	> 200 & < 300
	6	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	6	> 500 & < 600	> 300 & < 400	> 300 & < 400	> 600
	7	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	7	> 300 & < 400	> 100 & < 200	> 100 & < 200	> 300 & < 400
	8	> 600	> 600	> 600	> 600	8	> 600	> 600	> 600	> 600
	9	> 600	> 600	> 600	> 600	9	> 600	> 500 & < 600	> 500 & < 600	> 500 & < 600
<u>Klebsiella spp.</u>	1	> 600	> 600	> 600	> 600	1	> 300 & < 400	> 200 & < 300	> 200 & < 300	> 500 & < 600
	2	> 600	> 600	> 600	> 600	2	> 600	> 200 & < 300	> 200 & < 300	> 600
	3	> 600	> 600	> 600	> 600	3	> 600	> 500 & < 600	> 500 & < 600	> 600
	4	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300	4	> 500 & < 600	> 300 & < 400	> 500 & < 600	> 600
	5	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300	5	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
<u>Pseudomonas spp.</u>	1	> 600	> 600	> 600	> 600	1	> 600	> 500 & < 600	> 500 & < 600	> 600
	5	> 500 & < 600	> 600	> 500 & < 600	> 500 & < 600	5	> 600	> 100 & < 200	> 100 & < 200	> 600
	6	> 500 & < 600	> 600	> 600	> 500 & < 600	6	> 200 & < 300	> 50 & < 100	> 200 & < 300	> 200 & < 300
<u>Staph aureus</u>	1	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	1	> 200 & < 300	> 100 & < 200	> 100 & < 200	> 200 & < 300
	2	> 600	> 600	> 600	> 600	2	> 600	> 300 & < 400	> 300 & < 400	> 600

MIC values determined in triplicate in each case.

TABLE - 27

EFFECT OF pH ON MIC OF ANTIBIOTICS

Organism	Strain No	Streptomycin MIC at pH				Strain No	Gentamycin MIC at pH			
		Control(7.5)	6.5	7.0	8.0		Control (7.5)	6.5	7.0	8.0
<u>E.coli</u>	1	> 600	> 600	> 600	> 600	1	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	2	> 600	> 500 & < 600	> 600	> 600	2	> 50 & < 100	> 500 & < 600	> 100 & < 200	> 50 & < 100
	3	> 600	> 600	> 600	> 600	3	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	4	> 600	> 600	> 600	> 600	4	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	5	> 600	> 500 & < 600	> 600	> 600	5	> 200 & < 300	> 400 & < 400	> 300 & < 400	> 300 & < 300
	7	> 600	> 600	> 600	> 600	7	> 600	> 600	> 600	> 500 & < 600
<u>Proteus spp.</u>	1	> 600	> 600	> 600	> 600	1	> 100 & < 200	> 300 & < 400	> 300 & < 400	> 100 & < 200
	2	> 600	> 500 & < 600	> 600	> 600	2	> 100 & < 200	> 500 & < 600	> 200 & < 300	> 100 & < 200
	3	> 600	> 600	> 600	> 600	3	> 200 & < 300	> 400 & < 500	> 300 & < 400	> 200 & < 300
	4	> 600	> 500 & < 600	> 600	> 600	4	> 500 & < 600	> 600	> 600	> 500 & < 600
	5	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300	5	> 50 & < 100	> 100 & < 200	> 50 & < 100	> 50 & < 100
	6	> 600	> 600	> 600	> 600	6	> 200 & < 300	> 300 & < 400	> 200 & < 300	> 200 & < 300
	7	> 500 & < 600	> 600	> 300 & < 400	> 500 & < 600	7	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400
	8	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	8	> 100 & < 200	> 100 & < 200	> 100 & < 200	> 100 & < 200
	9	> 300 & < 400	> 500 & < 600	> 300 & < 400	> 200 & < 300	9	> 500 & < 600	> 600	> 500 & < 600	> 400 & < 500
<u>Klebsiella spp.</u>	1	> 300 & < 400	> 300 & < 400	> 300 & < 400	> 300 & < 400	1	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
	2	> 600	> 600	> 600	> 600	2	> 200 & < 300	> 500 & < 600	> 300 & < 400	> 200 & < 300
	3	> 500 & < 600	> 500 & < 600	> 500 & < 600	> 500 & < 600	3	> 500 & < 600	> 600	> 500 & < 600	> 500 & < 600
	4	> 600	> 600	> 600	> 600	4	> 200 & < 300	> 300 & < 400	> 300 & < 400	> 200 & < 300
	5	> 100 & < 200	> 100 & < 200	> 100 & < 200	> 100 & < 200	5	> 50 & < 100	> 50 & < 100	> 50 & < 100	> 50 & < 100
<u>Pseudomonas spp.</u>	1	> 600	> 600	> 600	> 600	1	> 50 & < 100	> 100 & < 200	> 50 & < 100	> 50 & < 100
	5	> 400 & < 500	> 500 & < 600	> 400 & < 500	> 400 & < 500	5	< 50	< 50	< 50	< 50
	6	> 600	> 600	> 600	> 600	6	< 50	< 50	< 50	< 50
<u>Staph aureus</u>	1	> 600	> 600	> 600	> 600	1	> 200 & < 300	> 200 & < 300	> 200 & < 300	> 200 & < 300
	2	> 600	> 600	> 600	> 600	2	> 600	> 600	> 600	> 200 & 300

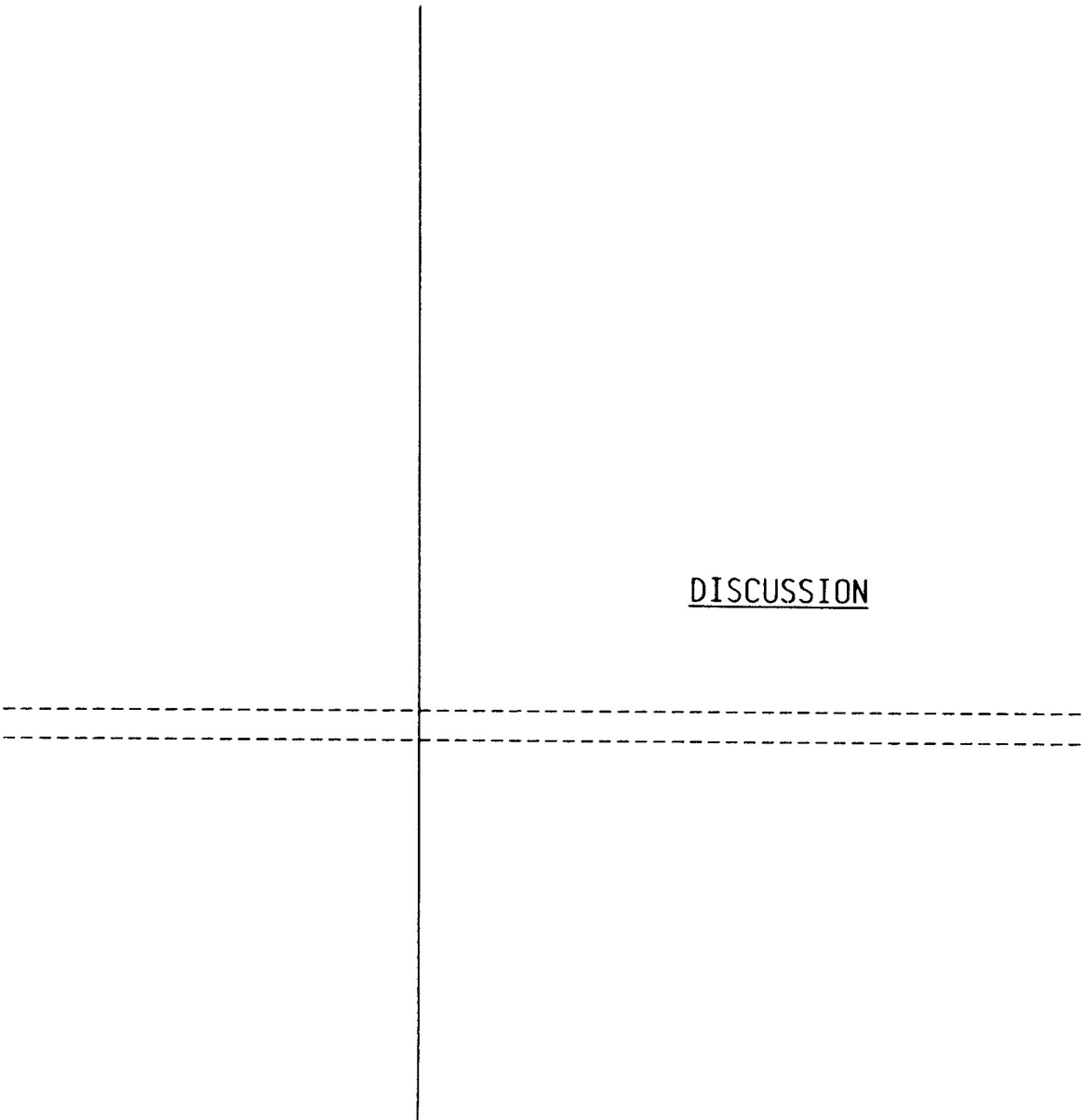
MIC values determined in triplicate in each case.

TABLE 28

EFFECT OF pH ON MIC OF 4 ANTIBIOTICS FOR DIFFERENT
ORGANISMS

Sr. No.	Organism	Strains Tested	Effect of pH	Number of Strains Showing Effect on MIC of			
				Chl.	Tetr.	Str.	Gent.
1	<u>E.coli</u>	6	< 7.5	2	4	2	2
			> 7.5	3	0	0	1
2	<u>Proteus</u> spp.	9	< 7.5	1	8	4	7
			> 7.5	0	4	1	1
3	<u>Klebsiella</u> spp.	4	< 7.5	0	4	0	3
			> 7.5	0	2	0	0
4	<u>Pseudomonas</u> spp.	3	< 7.5	2	3	1	1
			> 7.5	0	1	0	0
5	<u>Staph aureus</u>	2	< 7.5	0	2	0	0
			> 7.5	0	0	0	1

Chl. - Chloramphenicol
Tetr - Tetracycline
Str. - Streptomycin
Gent - Gentamycin



DISCUSSION

DISCUSSION

The increasing incidence of bacterial resistance to commonly prescribed antibiotics has markedly limited the usefulness of many and has necessitated the prescription of some of the more toxic agents when less toxic ones are apparently ineffective. Hence in use of antibiotics, particularly aminoglycosides clinically relevant results in sensitivity testing are of great importance because of potential toxicity of these drugs (16).

The rational use of antimicrobial agents depends to a large extent, on reliability of in-vitro testing of susceptibility and solving the problem of discrepancy in in-vitro testing of susceptibility and in-vivo response to the drug.

There are a number of host factors which can influence in-vivo susceptibility of microorganisms to the antimicrobial agents. These factors are not taken into consideration while carrying out routine sensitivity test and hence extrapolating in-vitro sensitivity test results and directing chemotherapy can pose problems.

As cited earlier these factors can be (i) pathophysiological state of the host (ii) severity of infection (iii) need of other supplementary measures (iv) concentration of the drug in body fluid being not necessarily the same as used in testing (v) location of infection and hence variations in conditions affecting the degree of susceptibility within body of the host.

Without realising these factors if in-vitro tests are relied upon

following situations can arise -

(a) Pathogen seemingly sensitive to the action of a particular drug does not respond to the treatment with such a drug in infectious state within the body of the host.

(b) Apparently inactive antibiotics may have potential activity in in-vivo situations but are unnecessarily denied use, based on in-vitro results.

(c) Doses of some toxic antibiotics can be reduced and same effect can be obtained without toxic side effects.

Out of the various factors cited, concentration of antibiotic at the site of infection vis-a-vis in blood and location of infection and accordingly varying conditions are the ones which play a major role in the discrepancy in the in-vitro and in-vivo situations.

Concentration of Antibiotic at the Site of Infection vis-a-vis in Blood

Antibiotic sensitivity tests of Kirby and Baur (49) are designed with the assumption of certain antibiotic concentration being reached and maintained in blood and accordingly the possible in-vivo effects are predicted. As is seen earlier (2, 14 - 17) concentrations in urine, in cerebrospinal fluid, in bile, in tissue differ far from those achieved in blood. Hence correlation between in-vitro tests which are based on the assumption of certain concentration of drug in blood and the in-vivo effects of the drug would not be seen if interactions occurring between the drug and the microbe at the infection sites is different due to different concentration of the antibiotic reached. Thus it becomes a serious point of consideration as to how far penetration of the drug

and concentration of the drug is possible in different parts of the body and in different body fluids.

Location of Infection :

Conditions prevalent at different sites of infection are not at all reflected in sensitivity tests in culutre plates.

Thus factors such as pH, aerobic or anaerobic conditions, action of different enzymes released by tissues or present in body fluids, detergent like action of bile acids, presence or absence of certain inorganic ions or variations in their concentrations, chelating agents and their action in removal of ions, accessibility of pathogens to phagocytic actions and products of host immune responses etc. have their effect on microbe-antibiotic interaction (10 - 13). The immediate physico-chemical environments of microbes vary considerably at different locations of infections such as gastrointestinal tract, urinary tract, hepatobiliary tree, respiratory tract and wounds etc. Therefore in-vivo susceptibility of the same organism to the same antibiotic may be different at different locations of infection and it may also be different from that observed in culture plate.

Keeping in view the fact that (1) concentration of antibiotic at site of infection (2) and location of infection and it's conditions are the major cause of discrepancy between in-vitro susceptibility and in-vivo response, we have tried to mimic some of these and study their influence on in-vitro results. Here an attempt has been made to incorporate in the in-vitro tests some of the factors that may play a role in modifying in-vivo susceptibility of microbe to antibiotics. This was done with an expectation that the results would throw some

light on the relevance of these factors while planning a particular drug treatment. In our view such susceptibility tests would be closer to some of the in-vivo situations and hence be realistic. Also we have undertaken these tests particularly on multidrug resistant pathogens which pose a serious threat.

Bile acids are present in intestine, liver, gall bladder. Cholic acid is a major component in this and the other derivatives present are deoxycholic acid, taurocholic acid, lithocholic acid, chenodeoxycholic acid. 5 - 15 gms of these acids are daily secreted (51). It is true that most microorganisms are resistant to intestinal concentrations of bile but some antibiotics can have better efficacy in the presence of bile acids (36). Penetration of antibiotic in the biliary tree may be a problem while treating hepatobiliary infections. Hence treatment of microorganisms in-vitro with bile acids, and subsequent susceptibility testing by finding out MIC for different antibiotics can tell us about in-vivo responses of the same antibiotics in the anatomic site containing bile acids.

In our study, cholic acid, sodium deoxycholate and sodium taurocholate individually were found to be effective in reduction of MIC of all the 4 antibiotic studied (i.e. chloramphenicol, tetracycline, streptomycin and neomycin). Effect was observed on E.coli, Proteus and Klebsiella spp but not on Pseudomonas, Staph aureus and Salmonella spp. Whenever treatment was effective reduction in MIC was 2 - 4 fold. This is much lower in comparison to the reported (33) 40 fold reduction in MIC of different antibiotics.

Combination of any two or more than two of these bile acids when

used for treatment was not found to be more effective in reduction of MIC of antibiotics than the individual bile acids.

pH is low in stomach and does not permit development of any organisms. pH is also low in urinary tract (24, 32), in inflamed tissues (3) and in CSF (16). pH is high in small intestine (51). Hence effect of different pH on activity of different antibiotics against pathogens is a significant information and can assist in judgement of chemotherapy of infection in different locations.

From our studies of effect of pH on MIC of antibiotics it can be said that acidic pH (6.5) had notable effect on MIC of tetracycline and gentamycin. Acidic pH increased MIC of gentamycin for 13 out of 24 different bacterial strains studied. Our observation of increase in MIC of gentamycin (which was 2 - 8 fold) concurs with earlier reports. In case of tetracycline, MIC decreased for 20 out of 24 different bacterial strains studied and reduction was 2 - 4 folds. Apart from these two sets of outstanding data, changes in pH, either to the acidic side (6.5) or alkaline side (8.0) had marginal influence on MIC of the other antibiotics. Earlier report (24) of alkalizing medication in treatment of urinary tract infection by gentamycin (claiming 32 - 128 times reduction) is substantiated by our study.

Proteolytic enzymes are present in intestinal secretions and also in inflamed areas (enzyme released from phagocytic cells). At these sites enzymes certainly can have effect on microbe-drug interaction. So, if effect of enzyme treatment is seen on MIC of different antibiotics, in the in-vitro tests, one should find it's usefulness in suggesting the possible role of enzymes in microbe-drug interactions in in-vivo

situations.

With this logic we have studied effect of papain, pancreatin and trypsin on MIC of 4 antibiotics (i.e. chloramphenicol, tetracycline, streptomycin and gentamycin). In our observations, effect of proteolytic enzyme treatment on susceptibility of organisms to antimicrobial agents was not uniform. Proteolytic enzyme treatment was ineffective in changing the MICs of aminoglycosides. But they were effective in the range of 4 out of 24 to 10 out of 24 strains tested in reducing the MIC's of chloramphenicol and tetracycline. Whenever the enzyme treatment was found to be effective, the reduction in MIC was 2 - 6 fold. Earlier reports (37) claim effect of papain treatment on MIC of streptomycin also, which we have not observed. The same reports have suggested use of papain in clinical practice to increase efficacy of some antibiotics.

Magnesium, sodium, calcium, phosphate, Fe^{++} ions are present, in different concentrations in intestinal fluid, in CSF, in urine, in blood and in inflamed areas (52) and these can certainly influence drug-microbe interaction in-vivo. Hence study of effect of these ions on MIC of antibiotics in-vitro will be of value to understand in-vivo response. Also, higher osmolarity of urine, colonic contents and the contents of phagocytic lysosomes can influence the response of microbes to antibiotics in these in-vivo sites. Most of the studies of effect of ions reported earlier (12, 41 - 47) are open to criticism that they lack in precision. This is because in study of effect of these ions results are expressed in terms of additional reduction in the number of viable organisms on incorporation of a given factor along with the antibiotic. We have on the other hand studied, effect of different ions

on the actual MIC values of the antibiotics, and such data is of greater practical importance.

In earlier reports Fe^{++} (40) is showed to suppress action of tetracycline (may be because of the interaction of the ions with the drug and removing it from the site of action or unavailability of cations for action of essential enzymes). Here, in our observation Fe^{++} decreases MIC of all the four antibiotics studied (chloramphenicol, tetracycline, streptomycin and gentamycin) in 4-6 out of 24 different bacterial strains tested. And there is no increase in MIC value observed for any microbe-drug interaction in presence of Fe^{++} ions. In our opinion, Fe^{++} by chelation maintains high concentrations of antibiotics in and around cells and therefore may be useful in reduction of MIC of antibiotic for orgaanisms.

Effect of magnesium ions was quite variable and increase or decrease in MIC was observed in 1-4 out of 24 different bacterial strains studied. Effect was 2 to 6 fold whenever present. Earlier reports (42) claim greater resistance by organisms to streptomycin and gentamycin in presence of magnesium ions.

Effect of phosphate ions was notable on tetracyline. They decreased the MIC of the antibiotic for 6 out of 24 bacterial strains tested. In contrast phosphate ions increased MIC of neomycin for 9 out of 24 bacterial strains studied. For chloramphenicol and streptomycin the effect of phosphate ions on MIC was negligible.

Potassium chloride increased MIC of gentamycin for 6 out of 24 bacterial strains studied. Except for this observation potassium

chloride showed negligible effect on MIC of other antibiotics studied.

Study of effect of chelating agents on MIC of antibiotics of organisms can tell us about their possible role in depriving ions from drug-microbe interaction sites and influence (positive or negative) the drug actions. Effect of EDTA on MIC of antibiotics was studied for these reasons. Earlier reports (39) claim complete reversal of resistance of Pseudomonas aeruginosa for tetracycline and reduction of MIC of chloramphenicol and tetracycline for E.coli, Proteus spp. In our study reduction or increase in MIC was seen in 0 - 3 out of 24 bacterial strains studied for any of the four antibiotics.

In general it can be seen that bile acids, proteolytic enzymes and reduction in pH can have a profound effect on the susceptibility of microorganisms to different antibiotics. As a result in such situations the organism can respond to chemotherapy even when reported resistant by standard in-vitro tests. A direct need created by this observation is that the factors causing modification of susceptibility be incorporated in designing in-vitro tests, or considered while treating infections.

Similar investigations making corrections for factors such as anaerobiosis, osmolarity, treatment of bacterial cells with enzymes from neutrophils and macrophages, tissue breakdown products, antibodies, complement components, etc. may give a different picture in antibiotic resistance pattern as judged by in-vitro tests. And such correlation could increase the value of in-vitro tests in predicting a satisfactory in-vivo response.



SUMMARY AND CONCLUSIONS

+++++

SUMMARY AND CONCLUSIONS

Acquired drug resistance is an important limitation to the use of antibiotics. Avoidance of indiscriminate use of antibiotics can solve the problem of emergence of drug resistant pathogens to certain extent. But selection of appropriate antibiotic is also very crucial, for successful treatment of severe infections is today done by in-vitro susceptibility tests of Kirby and Baur. But very often we encounter discrepancy between in-vitro susceptibility and in-vivo response. Thus organism apparently resistant by in-vitro tests may show an in-vivo response or organism apparently sensitive by in-vitro tests may fail to respond in-vivo. These discrepancies arise due to the host factors which are not considered in today's routine susceptibility tests. Therefore it is logical to expect changes in susceptibility if in-vitro tests are modified by inclusion of host factors and then results can be close to reality.

Here we have selected 24 drug resistant strains and identified them as multidrug resistant by conventional Baur and Kirby's method. Further we have carried out treatment of these strains with factors such as bile acids, proteolytic enzymes, changes in pH, different ions and chelating agents, and then studied effect of such treatment on MIC of antibiotics, namely chloramphenicol, tetracycline, streptomycin, neomycin and gentamycin.

Findings of our studies show that bile acids, proteolytic enzymes and pH have an effect on some of the strains tested. Effect is quite

profound as it changes MIC 2 - 8 fold. The effect is not necessarily characterised by reduction in MIC, e.g. for gentamycin acidic pH increased the MIC.

Thus in our opinion these and several other host factors may be significant in antibiotic sensitivity of pathogens in in-vivo situations and therefore should be incorporated in in-vitro tests to overcome the problem of discrepancy. This will make selection of appropriate antibiotic meaningful and successful. Wherever incorporation of a factor in routine tests is practically difficult at least treatment policy should take into consideration their effects in-vivo.



APPENDIX - I

APPENIX - I**1) Nutrient Broth :**

Peptone	1.0 gm.
Meat Extract	0.5 gm
Sodium Chloride	0.5 gm.
Distilled water	100 ml.
pH	7.4
Sterilized at 121 °C for 15 min.	

2) Phosphate Buffer Saline :

	pH 6.0	pH 8.0
KH_2PO_4	1.8 gm.	0.75 gm.
K_2HPO_4	8.2 gm	16.40 gm.
NaCl	8.75 gm.	8.75 gm.
Distilled water	1000 ml	1000 ml

Sterilized at 121 °C for 15 min.

3) Mueller and Hinton Agar :

Meat Extract	1.0 gm
Bactocasamino acids	1.75 gm
Starch	0.15 gm
Bacto Agar	1.70 gm
Distilled Water	100 ml
pH	7.5

Sterilized at 121 °C for 15 min.

BIBLIOGRAPHY

#####

BIBLIOGRAPHY

1. Franklin, T.J. and Snow, G.A.
Problems of resistance to antimicrobial drugs. In " Biochemistry of antimicrobial action " Chapman and Hall, Publ. London, 175 - 207, 1980.
2. Barry, A.L.
Introduction and Definition of terms " Resistant " and "Susceptible"
In " Antimicrobial susceptibility test : Principles and Practices" Henry Kimpton Publ. London 3 - 21, 1976
3. Goldstein, A., Arnow, L. and Kalman, S.M.
Drug Resistance In " Principles of drug action : basis of pharmacology " Wiley Biomedical Health Publ., Newyork, 517 - 560, 1974.
4. Gale, E.F., Cundliffe, E., Reynolds, P.E., Richmond, M.H. and Waring, M.J.
Bacterial Resistance to Antibiotics. In " The molecular basis of antibiotic action ", John Wiley Publ., New York, 548 - 595, 1981.
5. Benveniste, R. and Davies, J.
" Mechanisms of antibiotic resistance ", Annual Review of Biochemistry, 42 : 471 - 506, 1973.
6. Watnabe, T.
Infective heridity of multiple drug resistance in bacteria. Bacteriol. Rev. 27 : 87 - 115, 1963.
7. Hayes, W.
Sex factors and other plasmids. In " The Genetics of bacteria and their viruses ", John Wiley & Sons., Publ. New York, 760 - 775, 1976.
8. Lewin, B.
Transposable elements in bacteria , In " Genes " . Wiley Eastern Limited Publ. New Delhi., 589 - 608, 1986.
9. International collaborative study on antibiotic sensitivity testing.
Acta Patha. Micro. Scand (B) Suppl. 217 : 1 - 90, 1971.
10. Lee Hand, W., King-Thompson, N.L. and Johnson, J.D.
Influence of bacteria-antibiotic interactions on subsequent anti-

- microbial activity of alveolar macrophages. J. Infect. Dis. 149 : 271 - 276, 1984.
11. Bryant, R.E., Rahad, A.L., Mazza, J.A. and Hammond, D.
Beta-lactamase activity in human pus. J. Infect. Dis. 142 : 594 - 601, 1980.
 12. Vaudaux, P. and Waldvogel, F.A.
Gentamycin inactivation in purulent exudates. J. Infect. Dis. 142 : 586 - 593, 1980
 13. Bryant, R.E. and Hammond, D.
Interaction of purulent material with antibiotics used to treat Pseudomonas infections. Antimicrob. Agents Chemother. 6 : 702-707, 1974.
 14. Musher, D.M., Minuth, J.N., Thorsteinsson, S.B. and Holmes, T.
Effectiveness of achievable urinary concentration of tetracycline against " tetracycline resistant " pathogenic bacteria. J. Infect. Dis. (Suppl.) 131 : S-40 - S-44, 1975.
 15. Smithivas, T., Hyams, P.J. and Rahl, J.J. Jr.
Gentamycin and ampicillin in bile. J. Infect. Dis. 124 : S-106 - S-108, 1971.
 16. Strausbaugh, L.J. and Sande, M.A.
Factors influencing therapy of expt. Proteus mirabilis meningitis in rabbits. J. Infect. Dis. 137 : 251 - 260, 1978.
 17. Pennington, J.E. and Reynolds, H.Y.
Concentrations of gentamycin and carbenicillin in bronchial secretions. J. Infect. Dis. 128 : 63 - 68, 1973.
 18. Mett, H., Ozak, K.G. and Vosbeck, K.
Duodenopancreatic secretions enhance bactericidal activity of antimicrobial drugs. Antimicrob. Agents Chemother. 26 : 35 - 38, 1984.
 19. Verklin, R.M. and Mandell, G.L.
Alteration of effectiveness of antibiotics by anaerobiosis. J. Lab. Clin. Med. 89 : 65 - 71, 1977.
 20. Reynolds, A.V., Hamilton, J.M.T. and Burnfitt, M.W.
Diminished effect of gentamycin under anaerobic and hypercapnic conditions. Lancet I (Jan-March) 447-449, 1976.

21. Minuth, J., Musher, D. and Thorsteinson, S.B.
Inhibition of antibacterial activity of gentamycin by urine. J. Infect. Dis. 133 : 1 - 14, 1976.
22. Lipton, J.
Gentamycin in gram negative infection of urinary tract. Antimicrob. Agents Chemother. 6 : 172 - 174, 1966.
23. Rubenis, M., Kozij, V.M. and Jackson, G.G.
Laboratory studies on gentamycin. Antimicrob. Agents Chemother. : 153 - 156 , 1963.
24. Sabath, L.D., Gerstein, D.A., Leaf, C.D. and Finland, M.
Increasing the usefulness of antibiotics treatment of infectious diseases caused by gram negative bacilli. Clin. Pharmacology Ther. 11 : 161 - 167, 1970.
25. Levy, J., Smith, A.L., Kenny, M.A., Ramsey, B. and Schoenknecht, F.
Bioactivity of gentamycin in purulent sputum from patients with cystic fibrosis and bronchiectasis. J. Infect. Dis. 148 : 1069 -1076, 1983.
26. Garrod, L.P. and Waterworth, P.M.
Effect of medium composition on apparent sensitivity of Pseudomonas aeruginosa to gentamycin. J.Clin.Path. 22 : 534 - 538, 1969.
27. Washington, J.
Effect of cation content of agar on activity of aminoglycosides against Pseudomonas aeruginosa. J. Infect. Dis. 137 : 103 - 111, 1978.
28. Berkman, S., Henry, R.J. and Housright, R.D.
Studies on streptomycin. Factors influencing activity of streptomycin. J. Bacteriology. 53 : 567 - 574, 1947.
29. Jawer, Z.E.
Antimicrobial drugs : Mechanisms and factors influencing their action. In " Antimicrobial Therapy ". Kagan B.M., W.B. Saunder's Company Publ. Philadelphia, London, Toronto. 8 - 10, 1980 .

30. Abraham, E.P. and Duthie, E.S.
Effect of pH of medium on activity of streptomycin and penicillin.
Lancet I (Jan - June), 455 - 459, 1946.
31. Sabath, L.D. and Tuftegard, T.
Rapid micro-assays for clindamycin and gentamycin when present together and effect of pH and of each on antibiotic activity of other. Antimicrob. Agents Chemother. 6 : 54 - 59, 1974.
32. Brumfitt, W.
Adjustment of urine pH in chemotherapy of urinary tract infections. Lancet 1 : 186 - 190, 1962.
33. Klimnyuk, S.I.
Screening of effective agents decreasing antibiotic resistance in microorganisms. Antibiotiki 29 : 182 - 184, 1984.
34. Tvorko, M.S., Kalashnik, S.A. and Kovanova, E.N.
Effect of bile and bile acids on antibiotic resistance of bacteria. Antibiotiki. 29 : 257 - 260, 1984.
35. Tkachuk, N.I.
Combined effects of nitrofurantoin drugs and bile acids on staphylococci. Antibiotiki. 29 : 188 - 191, 1984.
36. Sytnik, I.A.
Microbiological aspects of development of rational antibiotic therapy of bacterial infections of biliary tract. Antibiotiki. 29 : 95 - 99, 1984.
37. Baskanchiladze, G.SH., Khurtsilava, L.A., Gelovani, I.A., Asatiani, M.V. and Rossinsky, V.A.
Chemotherapeutic efficacy of some antibiotic combinations with papain in experimental septicemia. Antibiotiki. 29 : 33 - 35, 1984.
38. Dall, L., Barnes, W.G., Lane, J.W. and Mills, J.
Enzyme modification of glycocalyx in treatment of experimental endocarditis due to viridans streptococci. J. Infect. Dis. 156 : 736 - 740, 1987.
39. Weiser, R., Asscher, A.W. and Wimpenny, J.
In-vitro reversal of antibiotic resistance by EDTA. Nature (London) 219 : 1365 - 1366, 1968.

40. Weinberg, E.
Effect of metallic ions. Bacteriol. Rev. 21 : 46 - 48, 1957.
41. Gilbert, D.N., Kutscher, E., Barnett, J.A., Sanford, J.P. and Ireland, P.
Effect of concentrations of calcium and magnesium ions on in-vitro susceptibility testing of Pseudomonas aeruginosa to gentamycin. J. Infect. Dis. (Suppl.) S-37 - S-45, 1971.
42. Zimilis, V.M. and Jackson, G.G.
Activity of aminoglycosidic antibiotics against Pseudomonas aeruginosa : Specificity and site of calcium and magnesium antagonism. J. Infect. Dis. 127 : 663 - 669, 1973.
43. Medeiros, A.A., O'Brien, T.F., Wacker, W.E.C. and Yulung, N.F.
Effect of salt concentrations on apparent in-vitro susceptibility testing of Pseudomonas and other gram negative bacilli to gentamycin. J. Infect. Dis. (Suppl.) 124 : S-59 - S-64, 1971.
44. D'Amato, R.F., Thornsberry, C., Backer, C.N., and Kirven, L.A.
Effect of calcium and magnesium ions on susceptibility of Pseudomonas spp. to tetracycline, gentamycin, polymyxin B and carbenicillin. Antimicrob. Agents Chemother. 7 : 596 - 600, 1975.
45. Beggs, W.H. and Andrews, F.A.
Role of ionic strength in salt antagonism of aminoglycoside action on Escherichia coli and Pseudomonas aeruginosa. J. Infect. Dis. 134 : 500 - 504, 1976.
46. Kenny, M.A., Pollack, H.M., Minshew, B.H., Casillas, E. and Schoenkuecht, F.D.
Cation components of Mueller and Hinton agar affecting testing of Pseudomonas aeruginosa susceptibility to gentamycin. Antimicrob. Agents Chemother. 17 : 55 - 62, 1980.
47. Young, C. and Hewitt, W.L.
Activity of aminoglycoside antibiotics in-vitro against gram negative bacilli and Staphylococcus. Antimicrob. Agents Chemother. 4 : 617 - 625, 1973.
48. Cowan, S.T.
Gram negative facultative anaerobic rods. Gram negative aerobic rods and cocci. Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith. In " Bergey's Manual of Determinative Bacteriology " editors Buchanan, R.E. and Gibbons, N.E., Williams

- and Wilkins Company Publ. 290 - 340, 1975.
49. Baur, A.W., Kirby, W.M.M., Sherris, J.C. and Turck, M.
Antibiotic susceptibility testing by standardized single disc
method. Am. J. Clin. Path. 45 : 493 - 496, 1966.
50. Bell, J.B., Elliot, G.E. and Smith, D.W.
Influence of sewage treatment and urbanization on selection of
multiple resistance in fecal coliform populations. Appl. and Envi.
Microb. 46 : 227 - 232, 1983.
51. Siegel, S.
The case of two independent samples In " Nonparametric statistics
for the behavioural sciences " McGraw Hill Kogakusha Ltd.
Publ. Tokyo. 116 - 126, 1956.
52. White, A., Handler, P., Smith, E.L. and Stetten, D.W.
Specialized extracellular fluids. In " Principles of Biochemistry
McGraw-Hill Book Company, Inc. Publ. New York, 713 - 734, 1959.



