STUDIES ON THE ANTIBIOTIC RESISTANCE PATTERN AND THE IMPACT ON HUMAN BODY TEMPERATURE OF *E.coli*

By

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A Thesis submitted to Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University), Coimbatore, in partial fulfilment of the requirement for the degree of Doctor of Philosophy

May 2001

I

CERTIFICATE

This is to certify that this dissertation entitled **" STUDIES ON THE ANTIBIOTIC RESISTANCE PATTERN AND THE IMPACT ON HUMAN BODY TEMPERATURE OF E.coli "**, as a piece of work in the field of microbiology, submitted to Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University), Coimbatore, for the award of the Degree of **Doctor of Philosophy in Life Sciences** is a record of original research work done by **M.S . PREMALATHA,** during the period of her study in the Department of Life Sciences , Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University) Coimbatore, under my supervision and guidance and the dissertation has not formed the basis for the award of any Degree / Diploma / Associateship/ Fellowship or other similar title to any candidate of any University and it represents entirely an independent work on the part of the candidate.

Signature of the supervisor

DECLARATION

I hereby declare that the matter embodied in this dissertation titled, **"STUDIES ON THE ANTIBIOTIC RESISTANCE PATTERN AND THE IMPACT ON HUMAN BODY TEMPERATURE OF E.coli"**,submitted to the Avinashilingam Institute for Home Science and Higher Education for Women(Deemed University) Coimbatore, in partial fulfilment of the requirements for the award of Degree of **DOCTOR OF PHILOSOPHY** in Life Sciences, is a record of original research work done by me under the supervision and guidance of **Dr. C.V.R. INDIRA, M.Sc .,Ph.D.**,Dean, Faculty of Science, Professor and Head of the Department of Life Sciences and that it has not formed the basis for the award of any Degree/ Diploma / Associateship/Fellowship or other similar title to any candidate of any university.

Supervisor

Signature of the Candidate

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INTRODUCTION

CHAPTER - I

INTRODUCTION

IDENTIFICATION

E.coli, a typical coliform organism of the intestinal tract is present in large numbers, invariably, in the faeces of man and animal. It is noncapsulated and usually motile. Its motility, in some cases, may be extremely difficult to detect. It appears usually as a short-rod of the size 0.5 μ to 2-5 μ. Shorter coccobacillary and long filamentous forms are occasionally observed in urine. It exhibits no special arrangement. It produces on solid media, relatively large colonies that are smooth and gustering. Variants, however, could be isolated from pyogenic infection which give rise to very minute colonies. These variants produce the normal type of colony with various organic sulphur compounds when grown on media.

PRESENCE

E.coli is a common inhabitant of the human large intestine and is usually harmless. The Escherichia organisms occur both as normal inhabitants of the intestinal tract and as intestinal pathogens or extraintestinal pathogens. Among *E.coli* bacilli groups, three namely enterotoxigenic (ETEC), enteropathogenic (EPEC) and enteroinvasive (ETEC) have been recognised as the cause of acute diarrhoeal diseases (Pushpa et al ., 1987).

E.coli bacilli infect the skin and the wound exudates by chance contamination. When these organisms predominate then they are responsible for the natural flora of the large intestine, in adults on mixed diet. This bacterial flora is affected by changes in the intestinal environment. For instance, if an adult diet were to be changed from a high protein to a high carbohydrate one then, *E.coli* becomes gradually insignificant. Thus *E.coli* is a conspicuous member of the indigenous flora of the human intestinal tract.

PROPERTIES

Certain pathogenic serotypes of *E.coli* cause diseases as they gain virulence. They (vide Review) are responsible for urinary tract infection and gastroenteritis. Young women and elderly adults who are more susceptible to urinary tract infection is commonly caused by *E.coli* and is frequently found even in healthy people. The disease may progress from simple urethritis to serious pyelonephritis. It is characterised by dysuria, frequency of urination, pyuria and occasionally hematuria. Gastroenteritis caused by *E.coli* ranges from simple diarrhoea to a more severe form resulting in a debilitating loss of fluids and electrolytes. Gastroenteritis is serious and sometimes fatal, in nutritionally deprived infants and elderly debilitated adults.

E.coli is usually non-haemolytic on Blood Agar. Haemolytic strains are ofcourse occasionally encountered. The great majority

of *E.coli* strains ferment lactose promptly i.e., within 24 hours producing of acid and gas. Glucose, Maltose and Mannitol are also fermented usually with gas production. *E.coli* does not liquify gelatin or produce hydrogen sulphide. It does not grow in media containing potassium cyanide.

E.coli is of considerable value as an indicator organism, in the bacteriological examination of water supplies. The presence of *E.coli* confirms that the water has been contaminated from faecal sources.

E.coli could colonize the human small intestine by plasmid mediation. There are plasmids that upset the ionic balance across the intestinal mucosa, causing an outflow of water into the intestinal lumen. The result is a severe diarrhoeal disease known as traveller's diarrhoea (Pushpa and Mahabirsingh, 1987).

R PLASMIDS

The drug resistant or R plasmids were originally isolated from the bacterium Shigella dysenteriae during an outbreak of dysentery in Japan (Watanabe, 1963). Their defining characteristics are: they confer on their host-cell resistance to a variety of antibiotics; they are usually self transmissible. Many R plasmids consist of two contiguous segments of DNA - One is Resistance transfer factor (RTF) and the other, R determinant (RD). RTF carries gene regulating DNA replication and copy number besides the transferee gene(s) and the gene for tetracycline resistance. RD is variable in size and carries the other

gene(s) for antibiotic resistance. This determinant is resistant to antibiotics (single or combined) like Penicillin (Pen) , Ampicillin (Amp), Chloramphenicol (Cam), Streptomycin (Str), Kanamycin (Kan) and Sulfonamide (Sul). This is noticed commonly(Watanabe, 1963).

Small drug resistant plasmids, lacking the ability to transfer but containing the gene for Tetracycline resistance, are also known. Genetic analysis has shown the possibility of multiple drug resistance by plasmid mediation, readily transferred between strains. R Plasmids are found in both Gram negative and Gram positive becteria. They are quite common in Enterobacteriaceae and in Staphylococci.

Antibiotic resistance, determined by plasmids was first discovered in Japan, in 1959. Until then it was assumed that antibiotic resistance in bacteria (a clinical problem) resulted from the selection of chromosomal mutants and multiple antibiotic resistance, from the sequential accumulation of mutations. Drug resistance may be mediated by 'R' factor. This is an important mode of transmission in drug resistance. There is evidence that many a time 'R' factor may lead to enhanced virulence of drug resistance. There is simultaneous transfer of resistance to a number of drugs eg., multiple resistance to chloramphenicol, streptomycin, tetracycline and sulfonamides etc. Multiple drug resistance was initially seen in bacteria causing diarrhoea, typhoid and urinary tract infection (Watanabe, 1963).

REASON FOR SELECTION OF *E.coli* FOR THE PRESENT STUDY

E.coli **has been selected as a principal type, in the present study, for the following reasons:**

1. ***E.coli*** **is disseminated throughout the environment in the faeces of human beings and animals.**

2. **It has the ability to survive for months outside the colon.**
3. **Pathogenic *E.coli*, reported to be causative agent of diarrhoeal diseases and urinary tract infection in human beings, deserves a study to determine the drug resistance pattern.**

NEED AND JUSTIFICATION OF THE PRESENT STUDY

1. **Antibiotic resistance in infectious pathogenic bacteria is a serious problem in human health care.**
2. **Although a variety of antibiotics are available a particular antibiotic, used for a prolonged period improperly and insufficiently becomes ineffective.**
3. **The indiscriminate use of antibiotics has been responsible for the high resistance exerted by bacteria.**
4. **The phenomenon of transfer of drug resistance among *E.coli* strains has led to the elimination of sensitive pathogenic strains and to the distribution of resistant ones into the environment.**
5. **The multiple drug resistant-isolates of - Among Enterobacteriaceae, have been reported throughout the world. This is becoming a problem in developing countries.**

6. Resistance is mediated mostly by 'R' factors resulting in the rapid spread of the resistance among the various bacteria of the environment.
7. It is generally observed that resistance to antibiotics is common in Gram negative bacteria.

These facts justify the study undertaken.

OBJECTIVES OF THE STUDY

1. To find out the biochemical characteristics of *E.coli* isolates.
2. To determine the impact of *E.coli* in the circadian variations of the host body temperature.
3. To confirm the pyrogen *E.coli* by animal inoculation.
4. To find out the reactions of the human host to the different parametric biochemical tests.
5. To prove the prevalence of multiple antibiotic resistance of *E.coli* organisms in different diarrhoeal samples .
6. To study the mechanism of the resistance pattern (molecular characterization) :
   a) Plasmid analysis of sensitive isolates.
   b) Plasmid analysis of resistant isolates.
   c) Restriction Fragment Length Polymorphism studies (RFLP).
   d) Ploymerase Chain Reaction studies (PCR).
7. Plasmid curing and their sensitivity to antibiotics.
8. To limit the study to therapeutic dimension

REVIEW OF LITERATURE

CHAPTER - II

REVIEW OF LITERATURE

The genus Escherichia was, formerly, subdivided into a number of species based on differences in sugar fermentation reactions. Later, only one species, Escherichia coli, came to be recognized. This was further subdivided into Serotypes and biotypes. The characteristics of Escherichia, distinguishing it from other Enterobacteria, are :

1. Motility
2. Formation of gas from glucose
3. Fermenting lactose
4. Production of indole
5. Giving a positive methyl-red reaction
6. A negative voges-proskauer reaction
7. Utilising ,citrate growing in KCN
8. Decomposing urea or liquefying gelatin

Some isolates differ from the typical, in one or two of these traits. But these are nevertheless accepted as *E. coli* (Sojka, 1965).

MORPHOLOGY

E.coli is a Gram negative non-sporing bacilli. Most strains (about 80 %) are motile, though motility is often feeble on primary isolation and most strains (about 80 %) are fimbriate. The fimbriate, in most of the fimbriate strains, are of type i.e., haemagglutinating, adhesive for epithelial cells and mannose sensitive. But different kinds of fimbriae, (type MRE), mannose - resistant, eluding haemagglutinating and with adhesive properties, are formed as well as type I fimbriae, in some strains. In some others, type I fimbriae is absent. A few strains are capsulate and many others form abundant loose slime when grown on sugar - containing medium, at 15^0 to 20^0 c (Duguid et al., 1985).

CULTURAL CHARACTERISTICS

Most strains ferment lactose rapidly. Therefore, the colonies on Mac - Conkey medium are smooth, glossy, translucent and rose - pink in colour. On cystine - lactose - electrolyte deficient (CLED) medium with bromothymol blue (much used for urinary bacteriology), the colonies are smooth and circular measuring about 1-1.5 mm in diameter. They are yellow opaque if lactose fermenting, and blue if nonlactose fermenting. Growth is either impaired or totally inhibited on deoxycholate citrate agar and colonies that do grow are small, pink and opaque. On blood agar, the colonies of some strains are surrounded by zones of haemolysis.

Most strains are prototrophic and grow on simple synthetic media such as glucose ammonium salts agar without supplementation with amino acids or vitamins, e.g. Davis and Mingioli medium. *E.coli* is uninhibited by bile salt, in Mac-Conkey medium, but is inhibited by the citrate in Leifson's DCA medium and by sodium selenite, sodium tetrathionate and brilliant green (Dugiud et al., 1985).

BIOCHEMICAL REACTIONS

It is mainly by the pattern of its biochemical reactions with certain substances that *E.coli* is outstanding among other Enterobacteria and is defined as a species. Carbohydrates are attacked fermentatively, with the production of acid and gas. A few strains are anaerogenic, producing acid but not gas. Traditionally, the prompt fermentation of lactose (with 24h and usually within 8h) was a quality essential for, the identification of a strain as *E.coli.* But it has been now accepted that the species includes some late lactose fermenting or lactose negative strains (Duguid et al., 1985).

For classifying Enterobacteria, isolated from drinking - water supplies, bacteriologists commonly use the 'IMVIC' reactions to distinguish faecal coliform *E.coli* from Klebsiellae, Enterobacters and other Coliforms derived from soil and vegetation through reactions such as indole positive, methyl-red positive, voges proskauer negative and citrate utilization negative.It is also distinguished from the other coliforms by its ability to form gas from lactose in Eijkman tests, incubated at 44^0 c (Sojka ,1965).

For general purposes, in clinical microbiology, the full range of tests necessary for the conclusive identification of an isolated species as *E. coli* is rarely conducted. A provisional identification, based on the pattern of reactions, in a restricted range of tests, is usually considered sufficient e.g., pink, non-mucoid colonies on Mac Conkey, fermentation of glucose and lactose, motility, indole production and non-utilization of citrate. There are still a few more reliable tests as colony appearance and Lactose fermentation. The isolate then should been described as a " Coliform bacillus" and not as *E. coli* (Sojka,1965).

VIABILITY

E. coli is, generally, similar to other Enterobacteria, in its susceptibility to inimical agents and conditions. It is, however, slightly more resistant to heat, to some chemicals and to drying than are Salmonellae and Shigellae. It is killed by moist heat at 60^0c within 30 minutes, although occasionally, some bacteria may survive pasteurization in milk at 62^0 to 63^0 c for 30 minutes. It can remain alive in water for several weeks or months, but it does not appear to grow in natural waters, outside the body. It can survive

for several days when dried on clothing or in dust. Pathogenic serotypes have been found to be viable and numerous in floor dust, in air and on clothing, napkins and ward equipment in hospitals housing infants with gastroenteritis (Rogers, 1951; Hutchison, 1957).

ANTIBIOTIC SENSITIVITY

E. coli , in its susceptibility to antibiotics resembles many other Enterobacteria. Different strains do differ markedly, in the degree of their sensitivity, to antibiotics commonly used to treat coliform infections. Strains of *E.coli* collected from the faeces of healthy persons or from infected persons urinary tract and from patients outside hospitals are commonly sensitive to readily attainable concentrations of a variety of antimicrobial drugs. Strains causing infections in hospitals are often resistant to some of these drugs (Duguid, 1985).

PENICİLLIN

The earliest known, clinically effective penicillin against *E. coli* was ampicillin (Minimum inhibitory concentration 8 mg/litre). Later, after its introduction, antibiotic resistance due to hydrolysis by lactamases was recognized. This is now a major clinical problem and 30 % or more of *E. coli* isolates are found to be

resistant to penicillin. The most important enzymes are the transmission electron microscope (TEM) type plasmid mediated β lactamases (Sojka,1965). Many *E. coli* strains are also sensitive to the related group of penicillins, carbenicillin (4 mg/litre), ticarcillin (4 mg/l), mezlocillin (2 mg/l), piperacillin (2 mg/l) and azlocillin (8 mg/l). But these antibiotics are unstable in their effectiveness towards several of the common clinically important β lactamase inhibitors like, clavulanic acid. Penicillin, theoretically, confers β lactamase stability on the previously susceptible drug . The first antibiotic combination, available for clinical use, was augmentin i.e., amoxycillin and clavulanic acid (Sojka,1965).

CEPHALOSPORINS

E. coli was found sensitive to the early cephalosporins (e.g. cephaloridine 4 mg/l, cephalothin 8 mg/l). The newer agents in this group not only more active but also more resistant to hydrolysis by β lactamases. The minimum inhibitory concentration of the second generation drugs namely cefuroxime, cefamandole, cefoxitin etc., is of the order of 0.5 - 2 mg/litre and of the third generation drugs namely, cefotaxime, latamoxef, ceftriaxone, ceftazidime etc., 0.05 - 1 mg/l (Kauffmann,1969).

AMINOGLYCOSIDES

These antibiotics are choice drugs like gentamicin, tobramycin and netilmicin. They are used for serious infections due to *E. coli* and resistance to them is uncommon in Britain (Duguid ,1985). The minimum inhibitory concentration of gentamicin, tobramycin and netilmicin is 0.1 - 1 mg/l and of amikacin 1-4 mg/l (Duguid ,1985).

OTHER ANTIMICROBIAL AGENTS

Antimicrobial agents are used mainly in clinical practice and in fact, almost exclusively, to treat urinary tract infections. Orally administered drugs, active against *E. coli*, include sulphonamides (although resistance is common), trimethoprim, cotrimoxazole (the combination of a sulphonamide with trimethoprim), tetracyclines, ciprofloxacin, nitrofurantoin and nalidixic acid (Cowan and Steel,1974).

DETECTION OF E. COLI IN CLINICAL SPECIMENS

The detection of *E. coli* in clinical microbiology is a public health issue with clear local responsibilities. Therefore, no official or standard international methods have been elaborated. In contrast to

the food and water microbiologist, the physician is not primarily interested in the number of *E. coli*. The latter's better concern is the identification of *E. coli* as a possible causative agent for intra - intestinal and extra-intestinal disorders. In addition, identification of this organism is often used in medicine for epidemiological purposes. The approach is, therefore , totally different (while the basic principles remain the same) with local variations. Manual for the identification of Medical bacteria (Cowan and Steel, 1974).

Large numbers of *E. coli* are expected in faeces and also in urine from suspected lower urinary tract infection. In these cases, samples can be plated directly on Mac Conkey agar or on eosin methylene blue (EMB) agar. In the case of suspected E. coli diarrhoea, high dilutions 10^{-7}, 10^{-8} of faeces are often directly plated on blood agar to isolate enteropathogenic or haemolytic E. coli that may be present in very large numbers (>/ g) or poorly on the selective plates (Martin, 1970).

On the other hand, very small numbers of bacteria are expected in blood samples obtained from patients with suspected *E.coli* bacteraemia. The blood samples are diluted 10-20 times, in order to dilute antibiotics that may be present. Usually *E. coli* will grow within 24 hours (Martin ,1970).

DETECTION OF ESCHERICHIA COLI AS A PATHOGEN

For the direct detection of *E.coli* as a potential pathogen, immunological methods and DNA hybridization are virtually the only alternatives to animal or tissue culture testing. The only important exception to this, is the direct plating method for the detection of sorbitol non-fermenting, H - glucuronidase negative, enterohaemorrhagic *E.coli* (Szabo et al., 1986).

Only a few immunological or DNA hybridization detection methods for pathogenic *E.coli* subgroups, suitable for use in routine diagnostics, have so far been developed. Strains producing the heat labile enterotoxin (LT) have served as models for the development of both immunological and nucleic acid hybridization tests of *E.coli*. A sensitive ELISA test for the detection of LT producing *E.coli* was developed by Svennerholm and Holmgren in1978.

DNA hybridization has also been successfully applied to the identification of heat stable enterotoxin (ST) producing *E. coli* in clinical specimens (Moseley et al., 1982). A synthetic oligonucleotide ST probe was used by Hill et al. (1985) for the identification of ST producing strains by colony hybridization.

E. coli IN HEALTHY INTESTINE

E. coli strains dominate the aerobic commensal bacteria present in the highly anaerobic gut environment (healthy). Strains of any 'O' group may be present as commensals. Some groups, e.g., 01, 02, 04, 06, 07, 018 and 075, appear to occur more commonly than others. Many serotypes may be present in an individual's intestine at any one time. But over a period the types change. Some types (residents) persist for relatively longer periods, say many months, whereas others (transients) persist for only a few days or weeks (Sears et al., 1950). Many previous researchers have found that a strain of a new serotype, deliberately swallowed in large numbers, rarely becomes established as a resident (Cooke, 1974).

E. COLI IN INFECTIONS

There is possibility of infection from intestinal commensal isolates of *E.coli* Spreading to other parts of the body. These isolates also cause urinary tract infections (cystitis, pyelitis and pyelonephritis) and are commonly present in appendix abscess, peritonitis, cholecystitis, septic wounds and bedsores. These might infect the lower respiratory passages or cause bacteriaemia and endotoxic shock, particularly

in surgical or otherwise debilitated patients who are treated with antibiotics to which the isolates are resistant. They also occasionally cause meningitis in neonates. The commensal isolates, however, do not act as primary pathogens in the intestine and are not known to cause gastroenteritis.

E. coli DIARRHOEA

Certain pathogenic serotypes of *E.coli* have a primary pathogenicity in the intestine and cause gastroenteritis. They fall into three distinct groups, associated with different disease syndromes.The O serogroup of Enteropathogenic strains such as 26,55,86,111,114,119,125,126,127,128 and 142 are associated with infantile diarrhoea. The O serogroup of Enterotoxigenic strains such as 6,8,15,25,27,63,78,115,148,153 and 159 are associated with infantile and adult diarrhoea. The O serogroup of Entreroinvasive strains such as 28ac, 112ac, 124,136,143,144,152 and 164 are associated with dysentery like diarrhoea. One more type is Enterohaemostic strain (Duguid, 1985).

E. coli URINARY TRACT INFECTION

E. coli is by far the commonest cause of urinary tract infection and the serogroups most often responsible are 01, 02, 04, 06, 09, 011, 018, 039 and 075 (Gruneberg and Bettelheim, 1969). The

serogroups associated with urinary tract infection correspond to those that predominate in the faeces. This correlation led to the "prevalence theory" that a limited number of O groups are involved simply because they are dominant in the colon which is the reservoir of the infecting organisms. The alternative, "special pathogenicity theory" postulates that the *E. coli* strains while causing infection become prevalent and are specially equipped to colonise the periurethral area and subsequently invade the urinary tract.

ACTION MECHANISM OF CLINICALLY USED ANTIMICROBIAL DRUGS

SELECTIVE TOXICITY OF DRUGS

An ideal antimicrobial agent (antibiotic) exhibits selective toxicity. The term selective toxicity implies that a drug is harmful to a parasite without being harmful to the host. Selective toxicity, often, is relative rather than absolute suggesting that, a drug in a certain concentration, tolerated by the host, may damage a parasite (Davies, 1979). Selective toxicity could be a function of a specific receptor required for drug attachment or could depend on the inhibition of biochemical events essential to the parasite but not to the host.

The action mechanism of many antimicrobial drugs has not been completely understood. The four recognisable mechanisms of action are:

I. Antimicrobial Action Through Inhibition of Cell Wall Synthesis.
Examples : Bacitracin, Cephalosporins, Cycloserine, Penicillins, Ristocetin, Vancomycin.

II. Antimicrobial Action Through Inhibition of Cell Membrane Function.
Examples : Amphotericin B, Colistin, Imidazoles, Nystatin, Polymyxins.

III. Antimicrobial Action Through Inhibition of Protein synthesis.
Examples : Chloramphenical, Erythromycins, Lincomycins, Tetracyclines;
Aminoglycosides : Amikacin, Gentamicin, Kanamycin, Neomycin, Netilmicin, Streptomycin, Tobramycin, Etc.

IV. Antimicrobial Action Through Inhibition of Nucleic Acid Synthesis.
Examples : Nalidixic Acid, Novobiocin, Pyrimethanine, Rifampin, Sulfonamides, Trimethoprim (Blumberg and Strominger, 1974).

ORIGIN OF DRUG RESISTANCE

The origin of drug resistance may be genetic or nongenetic.

GENETIC ORIGIN

Most of the drug resistant microbes emerge as a result of genetic change and subsequent selection process by antimicrobial drugs.

NONGENETIC ORIGIN

Active replication of bacteria is usually required for most antibacterial drug actions. Consequently, microorganisms that are metabolically inactive (non-multiplying) may be phenotypically resistant to drugs. However, their offsprings are fully susceptible.

Example : Mycobacteria often survive in tissues for many years after infection. But they are restrained by the host's defences and do not multiply. Such "persisting" organisms are resistant to treatment and cannot be eradicated by drugs. Yet, if they start to multiply (e.g., following corticosteroid treatment of the patient), they are fully susceptible to the same drugs (Finland,1979; Novick and Morse, 1967 and Tipper,1979).

KINDS OF RESISTANCE

A. CHROMOSOMAL RESISTANCE

Chromosomal resistance develops as a result of spontaneous mutation in a locus that controls susceptibility to a given antimicrobial drug. The presence of the antimicrobial drug serves as a selecting mechanism to suppress the susceptible organisms and favour the growth of the drug resistant mutants.

B. EXTRACHROMOSOMAL RESISTANCE

Bacteria often contain extrachromosomal genetic elements called plasmids. R factors are a class of plasmids that carry genes for resistance to one and often several antimicrobial drugs and heavy metals. Plasmid genes, for antimicrobial resistance, often control the formation of enzymes capable of destroying the antimicrobial drugs (Finland, 1979; Novick and Morse, 1967 and Tipper, 1979).

RESISTANCE TO ANTIBIOTIC DRUGS

Susceptibility of bacteria to antibiotics varies between species of microbe and between strains of any given species. Thus, some species and groups are inherently resistant to certain antibiotics. Resistance may also develop in normally susceptible

species. The development of resistance in hitherto susceptible organisms can reflect either mutation or the acquisition of resistance encoding genes. Whether inherent or acquired, resistance can usually be ascribed to one of the following four types of mechanisms :

1. Production of drug - inactivating enzymes
2. Reduced affinity of a target
3. Bypassing of a sensitive target or
4. Impermeability to the drug.

These mechanisms are of importance to various antibiotics in developed (mutational or plasmid mediated) resistance (Richmond and Sykes, 1973).

ASPECTS OF RESISTANCE

Mechanism of resistance can be examined conveniently from two aspects.

1. Inherent (Innate, natural, intrinsic) resistance
2. Acquired resistance

INTRINSIC RESISTANCE

Gram negative bacteria such as Pseudomonas and Enterobacteriaceae are usually less sensitive to a variety of chemically unrelated inhibitors than are Gram positive bacteria. One reason for this is the different structural and chemical composition of the outer layers of the cells. The cell wall of Gram positive bacteria is a thick layer of fibre, pressed against the cytoplasmic membrane. The wall consists mainly of an interspersed with lipids, teichoic and teichuronic acids. The peptidoglycan comprises at least 50 % of the dry weight of the walls. The cell envelope of gram negative bacteria is more complex. The inner (cytoplasmic) membrane is adherent to the peptidoglycan which is covalently linked to Braun's elongated lipoprotein. The peptidoglycan lipoprotein complex occupies partly the periplasmic space that exists between the outer and the inner hydrophobic membranes. Chemically, the outer membrane (OM) consists of lipopolysaccharide (LPS), phospholipids and proteins (Lambert,1983). The major outer membrane protein with the fastest electrophoretic mobility is the lipoprotein. Other major proteins in *E. coli* K12, are the outer membrane proteins A, C and F and Protein *a* (Lambert, 1983; Nikaido and Rosenberg, 1983; Hammond et al., 1984).

ACQUIRED RESISTANCE

Resistance to non-chemotherapeutic agents might be acquired by mutation, and selection by phenotypic (non-genetic) adaptation or via plasmid transfer. Klebsiella aerogenes adapted to grow in the presence of crystal violet and the surface of the resistant cells differed from those of sensitive ones (Lowick and James, 1957). Adaptation to chloroxylenol resistance in Pseudomonas aeruginosa though unstable was effected by Russell and Furr (1977).

Temporary resistance by phenotypic adaption to alcohol's is described by chopra (1987), who also considers that, in general, a non-genetic adaptive type of resistance is unlikely to play an important role in determining the long term survival of bacteria against antiseptics disinfectants.

Acquired chromosomal resistance by mutation has been described: chlorhexidine resistance with Providencia Stuartii (Chopra et al., 1987); alcohol's with *E. coli* (Fried and Novick, 1973); cationic bactericides with various organisms (Chopra, 1987).

TRANSFERABLE DRUG RESISTANCE

R factors were first demonstrated in 1959(Japan), in1962 (Britain) and in 1966 (USA) although the use of antibiotics had been known before these dates. It was shown that resistance to several antibiotics could be transferred as a unit between isolates of shigella and *E. coli* by conjugation. Resistance to sulphonamides, tetracycline, chloramphenicol, ampicillin, streptomycin, kanamycin, neomycin and gentamicin have all been found to be commonly extrachromosomal in Enterobacteria. It is also transferable (in vitro i.e., inside or in vivo i.e., outside) to drug sensitive bacteria. The actual incidence of drug resistance, in particular species, varies when different populations are studied. But it is found to be increasing in most pathogenic species, throughout the world; the incidence of strains with multiple resistance is similarly increasing (Hayes, 1968)

It is not known how or where extrachromosomal (plasmid-borne) resistance gene(s) originate. Whether they originate in chromosomal or in plasmid DNA, they are certainly liable to appear in R factors soon after a new antibiotic comes into general use. Complex R factors contain gene(s) for resistance to several antibiotics. Under suitable conditions, transferable multiple

resistance R factors can be built up. Resistance to as many as seven or eight different antimicrobial drugs has been found to be carried in a single R factor (Garrod et al., 1973).

It is often difficult to show the spread of R factors from one bacterial species to another , in the gut of a normal animal. The R factors are transferred to progeny, mainly by linear mode, during cell division. When an antibiotic is given, however, the number of drug sensitive organisms are rapidly killed. Watson (1970) worked on selected drug resistant, opportune organisms, that could multiply and transfer R factors. He found that a drug sensitive organism alone, which has R factor access alone thrives while others do not thrive.

The oral administeration of an antibiotic may result in a rapid increase of R factors , in the gut flora. R factors might have been present, originally, in only a small proportion of the cells of a single species. But it is possible, through antibiotic treatment, to make them present in a large proportion of the cells of many species. R factors may contain multiple resistance genes. The purpose of giving a single antibiotic may be to ensure that the majority of the Enterobacteria in the gut (commensal or pathogenic) possess an R factor that confers resistance(Hayes,1968).

Physicians and Veterinary doctors witness increasing drug resistance in pathogenic species. There is a tendency towards the administration of a greater range of antibiotics. In turn, this results in the build up of more complex R factors that get more widely distributed to commensal and pathogenic organisms(Hayes,1968).

The dangers of transferable drug resistance are obvious. If the incidence of multiple-resistance R factors continues to increase infections from pathogenic Coliforms, contributed by lactose fermenting enteric bacilli and expelled in the excreta, then they are more difficult to treat. Many infections are usually mild and do not require antibiotic therapy. In typhoid fever, for example, treatment with chloramphenicol is often life saving and there are only a few effective substitutes. R factors with chloramphenicol resistance had been common in food poisoning Salmonella for many years but did not appear in Salmonella typhi until 1972. Since then, epidemics of chloramphenicol resistant typhoid fever, in Mexico and Asia, have had higher mortality rates and have proved more difficult to control (Garrod et al., 1973; Hayes, 1968; Watson, 1970).

PLASMID MEDIATED RESISTANCE

Plasmids rarely mediate mercury resistance . But, Plasmids with acute sensitivity to mercuric chloride and cadmium in

E. coli have also been described (Weiss et al., 1978; Foster and Nakahara, 1979; Foster et al., 1979).

Plasmid mediated resistance to cadmium (Cd^{2+}) is dissimilar from resistance to mercury (Hg^{2+}). Plasmid-coded resistance to arsenic compounds has been observed in both Gram positive and Gram negative bacteria although the mechanism is unclear. Likewise, resistance to cobalt (Co^{2+}) and nickel (Ni^{2+}) in *E. coli* have also been recorded (Chopra and Ball 1982).

The role of plasmids in the resistance of Enterobacteria to antiseptics, disinfectants and preservatives is not clear. Ahonkai and Russell (1979) had studied the effects of the insertion of the RP_1 (Resistant Plasmid number) plasmid into a wild type (DCo) and its envelope mutant (DC_2) strains of *E. coli*. According to Ahonkai and Russell(1979), RP_1 does not show resistance to Hg^{2+} or to organomercury but does so to carbenicillin, tetracycline and neomycin / kanamycin.

The RP_1 strains of *E.coli* showed the same order of sensitivity to benzalkonium, chlorhexidine, cetrimide, phenol, cresol, chlorocresol, PMN and thiomersal as the isogenic R strain. Indeed , in some cases, the plasmid strains appeared to be slightly more sensitive although the difference was not as marked as that claimed by Klemperer et al. (1980). Curtis and Richmond (1974) had earlier found that a mutant, As19, of *E. coli* was lysozyme

– sensitive and that it became resistant to this enzyme after acquiring the RP_1 plasmids. Curtis and Richmond, (1974) had also added that several other plasmids had no effect on the latent resistance of *E. coli*.

Drug inactivating enzymes include the β lactamases, which hydrolyse certain related group of penicillins, cephalosporins, and the aminoglycoside - modifying enzymes. Numerous subtypes of these enzymes occur. Most bacteria have chromosomally determined β lactamases although these often are not significantly expressed. Other β lactamases are plasmid encoded. During the 1960s and 1970s such enzymes spread widely in Gram negative species - first amongst Enterobacteria and later to Pseudomonas aeruginosa, Haemophilus influenzae and Neisseria gonorrhoeae. (Richmond and Sykes, 1973). Aminoglycoside and chloramphenicol modifying enzymes are mostly mediated by plasmid (Foster, 1983).

Modified target affinity can also cause resistance. Many antibiotics, acting as analogues of their normal substrates, inhibit enzymes. For example, β lactamase inhibit peptidoglycan and transpeptidases as they resemble the peptidoglycan precursors that are the substrates of these enzymes; sulphonamides inhibit dihydropteroate synthase by mimicking its substrate, P aminobenzoate. Mutations which increase the ability of these enzymes to discriminate between inhibitors and their

specific substrates, can cause resistance. Other antibiotics, including streptomycin, macrolides and lincosamides, bind to bacterial ribosomes, inhibiting protein synthesis. Ribosomal lincosamide resistance arises when bacteria acquire a plasmid coded RNA methylase, which methylates the mRNA group to which these drugs usually bind (Ubukata et al., 1985).

PLASMIDS

The term "plasmid" was proposed by Lederberg (1952) for all extrachromosomal genetic structures that can replicate autonomously. Plasmids have many of the properties of a small chromosome. They contain similar genetic information for controlling replication and for ensuring segregation of one copy into each daughter cell at cell division. Plasmids are much smaller than the chromosome (e.g. 50 to 100 genes) and the genes contained are not normally essential to the cell. The extrachromosomal autonomous DNA molecules of plamids (i.e., replicons) are found in many bacterial cells. Plasmids have also been found in lower eukaryotes, e.g., the 2 kb plasmid of yeast. Plasmids have been found to be common in a number of species of Gram negative bacilli including Escherichia, Shigella, Klebsiella, Salmonella and Proteus (Stanisich, 1988).

Enterococcus faecalis is an indigenous gut species (Watanabe et al., 1981) which bears plasmids (Clewell, 1981).

The practical importance of bacterial plasmids was first recognized in the early 1960s when transferable drug resistance was discovered (Watanabe, 1963). Plasmids range in size from approximately 1 kb to more than 400kb (equivalent to C.10% of the *E.coli* chromosomes). The smallest have only enough DNA to accommodate two or three smaller genes e.g p15A (Change and Cohen , 1978). Nevertheless, small plasmids are relatively abundant; many confer no obvious phenotype on their host. Plasmids, capable of mediating conjugation, have a minimum size of about C.20Kb to 30Kb. Such plasmids (large or small) are said to be cryptic (Scott, 1984; Thomas and Smith, 1987).

RHYTHMICITY IN ORGANIC SYSTEMS AND INTERACTION WITH ENVIRONMENT

Rhythmicity refers to the periodicity or scheduled functioning of all universal systems including the human organism. Rhythmicity is characteristic of nature. There is no component of nature that does not follow a particular rhythm. "Every component of the universe from an electron to a galaxy is continuously in motion and this movement cannot proceed for ever in the same direction sooner or later the movement must complete a cycle or stop and return in the opposite direction". Therefore a cyclic or rhythmic periodicity is characteristic systems in universe-both mechanical as well as biological organisms. They have been and are in constant interactions with their environment and therefore with nature.

Hence, it follows that as an evolutionary adaptation to life in a rhythmic environment most of their physiological as well as behavioural process also come to follow a rhythmic pattern. Life, itself is a single rhythm or cycle, encomposing several smaller rhythms of varying duration such as daily rhythms (Circadian) monthly rhythms and yearly rhythms (circannual). When the internal rhythms ("Biological clocks") of man are disturbed , the result is physiological disturbances (Cloudsley Thompson, 1961). The circannual rhythm in the urinary excretion of 17-ketosteroids has been discovered following 15 years of study in the excretory pattern of a normal male(Hoffman, 1969).

HUMAN BODY TEMPERATURE
ITS CIRCADIAN RHYTHM

The term body temperature refers to the temperature of the deeper structures (e.g. liver and brain). The skin usually has a lower temperature than the depths of the body. This provides a temperature gradient which leads to heat loss from the depths to the surface of the body and thence from the surface to the environment. In hot countries these temperature gradients may be reversed (Adair, 1971).

The temperature regulating system consists of two major functional divisions; (a) a quick reacting system, characterized as a neural net with time constants of seconds or minutes and (b) a sloweracting neuroendocrine system with time constants of

minutes or hours (Carlson, 1973). The two systems have important interactions, both centrally and peripherally, in subserving thermoregulation. In the neural net, skin receptors for warmth and " coolth" (Bligh and Moore,1972) are projected by sensory fibers in the lateral spinal thalamic tract to synaptic relays in the reticular system of the medulla and thence to the posterolateral ventromedial nuclei of the thalamus. Projection to cortical areas presumably mediates conscious thermal sensations.

Thermal receptors exist also in the spinal cord (Jessen and Mayer, 1971 and Simon and Iriki, 1971), in veins (Hammel,1968), and in the abdominal viscera (Hammel, 1968 and Rawson and Quick, 1972). Both surface and deep body receptors are presumed to connect functionally with the preoptic anterior and posterior hypothalamus thermoregulating centres. Although specific pathways have not been identified, the ascending noradrenergic tracts connecting the brain stem with the hypothalamus may transmit the thermal sensory input (Ungerstedt, 1971).

In mammals, the regulation of a relatively constant body temperature over a wide range of environmental conditions demands a complex interplay among all heat gain and heat loss mechanisms.

1. **Mechanisms in the defence against cold include:**

(a). Heat produced by muscular shivering, exercise or other exothermic biochemical reactions which elevate basal metabolic rate.

(b). Heat conserved by cutaneous vasoconstriction or by growth of hair.

2. **Defence mechanisms against heat include:**

(a). Increase in heat loss by sweating , panting or salivation, as well as by a rise in extracellular fluid volume and in cutaneous blood flow, with attendant increase in peripheral thermal conductance.

(b). Lowering of metabolic heat production. In addition to these physiologic and metabolic adjustments , it is now generally recognized that behavioural thermoregulation is of equal or of greater importance in defense against heat and cold in most mammals, especially in man (Brown and Brengelman, 1970).

MATERIALS & METHODS

CHAPTER III

MATERIALS AND METHODS

The methodology adoptd for the study is discussed under the following heads:

1. Observation of body temperature of diarrhoeal patients and certain biochemical tests

2. Source of bacterial pathogens

3. Sterilization

4. Grams staining

5. Isolation of *E.coli*

6. Identification of biochemical characteristics

7. Preparation of antibiotic disc
    i. Readying stock solutions
    ii. Dilution of the antibiotics

8. Antibiotic sensitivity testing

   i. Disc potency

9. Determination of the antibiotic resistance of the clinical isolates

   Preparation of

   i. Bacterial cultures for antibiotic assay

   ii Antibiotic assay medium

   iii. Seeded agar medium

   iv. Assay of antibiotics

   v. Measuring antibiotic activity

10. Molecular characterization of the isolates

   i. Plasmid DNA analysis

   ii. Restriction enzyme digestion

   iii. PCR amplification of *E.coli* DNA

   iv. Agarose gel electrophoresis

   v. Plasmid curing and antibiotic sensitivity testing

11. Confirmation of the Pyrogen *E.coli* effect by animal inoculation

DETAILS OF THE METHODOLOGY

1. **Observation of body temperature and certain biochemical tests for non diarrhoeal group (Control) and diarrhoeal group(Experiment) of human beings.**

   a. **Experimental Set up**

   A set of 10 non diarrhoeal human beings were considered as control and another set of 22 diarrhoeal patients were taken up for experimental study.

   b. **Site of measurement**

   For the study of rhythm, the temperature measured must represent the thermal state of the body oral temperature in Fahrenheit measurement has been recorded with a clinical thermometer.

   c. **Thermometer used**

   Glass, clinical thermometer (mercury threaded) was used. Oral temperature was measured by placing the thermometer under the tongue.

   The thermometer was held in position for two minutes. The same thermometer was used throughout the experiment to minimize instrumental error arising from change of thermometer.

The thermometer was sterilized after every recording.

d. Time and interval of measurement of the body temperature

The 24 hour body temperature readings for both the control and the experimental groups were recorded,at one hour interval,continuously for 5 days. The temperature was observed and recorded from 06.00 A. M. of the first day to 06.00 A. M. of the second day.Inorder to observe even the slight oscillations, occuring each hour, the temperature was recorded for every one hour, continuously for 24 hours.

Graphs were drawn to depict the circadian rhythm, in the control and the experimental groups, by taking the mean value of all observations for a particular time hourwise and daywise.

e.Observation of certain biochemical factors

The biochemical tests were as under :

i) Blood pressure
ii) Estimation of glucose
iii) Estimation of serum cholesterol total
iv) Estimation of haemoglobin
v) Estimation of total protein and albumin

vi) Estimation of Bilirubin

vii) Estimation of [ASAT] Aspartate Amino Transferase SGOT

viii) Estimation of [ALAT] Alanine Amino Transferase SGPT

ix) Estimation of Alkaline phosphatase

x) Estimation of urea in urine

xi) Estimation of creatinine in urine.

(Procedure included in the appendix)

f. Statistical analysis

In the present study, 't' test was used. Based on the 't' value difference between the control and the experimental groups, the difference in values was stated to be significant or non significant by comparison.

2. SOURCE OF BACTERIAL PATHOGENS

A total number of 22 isolates of *E.coli* collected from various diarrhoeal samples have been used for the present investigation. These clinical isolates were labeled as isolate numbers 1-22. The isolation and identification of *E.coli* was done according to the standard techniques (Cruikshank et al., 1975). The isolates were maintained on MacConkey Agar Medium (Hi-media, Bombay). These isolates were subcultured

whenever needed. The assay medium used for the antibiotics Muller Hinton Agar Medium (Hi-Media, Bombay).

3. STERILIZATION

Glassware, culture media, filter paper and micropipettes used in this experiment were sterilized by autoclaving at 121^{0}C at 1 atmosphere for 15 minutes. Forceps were sterilized by flaming and instantly dipping in spirit.

4. GRAMS STAINING

To differentiate bacteria into 2 principle groups Gram positive and Gram negative based on their Gram's reaction.

PRINCIPLE

Grams staining was one of the differential staining methods that differentiates bacteria into two groups, Gram positive and Gram negative. Differential staining requires the use of more chemical reagents that are applied, sequentially to a fixed smear. In Gram staining procedure the first reagent used was called primary stain, crystal violet which imparts colour to all the cells. Crystal violet attaches to negatively choiced groups in the cell wall membrane and cytoplasm. Then Gram's iodine, the mordant fixes the primary stain to the cells forming crystal violet complex (CV-I).

In Gram positive bacteria CV-I complex binds tightly to the Magnesiumribonucleic acid component of cell wall and forming Mg-RNA CVI complex which was more difficult to remove than the CV-I complex. The third reagent used was the decolourizing agent. Based on the chemical composition of the cell wall, the decolourizing agent may or may not remove the CV- I complex. The decolourizing agent functions as a lipid solvent and as a protein dehydrating agent. In the cellwalls of Gram positive bacteria, the lipid content was very less and was dissolved by alchohol and causing minute pores in the cell wall which was immediately closed by protein dehydrating agent of the alcohol and thus the loss of Mg- RNA-CV-I complex was prevented.

In Gram negative bacteria, due to high lipid content, the alcohol posses large pores resulting in the loss of CV-I complex and the cells remain colourless. When the counter stain was used which as a contrasting colour, as that of the primary stain. After decolourization step, those bacterial cells which have losed the primary stain have taken up the counter stain while others retain the primary stain.

MATERIALS REQUIRED

Glass slide, inoculation needle, crystal violet, Grams iodine, ethanol and safranin and diarrhoeal samples.

PROCEDURE

(a).A clean glass slide was taken and a loop of motion sample was taken and a thin smear was made on the slide.

(b). The smear was airdried and heat fixed.

(c). Then the smear was flooded with crystal violet the primary stain and allow to stand for 30 seconds.

(d). The excess stain was washed off with tap water.

(e). The smear was flodded with Gram's iodine and allowed to stand for 60 seconds. The excess stain was washed off with tap water.

(f). To decolourize the smear, ethanol was added drop by drop until the alcohol runs clear.

(g). After washing with tap water, the smear was counterstained with safranin for 60 seconds.

(h). The excess stain was washed off, air dried and observed under oil immersion objective of microscope

(Composition of stains and reagents were included in the appendix)

5. ISOLATION OF *E.coli*

A loopful of the sample was streaked on specific isolating medium and incubated for 24 - 48 hours. The presence (or) absence of growth with specific characteristic was recorded and the colonies were taken for further analysis. Following media were used for isolating the *E.coli* :

1. Nutrient agar
2. Mac conkey agar
3. Blood agar
4. Eosin methylene blue agar

(Composition of media included in the appendix).

6. BIOCHEMICAL CHARACTERIZATION

Biochemical characterization was carried out through the following tests:

a) Imvic Tests
b) Sugar Fermentation
c) Catalase Test
d) Urease Test
e) Motility Test

(Composition and Procedures included in the appendix).

7. PREPARATION OF ANTIBIOTIC CONTAINING DISC

I. Preparation of stock solution of antibiotics

The stock solution of antibiotics were prepared by dissolving 100 mg of respective antibiotic in 10 ml of sterile distilled water. This makes 10,000 $\mu g\ ml^{-1}$ or 10,000 ppm solution.

II. Dilution of antibiotics

The dilutions of antibiotics have been prepared from the stock solution using sterile distilled water for dilution. There are five dilutions for each antibiotic such as 10, 20, 50, 100 and 250 μg ml^{-1}.

Discs of 6 mm diameter were cut from a standard whatmann No. 1 filter paper and sterilized by autoclaving before use. Each disc dipped in 10,20,50, 100 & 250 $\mu g ml^{-1}$ solutions of antibiotics absorbed the same strength of the antibiotic solution. For instance , a disc dipped in 10$\mu g ml^{-1}$ has absorbed 10 μg; a disc dipped in 20 $\mu g ml^{-1}$ has absorbed 20 μg; a disc dipped in 50 $\mu g ml^{-1}$has absorbed 50 μg ; a disc dipped in 100 $\mu g ml^{-1}$ has absorbed 100 μg and a disc dipped in 250 $\mu g ml^{-1}$ has absorbed 250 μg respectively. The discs were dried and stored before use under a jet of warm air.

8. ANTIBIOTIC SENSITIVITY TESTING

I. Disc potency

Standard antibiotic discs were not used in the present study for two reasons

i) Commercially available discs had only specified potency for certain concentrations such as 250 μg and 500 μg.

ii) The study was essentially aimed at identifying the intrinsic resistance (i.e., the condition in which intake of antibiotic in higher concentration leads to assimilation of the antibiotic and the pathogen's resistance power increases while the body's resistance power decreases) pattern in pathogenic isolates of *E.coli.*

The antibiotics used in the present study were obtained from a commercial source. The name of the antibiotics used in the present study are as follows:

S. No.	Name of the antibiotics	Manufactures
1.	Ampicillin	Hindustan antibiotics Ltd., India
2.	Chloramphenical	Samir Pharmaceuticals Ltd., India
3.	Ciprofloxacin	Ranbaxy Laboratories Ltd., India
4.	Erythromycin	Tamil Nadu Data Pharmaceuticals Ltd., India

5.	Gentamicin	Alembic pharmaceuticals Ltd., India
6.	Nalidixic acid	Ranbaxy laboratories Ltd., India
7.	Nitrofurantoin	Eskayef pharmaceuticals Ltd., India
8.	Norfloxacin	Cipla Ltd., India
9.	Streptomycin	Hindustan antibiotics Ltd., India
10.	Trimethoprim/sulpha methoxazole	Roche products Ltd., India

9. DETERMINATION OF ANTIBIOTIC RESISTANCE OF THE CLINICAL ISOLATES OF BACTERIA

In order to find out the widespread occurrence of antibiotic sensitivity or resistance among the clinical isolates of bacteria, separate assays were performed for each culture. The methods followed were essentially the same as described by Norris and Ribbons (1969). The procedures followed were furnished below :

I. Preparation of bacterial cultures for antibiotic assay

A loopful of the standard clinical culture of bacteria was transferred to 10 ml of nutrient glucose both dispensed in standard test tubes. The tubes were placed over a gyratory shaker for 24 hours at 30^0c. This culture of 24 hour growth was used for seeding the medium for antibiotic assay.

II. Antibiotic assay medium

For antibiotic assay, nutrient glucose agar medium with following concentrations was used:

Glucose	5.0g
Peptone	5.0g
Sodium chloride	5.0g
Beef extract	3.0g
Yeast extract	1.0g
Agar agar	15.0g
Distilled water	1000ml
p^H	7.0

III. PREPARATION OF SEEDED AGAR MEDIUM

The nutrient glucose agar medium was prepared, in 100 ml quantities, in 250 ml Erlenmeyer flasks. After sterilization, he medium was brought to bearable warmth (45 ^{0}c). Then, a quantity of 0.1 ml of the 24 hours old bacterial culture was inoculated to each flask aseptically and mixed well. This medium was poured on to sterile petriplates, approximately 15 ml per plate and allowed to solidify. These seeded agar plates were used for antibiotic testing.

IV. Assay of antibiotics

Within 30 minutes of solidification of the agar, the plates were used for antibiotic assay. Using flame sterilized forceps, the antibiotic soaked paper discs were carefully placed on the agar surface and the plates were incubated at 28^{0} c for 24 hours. Five discs were placed per petriplate.

V. Measuring antibiotic activity

The sensitivity of the test bacterium towards a selected antibiotic was expressed in the form of hollow or

inhibition zone around the antibiotic disc. After the incubation period, the diameter of the inhibition zones was measured and compared with the interpretive chart of Kirby – Bauer Corporation , U.S.A., Bloomfield, New Jersey and classified as resistant and sensitive.

10. MOLECULAR CHARACTERIZATION OF THE ISOLATES

Molecular characterisation of *E.Coli* was done using Plasmid DNA analysis and Restriction enzyme digestion, using the Restriction enzymes EcoRI and Hind III and PCR studies.

I.PLASMID DNA ANALYSIS

PROCEDURE

1. Microcentrifuge tube was filled with saturated bacterial culture grown in LB broth + antibiotics. The tube was spun in microcentrifuge for one minute to make sure that tubes were balanced.The tube was briefly drained on paper towel and the supernatant was drained.
2. Step 1 was repeated in the same tube, filling the tube again with more bacterial culture. The purpose of the step was to increase the starting volume of cells so that more plasmid

DNA could be isolated per preparation .The tube was spun in microcentrifuge for one minute . Supernatant was Poured off and the tube was drained on paper towel .

3. O.2 ml of ice-cold solution 1 was added to cell pellet and the cells resuspended as much as possible, using disposable transfer pipette.

- Solution 1 contained glucose, Tris and EDTA. Glucose was added to increase the osmotic pressure outside the cells. Tris was a buffering agent used to maintain a constant pH (= 8.0). EDTA protected the DNA from degradative enzymes (called DNAses); EDTA bound the divalent cations that were necessary for DNAse activity .

4. 0.4 ml of solution 2 was added to the tubes, caped and inverted five times, gently. The tubes were left at room temperature for five minutes.

- Solution 2 contained NaOH and SDS (a detergent). The alkaline mixtures ruptured the cells , and the detergent broke apart the lipid membrane and sloubilizes cellular proteins. NaOH also denatured the DNA into single strands .

5. 0.3 ml of ice-cold solution 3 was added to the tubes, caped and inverted five times, gently. The tubes were incubated on ice for ten minutes.

- Solution 3 contained a mixture of acetic acid and potassium acetate . The acetic acid neutralized the pH , allowing the DNA strands to renature . The potassium acetate also precipitated the SDS from solution , along with the cellular debris . The *E.coli* chromosomal DNA , a partially renatured tangle, at this step was also trapped in the precipitate . The plasmid DNA remained in solution .

6. Tubes were centrifuged for five minutes . Supernatant was transferred to fresh microcentrifuge tube using clean disposable transfer pipette. During the transfer care was taken to avoid white precipitate formation.

- This fractionation step separates the plasmid DNA from the cellular debris and the chromosomal DNA in the pellet.

7. Remainder of the centrifuge tube was filled with isopropanol and the tube was left at room temperature for two minutes.

- Isopropanol effectively precipitates nucleic acids , but much less effective with proteins. A quick precipitation can therefore purify DNA from protein contaminants.

8. The tubes were centrifuged for five minutes . A milky pellet was at the bottom of the tube . Supernatant was poured off without dumping out the pellet and the tube was drained on paper towel.

- This fractionation step further purifies the plasmid DNA from contaminants.

9. 1 ml of ice-cold 70% ethanol was added to the tube, caped and inverted several times. The tubes were spun for one minute. Supernatant (be carefull not to dump out pellet) was poured off and the tube, drained on paper towel.

- Ethanol helps to remove the remaining salts and SDS from the preparation.

10. The tubes were allowed to dry for five minutes then was added 50 µl of TE.

SOLUTIONS

SOLUTION 1	**per 500 ml:**
50 mM glucose	**9 ml 50 % glucose**
25 mM Tris HCl (pH 8.0)	**12.5 ml 1 M Tris HCl pH 8.0**
10 mM EDTA (pH 8.0)	**10 ml 0.5M EDTA pH 8.0**
SOLUTION 2	**per 500 ml :**
1 % SDS	**50 ml 10% SDS**
0.2 N NaOH	**100 ml 1 N NaOH**
TE	**per 500 ml :**
10 mM Tris HCl	**300ml 5M Potassium Acetate**
1 mM EDTA	**57.5ml glacial acetic acid**
	per 100ml :
HCl pH 8.0	**1 ml 1 M Tris HCl pH 8.0** **0.5ml 0.5 M EDTA pH 8.0**

II. RESTRICTION ENZYME DIGESTION

Restriction enzymes, EcoR1 and Hind III, were used for the experiments. One microgram of DNA was digested with twenty units of enzymes, in a twenty μl reaction mixture containing 33 mM potassium acetate , 10 mM magnesium acetate, 0.5 mM dithiothreitol and 100μl per ml bovine serum albumin . The reaction mixtures were incubated at 37°c for two hours and digestion was stopped by the addition of EDTA to a concentration of 10 mM.

III. PCR AMPLIFICATION OF *E.coli* DNA

Amplification was demonstrated by electrophoresis of DNA in 4% agarose gel in TBE buffer (Maniatis et al., 1982) at 7V cm –1 and staining with ethidium bromide . Amplified DNA was digested with restriction endonucleases in the PCR buffer (without prior ethanol precipitation as this was not required)and led to a loss of DNA. Digested DNA was electrophorised on 4% agarose gel .

IV.AGAROSE GEL ELECTROPHORESIS

PROCEDURE

1. The electrophoresis unit was arranged.
2. 0.7 percent Agarose was prepared with TBE buffer (35 ml) and poured into gel casting tray and allowed to solidify (Added Ethidium Bromide).

3. The acrylic comb was arranged before solidifying.
4. After solidifying the comb was removed and the gel was placed in the electrophoresis tank which contained TBE buffer.
5. The samples of DNA were loadaed as per the following composition;

   Sample DNA - 10 μ/lit

   Loading dye - 5 μ/lit

   (Bromophenol Blue)

6. The electrodes were connected at 20 volts and 30milliamps and the DNA was allowed to run over the gel for 3 hours.
7. The gel was viewed under UV-transillumination (photo-dyne) for observations.

V. (a). PLASMID CURING

Acridine orange treatment

Materials required : Acridine orange, Gel apparatus

Characteristics of Gel

For DNA, like plasmid, horizontal gel apparatus was used.

PROCEDURE

1. Culture of *E. coli*, was taken.

2. The cells of the culture were grown overnight at optimum temperature
3. Acridine orange, a curing agent, was added to the medium (500μg/ml).
4. 0.1 ml of the overnight grown culture was transferred to the test tubes containing the medium treated with acridine orange culture was grown for 24 hours at the optimal temperature.The cells were used for plasmid purification.
5. The cells cured with acridine orange were tested against the antibiotics selected for the study. The loss of plasmids was reflected in the sensitivity of the cells towards the antibiotics.
6. The overnight grown culture, treated with acridine orange, was loaded in the gel electrophoresis.
7. The culture was allowed to run over the gel for 3 hours.
8. The gel was viewed under UV- transillumination (Photodyne) and the observation for the occurrence of plasmids was made.
9. Purified plasmids were subjected to agarose gel electrophoresis, and tested for the presence of plasmids by observing the bands. This process was repeated several times to observe the removal of plasmids.
10. A suitable control was maintained (without acridine orange).

EXPANSION FOR THE CHEMICALS USED IN THE PLASMID ANALYSIS

1. TE - Tris EDTA

2. EDTA - Ethylene Diamino Tetra Acetic acid

3. SDS - Sodium Dodecyl Sulfate

4. EB - Ethidium Bromide

5. TAE - Tris Acetate EDTA buffer

6. TBE - Tris Boric acid EDTA buffer

7. BPB - Bromo Phenol Blue

(b). Antibiotic Sensitivity Testing

After the removal of plasmids, the cultures of *E. coli* were again tested for antibiotic resistance or sensitivity. The methods followed were essentially the same as described by Norris and Ribbons (1969).

11. PYROGEN TESTING

To confirm the pyrogenic activity of *E.coli.*

PRINCIPLE

Pyrogens are substances that induce fever in animals,on parenteral administeration. Pyrogens originate from either exogenous or endogenous sources such as bacteria or leucocytes, respectively. Pyrogen can be tested by injecting it into mice and recording the temperature rise.

REQUIREMENTS

20 numbers of mice of 2-3 months age

Pyrogen- free sterile saline

0.25% of *E.coli* suspension

Clinical thermometer

PROCEDURE

Before proceeding to detect the pyrogen in test sample, a Sham test was conducted to find out the suitability of the animal used.

SHAM TEST

This was done one day before the main test.

The rectal temperature of the animal was recorded.

10 ml of pyrogen- free saline was intravenously injected into the mice and the rectal temperature was recorded for every 30 minutes, for 3 hours. The difference between the initial and the subsequent readings was noted. The animal showing 0.6^{0}c or more as variation in temperature was considered not suitable for the main test.

MAIN TEST

The initial rectal temperature (of the animals) was recorded and after 5 minutes the temperature was again recorded. The initial temperature of the animal was the mean of the 2 (two) readings, taken for each animal. The test animals were injected with 0.2ml of 0.25% *E.coli* suspension, subcutaneously. The rectal temperature of the animals, for a duration of 3 hours, for every half an hour was recorded. The initial temperature was subtracted from the final temperature to find out the difference.

SUBCUTANEOUS INJECTION

PROCEDURE

The left hand thumb and finger were used to pick up a fold of skin over the neck of the animal. The needle was inserted to a depth of about 7mm through the hollow formed below and between the fingers. The skin was released and the needle moved to check if it was placed subcutaneously for injection. The needle was then pushed through the skin with the finger and the thumb and squeezed.

RESULTS

CHAPTER - IV

RESULTS

I (i) DIFFERENTIATION OF BACTERIA

In the present study on *E.coli* , clinical samples from diarrhoeal patients were collected and stained employing Grams stain method. The stains were observed under the microscope for differentianting Gram positive OR Gram negative bacteria. Gram stained smear showing pink coloured, rod shaped bacteria were identified as Gram negative (Fig.1).

(ii) ISOLATION OF *E.coli*

The specific bacteria were isolated from the samples that were streaked on specified medium. After incubation, the cultural characteristics of the samples confirmed that they were *E.coli* (Fig.2 ,3, 4 & 5).

CULTURAL CHARACTERISTICS OF *E.coli*

1.Nutrient agar medium - Large, Thick, Greyish, White, Highly opaque, Circular, Dome shaped and Smooth colonies.

2.Macconkey agar medium- Lactose fermenting colonies Pink and Dome shaped.

3.Blood agar medium - Grey color, Non haemolytic colonies.

4.Eosin methylene blue agar medium - Metallic Sheen colonies.

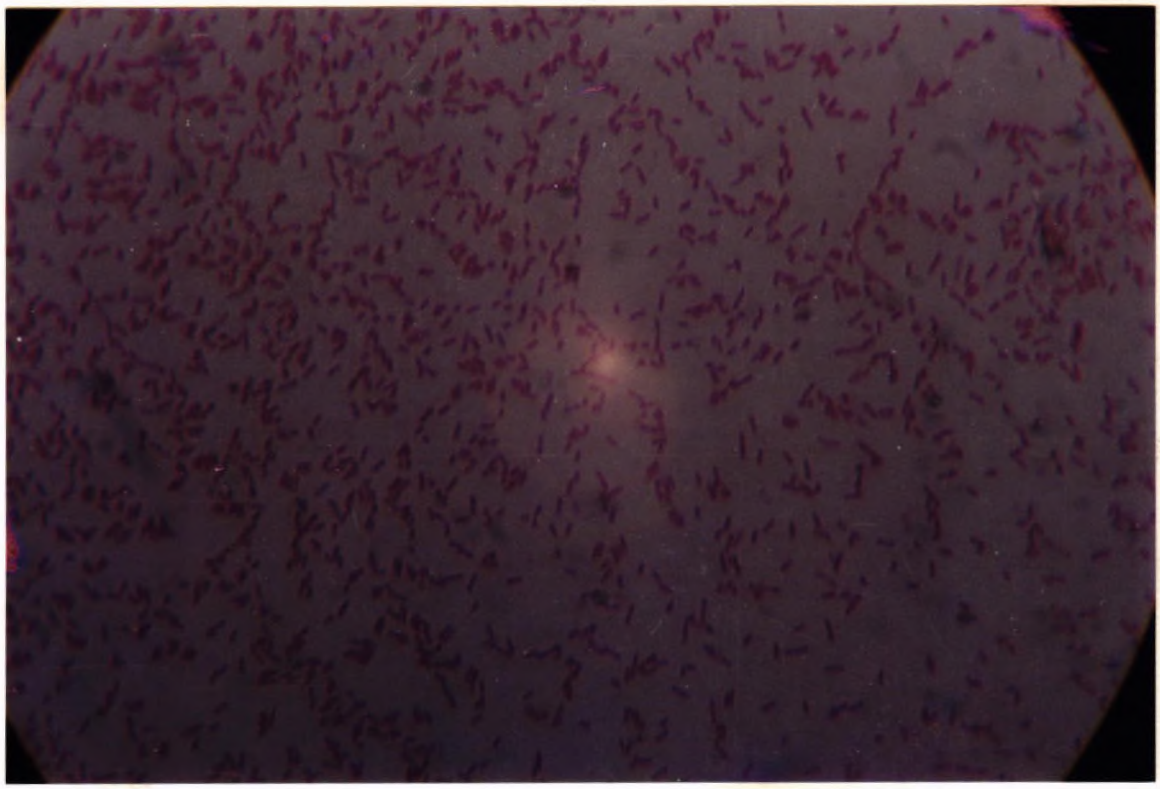


Fig.1 Microphotographic plate shows Gram negative Rods with E.coli

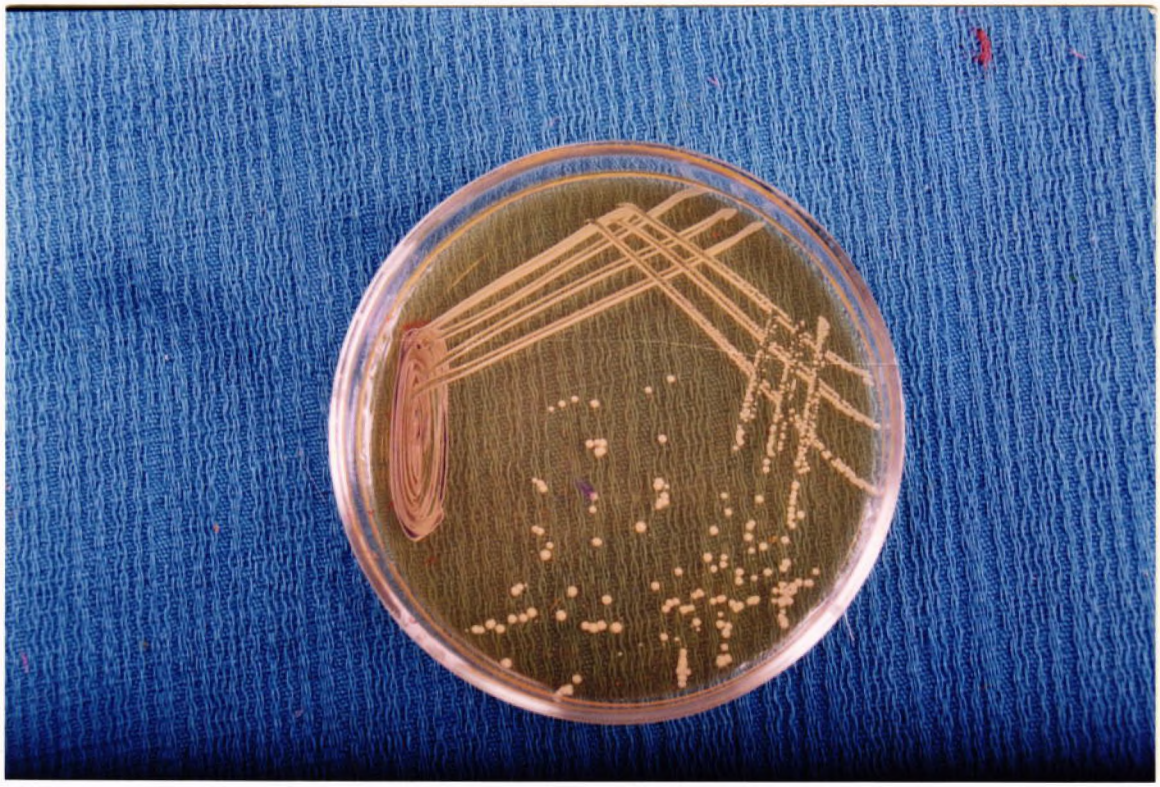


Fig. 2 Photographic Plate shows Nutrient Agar with E.coli

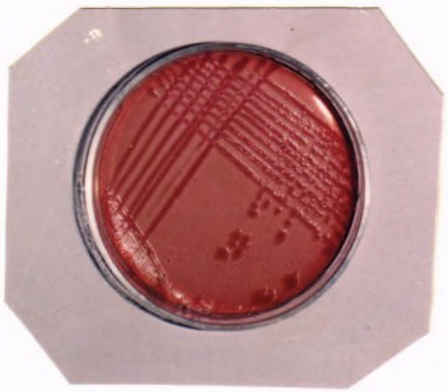


Fig.3 Photographic Plate shows Macconkey Agar with lactose Fermenting pink colour E.coli Colonies

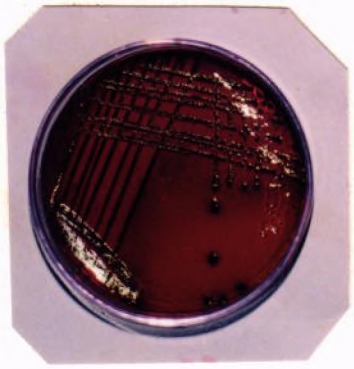


Fig.4 Photographic Plate shows EMB Agar with Metallic Sheen colonies of *E.Coli*

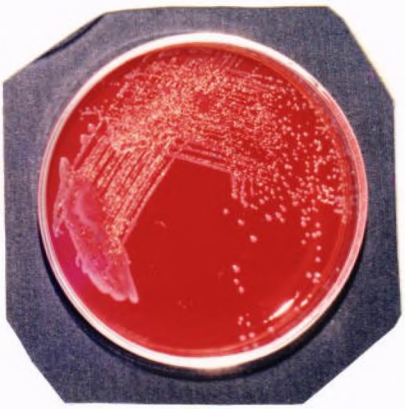


Fig.5 Photographic Plate shows Blood Agar with Non Haemolytic colonies of *E.Coli*

II. CIRCADIAN VARIATIONS OF BODY TEMPERATURE

The body temperature of the diarrhoeal patients (Experimental group) and a group of 10 non diarrhoeal human beings (control group) were observed periodically to find out the variations in their body temperature. Certain biochemical tests related to blood pressure, blood sugar, cholesterol, haemoglobin, albumin, globulin, phosphatase were done. Urine tests for urea and creatinine were also done. The pyrogenic activity of E.coli was confirmed by inoculating mice. These isolates were tested for sensitivity and resistance against 10 selected, commonly used antibiotics. The antibiotics used were Ampicillin, Trimethoprim / Sullpha metholazole, Ciprofloxacin, Chloramphenical, Erythromycin, Nitrofurantoin, Genatamicin, Nalidixicacid, Norfloxation and Streptomycin.

(i). THE BODY TEMPERATURE RHYTHM OF THE CONTROL GROUP - NON DIARRHOEAL

A group of 10 non diarrhoeal human beings were considered as control group and the hourwise body temperature observations showed a rhythmic pattern. But no marked variation was observed during the 3 days. A minima of 98.2^0 F and a maxima of 98.7^0 F were recorded (Table I ; Fig. 6, 7 & 8).

TABLE I

HOURWISE / DAYWISE AVERAGE OF BODY TEMPERATURE °F READINGS- CONTROL GROUP – NON DIARRHOEAL

	Days / Hours	1	2	3
AM	06.00	98.4 ± 0.1	98.5 ± 0.2	98.3 ± 0.04
	07.00	98.4 ± 0.01	98.3 ± 0.03	98.4 ± 0.5
	08.00	98.5 ±0.02	98.5 ± 0.01	98.6 ± 0.3
	09.00	98.4 ± 0.06	98.4 ± 0.01	98.4 ± 0.5
	10.00	98.5 ±0.01	98.5 ± 0.02	98.5 ± 0.02
	11.00	98.5 ± 0.01	98.6 ± 0.06	98.6 ± 0.09
	12.00	98.6 ±0.02	99.6 ± 0.03	98.7 ± 0.06
PM	13.00	98.7 ± 0.03	98.7 ± 0.01	98.6 ± 0.6
	14.00	98.6 ± 0.05	98.7 ± 0.06	98.6 ± 0.01
	15.00	98.5 ±0.4	98.2 ± 0.07	98.4 ± 0.03
	16.00	98.4 ± 0.03	98.4 ± 0.06	98.4 ± 0.03
	17.00	98.4 ± 0.01	98.5 ± 0.01	98.6 ± 0.01
	18.00	98.4 ± 0.03	98.5 ± 0.3	98.4 ± 0.03
	19.00	98.2 ± 0.01	98.2 ± 0.4	98.2 ± 0.04
	20.00	98.4 ± 0.03	98.3 ± 0.01	98.3 ± 0.1
	21.00	98.5 ± 0.3	98.4 ± 0.03	98.2 ± 0.3
	22.00	98.4 ± 0.1	98.4 ±0.01	98.4 ± 0.01
	23.00	98.5 ± 0.6	98.5 ± 0.2	98.6 ± 0.3
	24.00	98.6 ± 0.3	98.4 0.06	98.4 ± 0.02
AM	01.00	98.4 ± 0.01	98.3 ± 0.01	98.2 ± 0.3
	02.00	98.6 ± 0.01	98.5 ± 0.7	98.3 ± 0.5
	03.00	98.3 ± 0.01	98.4 ± 0.01	98.4 ± 0.03
	04.00	98.3 ± 0.04	98.5 ± 0.07	98.4 ± 0.3
	05.00	98.6 ± 0.05	98.4 ± 0.02	98.2 ± 0.2

N – 10

Twenty four observations each

Mean ± S.E readings are calculated for every one hour recording continuously for three days.

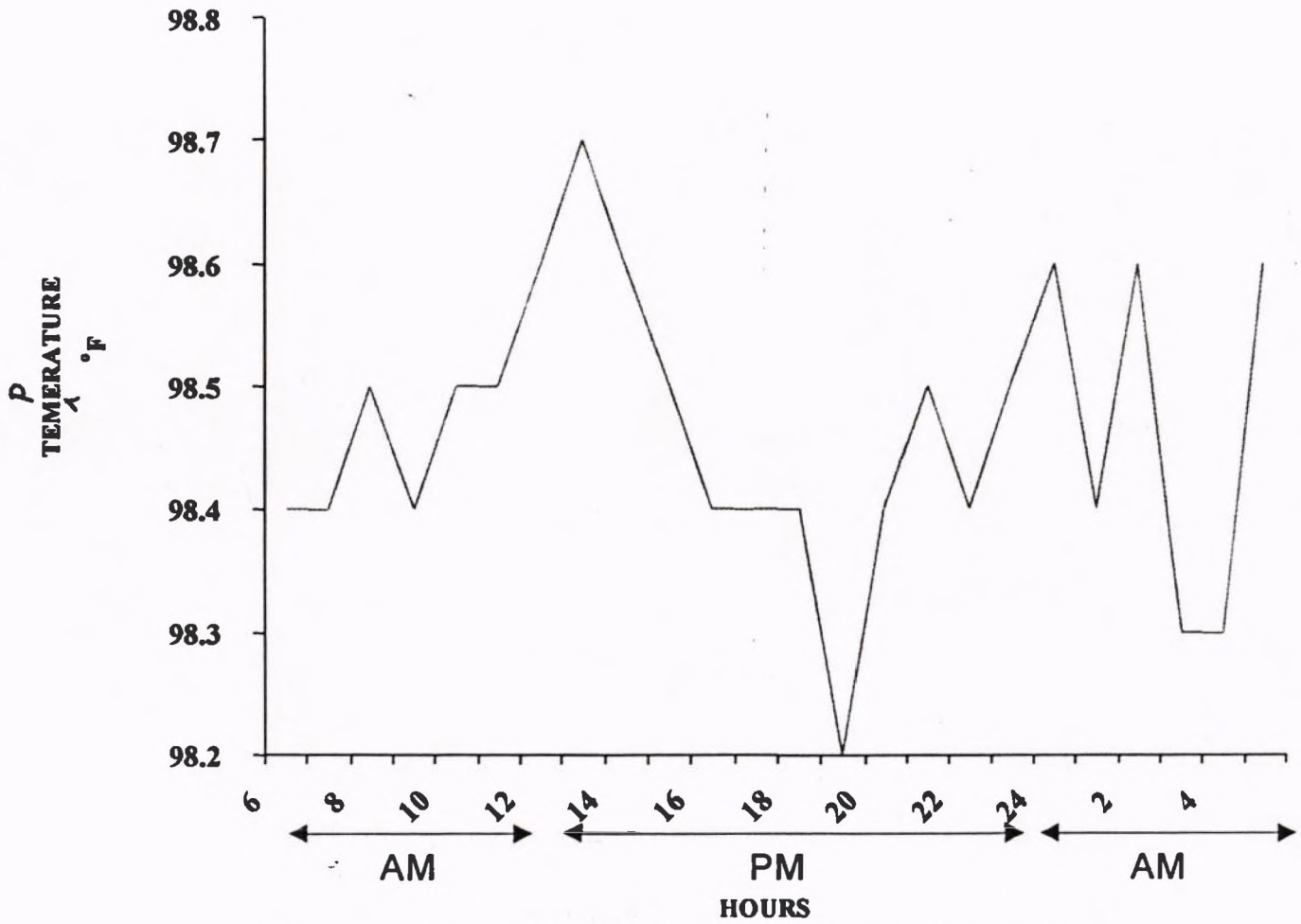


Fig. 6 HOURWISE BODY TEMPERATURE READINGS OF THE CONTROL GROUP - NON DIARRHOEAL DAY 1

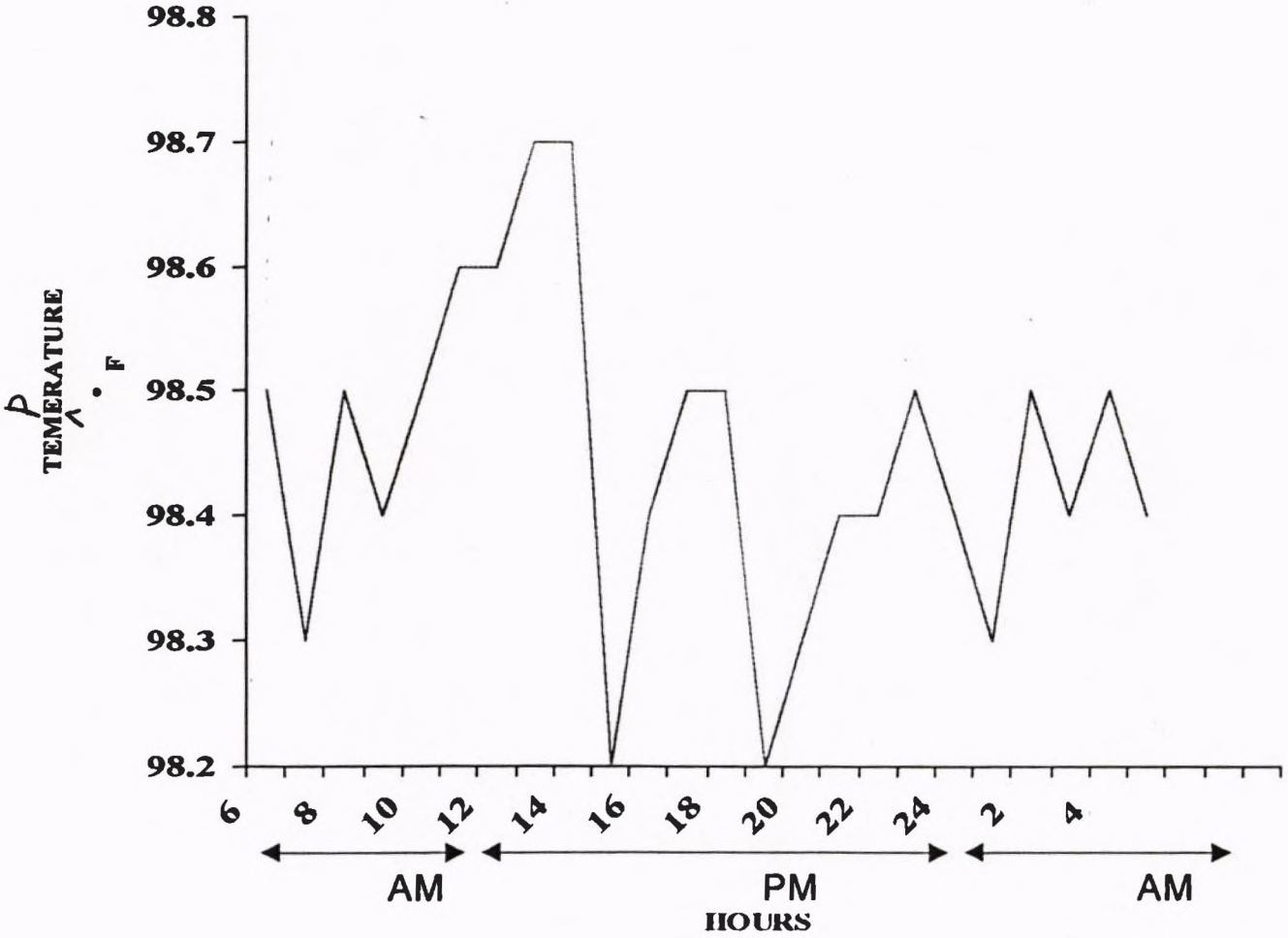


Fig. 7 HOURWISE BODY TEMPERATURE READINGS OF THE CONTROL GROUP NON DIARRHOEAL DAY 2

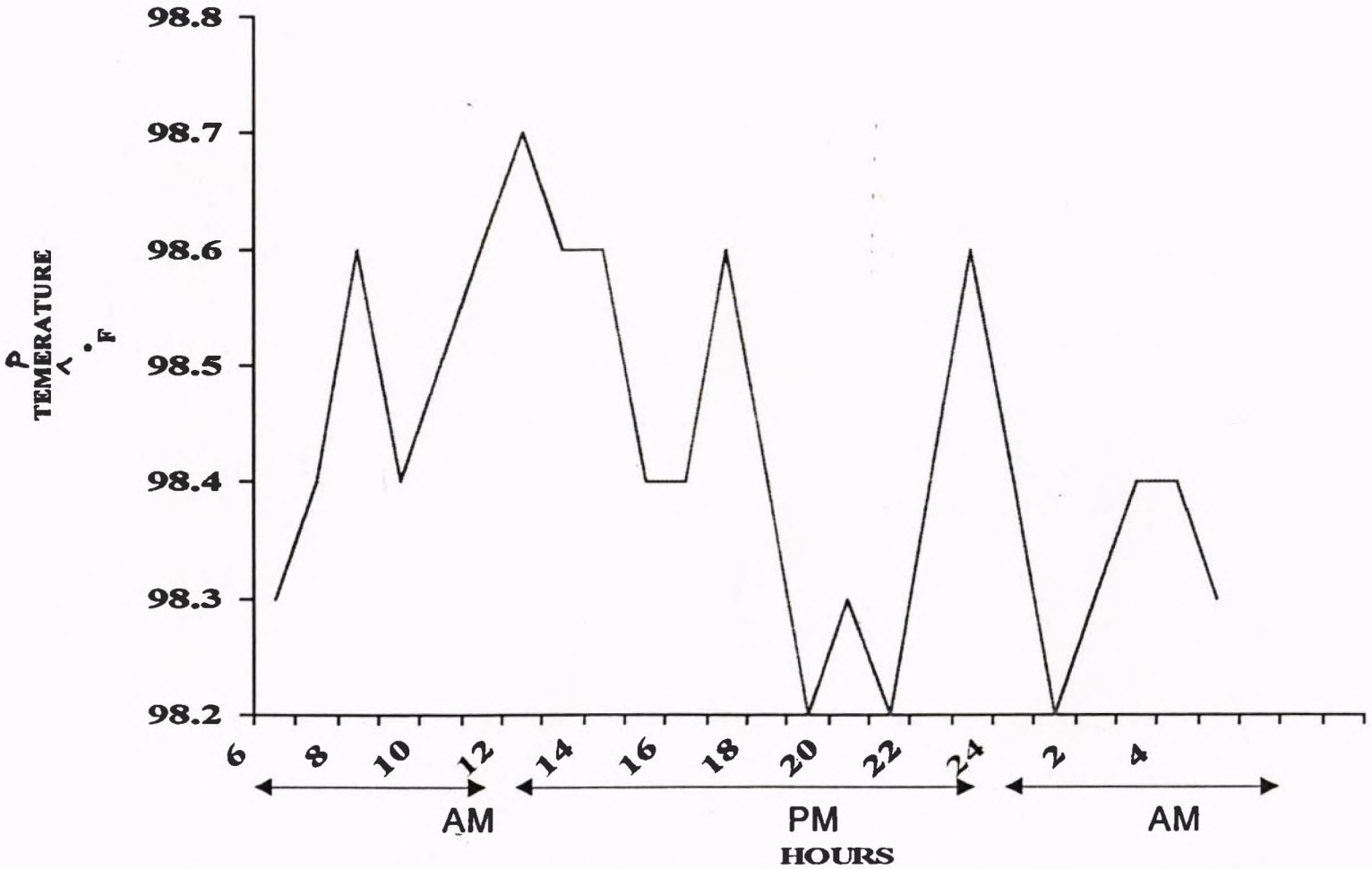


Fig. 8 HOURWISE BODY TEMPERATURE READINGS OF THE CONTROL GROUP NON DIARRHOEAL DAY 3

(ii). THE BODY TEMPERATURE RHYTHM OF THE EXPERIMENTAL GROUP - SENSITIVE ISOLATES

Among the 22 isolates of E.coli , 6 were sensitive towards the selected antibiotics. In this group, a minima of 98.2^0 F ± 0.03 at 7.00 hours , followed by a small rise of 98.5^0 F ± 0.04 between 10.00 and 11.00 hours and a maxima of 98.6^0 F ±0.01 at 12.00 hours on Day 3 has been observed. This was followed by a gradual fall in the body temperature such as 98.4^0 F ± 0.01 at 15.00 hours (Table II; Fig. 9,10 & 11).

(iii). THE BODY TEMPERATURE RHYTHM OF THE EXPERIMENTAL GROUP - RESISTANT ISOLATES

The body temperature of resistant isolates was observed. In this group, a minima of 98.1^0 F ± 0.3 at 06.00 hours, followed by a gradual increase and reached a maxima of 100.1^0 F ± 0.01 at 12.00 hours and a gradual fall in the body temperature recording as 98.4^0 F ± 0.01 at 02.00 hours on Day 1 was observed.

Compared to control and resistant isolates, the conspicuous occurrence of maxima and minima of body temperature recordings on Day1 itself in the resistant isolates could be due to the resistant nature of *E.coli* towards antibiotics (Table III ; Fig.12,13 & 14).

TABLE II

HOURWISE / DAYWISE AVERAGE OF BODY TEMPERATURE °F READINGS- EXPERIMENTAL GROUP – SENSITIVE ISOLATES

	Days / Hours	1	2	3
AM	06.00	98.4 ± 0.01	98.5 ± 0.03	98.4 ± 0.05
	07.00	98.5 ± 0.04	98.4 ± 0.05	98.2 ± 0.03
	08.00	98.5 ±0.03	98.7 ± 0.05	98.4 ± 0.2
	09.00	98.6 ± 0.03	98.7 ± 0.01	98.4 ± 0.06
	10.00	98.5 ±0.06	98.7 ± 0.06	98.5 ± 0.01
	11.00	98.6 ± 0.06	98.5 ± 0.01	98.5 ± 0.0
	12.00	98.5 ±0.07	99.7 ± 0.01	98.6 ± 0.5
PM	13.00	98.8 ± 0.08	98.6 ± 0.4	98.5 ± 0.01
	14.00	98.6 ± 0.06	98.4 ± 0.5	98.5 ± 0.3
	15.00	98.4 ±0.03	98.4 ± 0.3	98.4 ± 0.01
	16.00	98.5 ± 0.03	98.4 ± 0.01	98.4 ± 0.01
	17.00	98.4 ± 0.01	98.5 ± 0.5	98.6 ± 0.01
	18.00	98.4 ± 0.03	98.5 ± 0.3	98.4 ± 0.3
	19.00	98.5 ± 0.01	98.4 ± 0.3	98.6 ± 0.06
	20.00	98.5 ± 0.03	98.4 ± 0.01	98.4 ± 0.01
	21.00	98.5 ± 0.01	98.5 ± 0.1	98.4 ± 0.01
	22.00	98.5 ± 0.3	98.6 ±0.6	98.5 ± 0.3
	23.00	98.5 ± 0.01	98.5 ± 0.01	98.5 ± 0.7
	24.00	98.8 ± 0.01	98.4 0.03	98.6 ± 0.03
AM	01.00	98.6 ± 0.03	98.7 ± 0.2	98.5 ± 0.3
	02.00	98.5 ± 0.01	98.4 ± 0.3	98.5 ± 0.6
	03.00	98.4 ± 0.3	98.3 ± 0.01	98.6 ± 0.01
	04.00	98.6 ± 0.5	98.3 ± 0.03	98.4 ± 0.03
	05.00	98.5 ± 0.01	98.4 ± 0.04	98.3 ± 0.4

N – 6

Twenty four observations each

Mean ± S.E readings are calculated for every one hour recording continuously for three days.

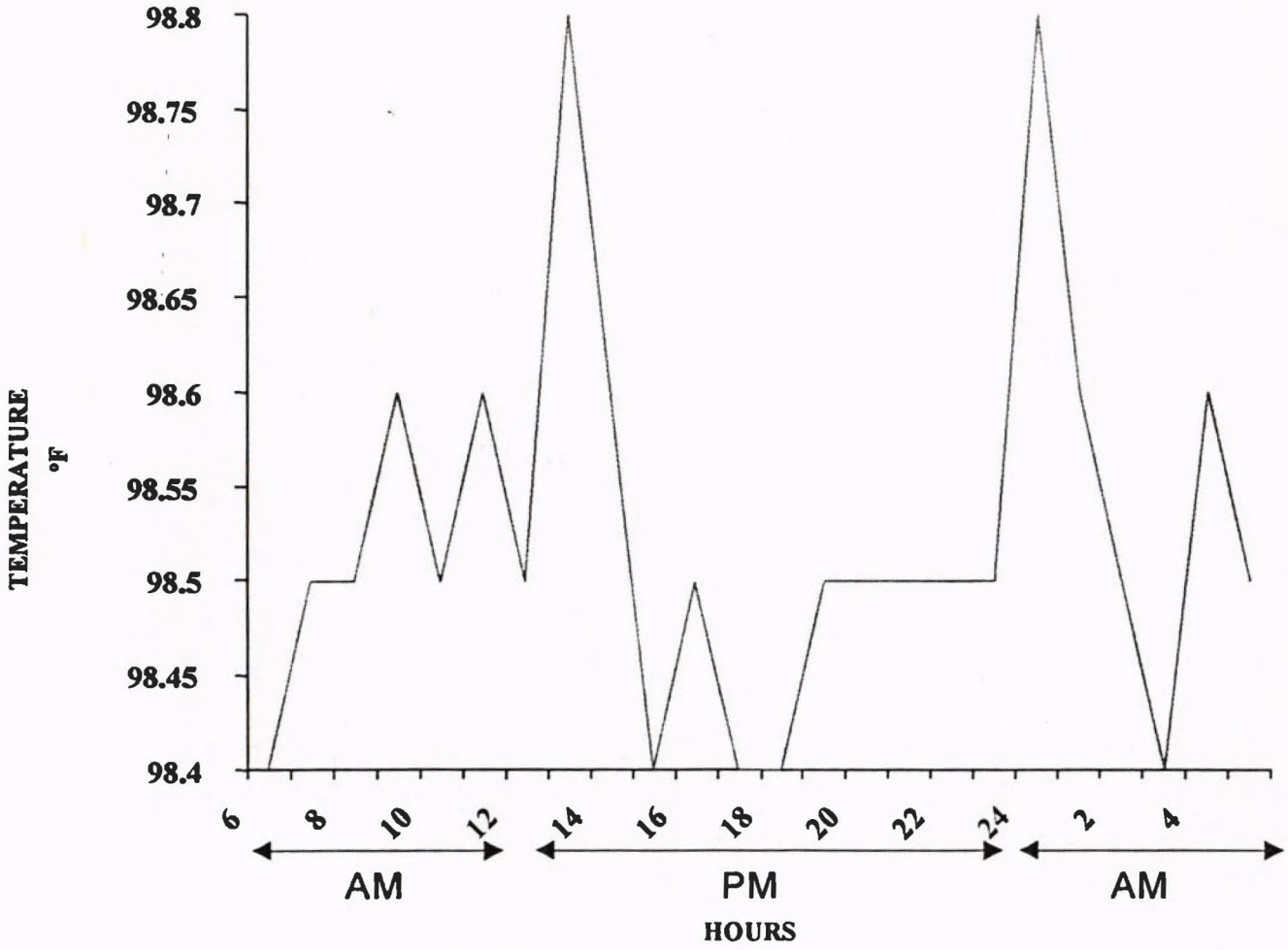


Fig. 9 HOURWISE BODY TEMPERATURE READINGS OF THE EXPERIMENTAL GROUP - SENSITIVE ISOLATES DAY 1

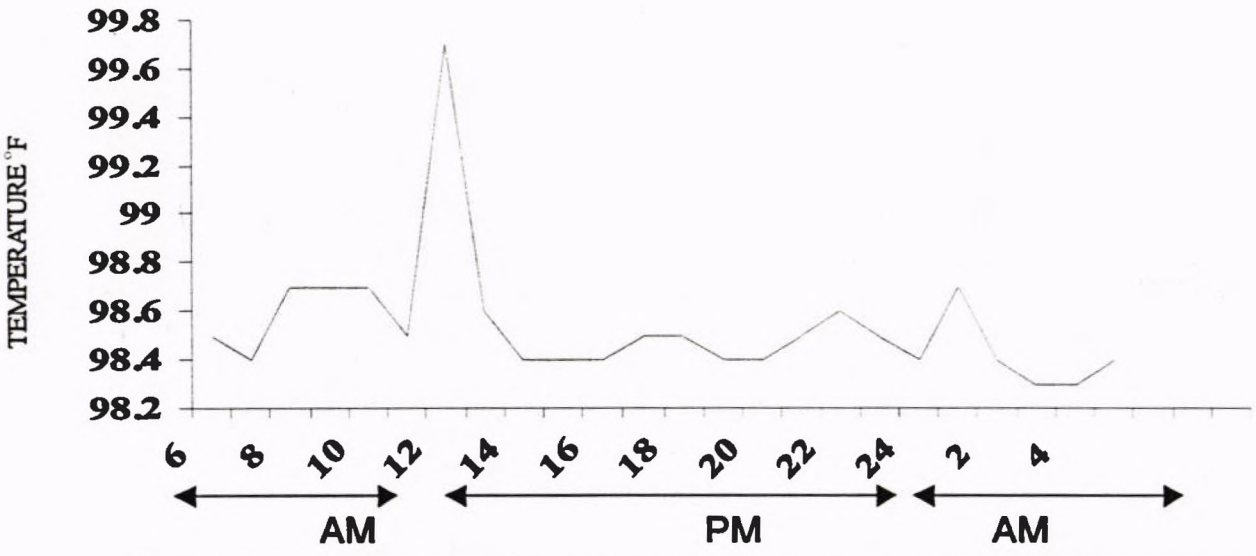


Fig. 10. HOURWISE BODY TEMPERATURE READINGS OF THE EXPERIMENTAL GROUP - SENSITIVE ISOLATES DAY 2

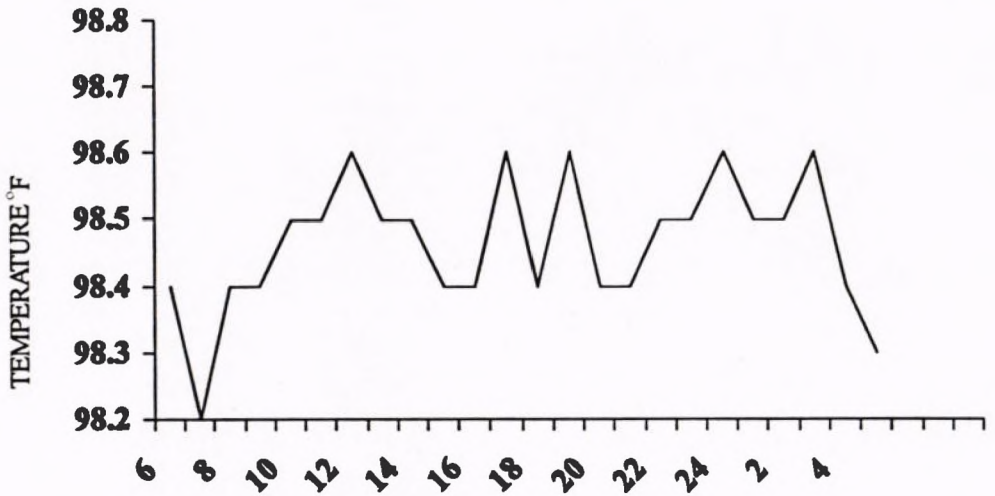


Fig. 11. HOURWISE BODY TEMPERATURE READINGS OF THE EXPERIMENTAL GROUP - SENSITIVE ISOLATES DAY 3

TABLE III

HOURWISE / DAYWISE AVERAGE OF BODY TEMPERATURE °F READINGS- EXPERIMENTAL GROUP - RESISTANT ISOLATES

	Days / Hours	1	2	3
AM	06.00	98.1 ± 0.3	98.6 ± 0.5	98.2 ± 0.2
	07.00	98.6 ± 0.01	98.5 ± 0.09	98.2 ± 0.06
	08.00	98.7 ±0.01	98.4 ± 0.6	98.4 ± 0.3
	09.00	98.8 ± 0.03	98.8 ± 0.3	98.5 ± 0.06
	10.00	98.6 ±0.02	98.6 ± 0.05	98.5 ± 0.3
	11.00	98.7 ± 0.3	98.5 ± 0.03	98.7 ± 0.01
	12.00	100.1 ±0.01	99.6 ± 0.03	99.8 ± 0.02
PM	13.00	98.6 ± 0.08	99.0 ± 0.01	99.4 ± 0.01
	14.00	98.6 ± 0.01	98.5 ± 0.03	98.7 ± 0.6
	15.00	98.5 ±0.03	98.5 ± 0.3	98.5 ± 0.02
	16.00	98.5 ± 0.01	98.4 ± 0.01	98.6 ± 0.02
	17.00	98.5 ± 0.01	98.5 ± 0.02	98.4 ± 0.6
	18.00	98.8 ± 0.02	98.8 ± 0.02	98.7 ± 0.01
	19.00	98.6 ± 0.04	98.6 ± 0.04	98.5 ± 0.03
	20.00	98.5 ± 0.01	98.5 ± 0.01	98.6 ± 0.6
	21.00	98.7 ± 0.3	98.7 ± 0.3	98.6 ± 0.01
	22.00	98.6 ± 0.01	98.6 ±0.01	98.5 ± 0.03
	23.00	98.8 ± 0.01	98.8 ± 0.01	98.6 ± 0.03
	24.00	99.0 ± 0.01	99.0 0.01	98.6 ± 0.03
AM	01.00	98.8 ± 0.03	98.8 ± 0.3	98.6 ± 0.06
	02.00	98.4 ± 0.01	98.4 ± 0.01	98.4 ± 0.01
	03.00	98.5 ± 0.01	98.5 ± 0.01	98.5 ± 0.02
	04.00	98.5 ± 0.03	98.3 ± 0.03	98.4 ± 0.03
	05.00	98.6 ± 0.01	98.6 ± 0.01	98.3 ± 0.2

N – 16

Twenty four observations each

Mean ± S.E readings are calculated for every one hour recording continuously for three days.

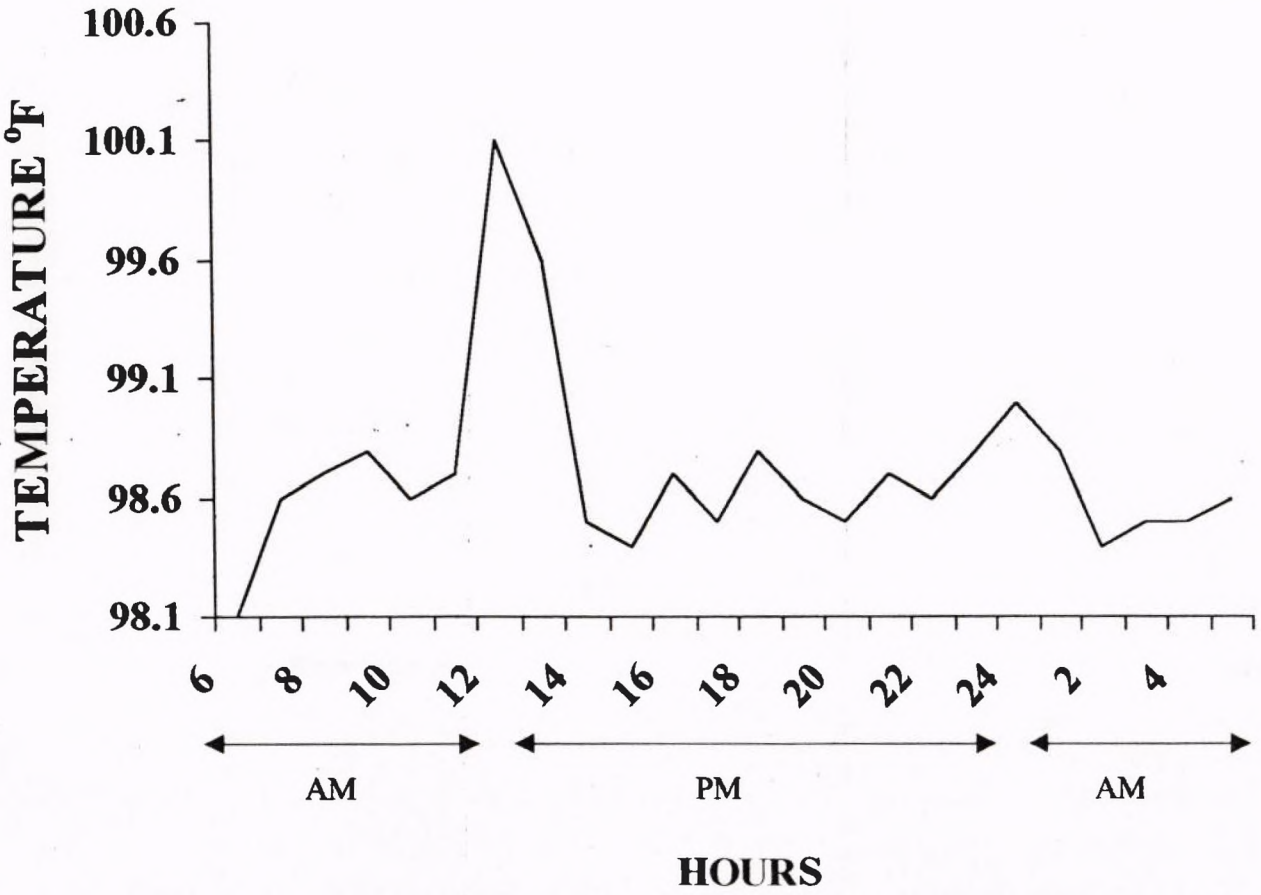


Fig. 12. HOURWISE BODY TEMPERATURE READINGS OF THE EXPERIMENTAL GROUP - RESISTANT ISOLATES DAY 1

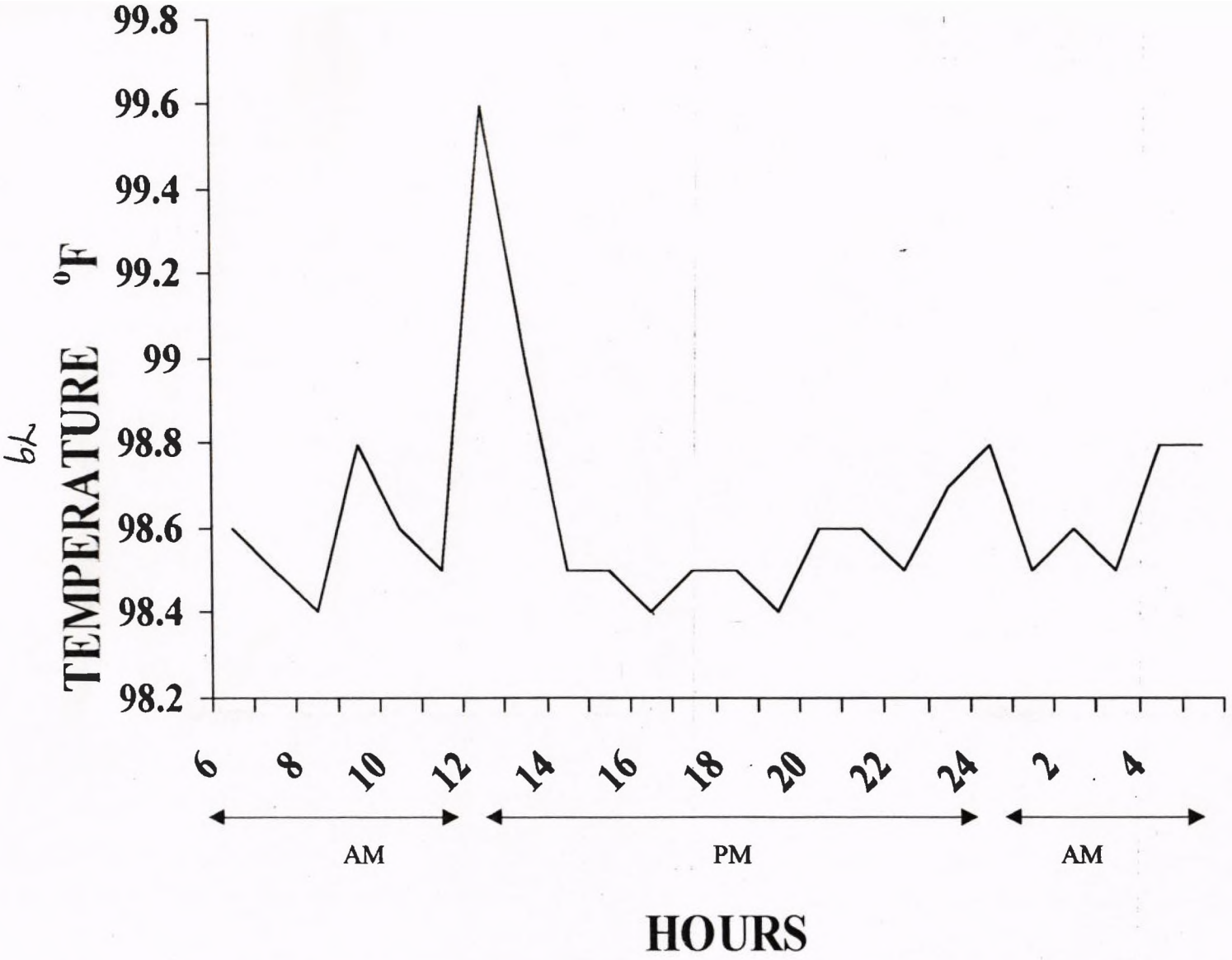


Fig. 13. HOURWISE BODY TEMPERATURE READINGS OF THE EXPERIMENTAL GROUP -RESISTANT ISOLATES DAY 2

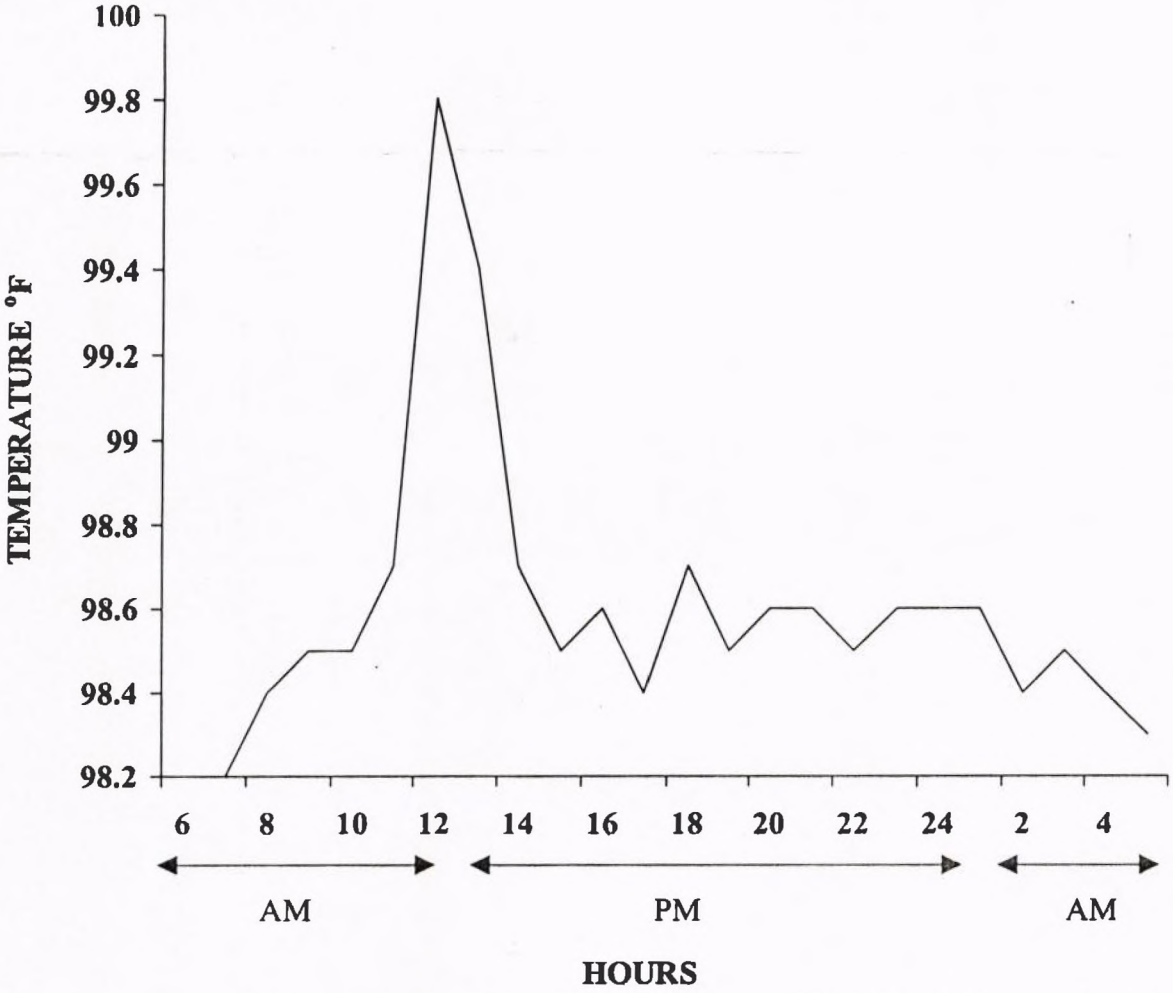


Fig. 14. HOURWISE BODY TEMPERATURE READINGS OF THE EXPERIMENTAL GROUP -RESISTANT ISOLATES DAY 3

(iv) COMPARISON OF THE BODY TEMPERATURE RHYTHM OF THE CONTROL AND THE EXPERIMENTAL GROUP - SENSITIVE ISOLATES

The body temperature readings of the sensitive isolates were slightly higher than that of the control group. By this observation it has been clear that the presence of *E.coli* in the experimental group has raised the body temperature (Table IV ; Fig.15).

(v) COMPARISON OF THE BODY TEMPERATURE RHYTHM OF THE CONTROL AND THE EXPERIMENTAL GROUP - RESISTANT ISOLATES

In the control group , a rhythmic pattern in body temperature readings was observed whereas in the experimental group there was sudden increase and decrease in body temperature. This implicates that the body temperature readings were arhythmic in the experimental group. Comparison of the mean of the body temperature of these two groups, Resistant isolates revealed that the body temperature of the experimental group was significantly higher than that of the control group. The maximum body temperature reading with

TABLE IV
COMPARISON OF THE MEAN BODY TEMPERATURE OF THE CONTROL AND THE EXPERIMENTAL GROUPS – SENSITIVE ISOLATES

Days	Control Group Mean ± S.E °F	Experimental Group sensitive isolates Mean ± S.E °F	't₀' Value	significant / Non Significant ($t_{0.05 - 1.960}$)
1	98.5 ± 0.03	98.7 ± 0.01	1.985	**
2	98.4 ± 0.04	98.6 ± 0.3	1.976	**
3	98.4 ± 0.02	98.5 ± 0.02	1.969	**

** - Significant

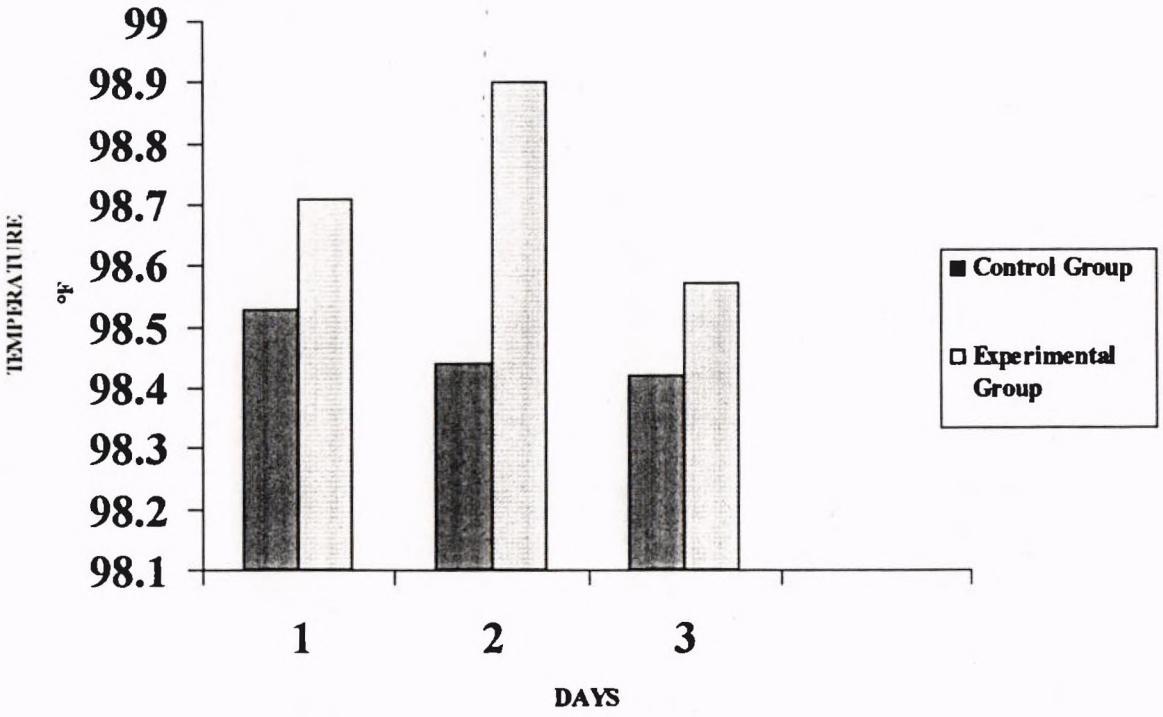


FIG. 15 COMPARISON OF THE BODY TEMPERATURE READINGS (MEAN) OF THE CONTROL AND THE EXPERIMENTAL GROUP – SENSITIVE ISOLATES

experimental group was 100.1^0 F while in the control group it was 98.7^0 F (Table V ; Fig.16).

(vi) COMPARISON OF THE BODY TEMPERATURE RHYTHM OF THE EXPERIMENTAL GROUPS - SENSITIVE ISOLATES AND RESISTANT ISOLATES

Comparison of the body temperature of the two experimental groups of isolates (both Sensitive and Resistant) showed that the body temperature of the resistant isolates was higher than that of the sensitive ones. In the sensitive isolates the maximum body temperature was only 98.8^0 F whereas in the resistant isolates the maximum body temperature was 100.1^0 F. This clearly shows that the body temperature of the resistant isolates is significantly higher than that of the sensitive isolates . The body temperature of the experimental groups was also compared with that of the control groups, body temperature readings of the experimental groups were significantly higher than that of the control group
(Table VI ; Fig.17).

TABLE V
COMPARISON OF THE MEAN BODY TEMPERATURE OF THE CONTROL AND THE EXPERIMENTAL GROUPS – RESISTANT ISOLATES

Days	Control Group Mean ± S.E °F	Experimental Group Resistant isolates Mean ± S.E °F	't' Value	Significant / Non Significant ($t_{0.05} = 1.960$)
1	98.5 ± 0.03	98.8 ± 0.08	2.653	**
2	98.4 ± 0.04	98.7 ± 0.04	2.545	**
3	98.4 ± 0.02	98.6 ± 0.06	2.437	**

** - Significant

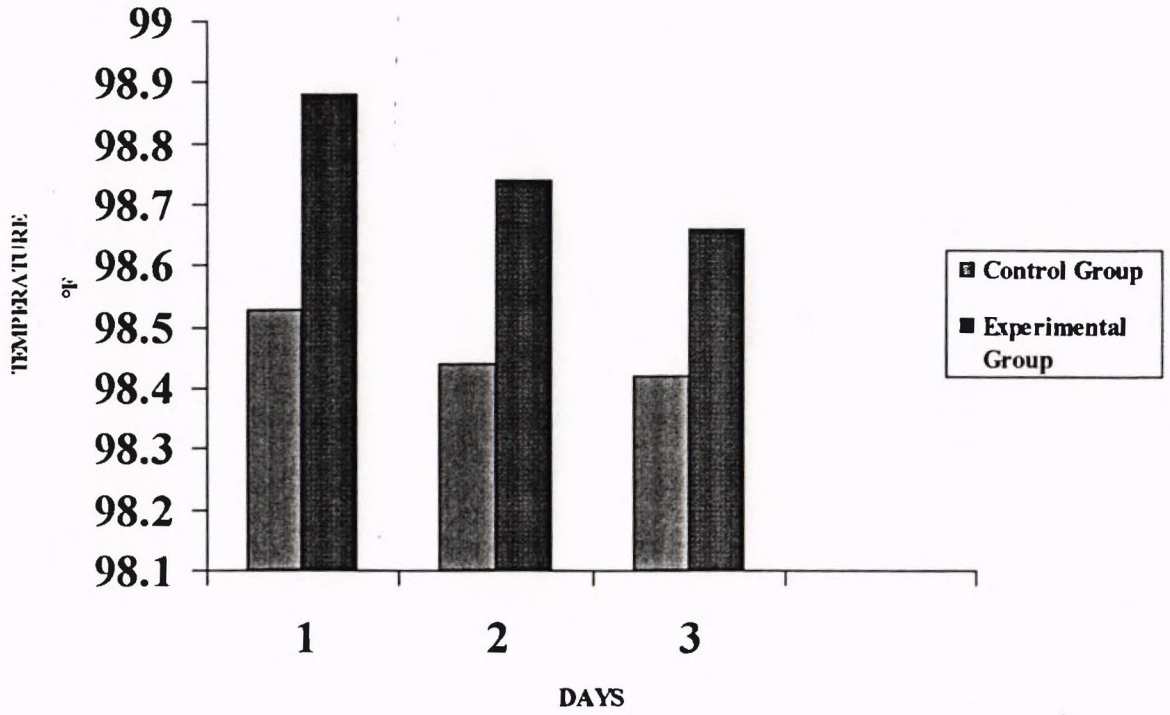


FIG. 16 COMPARISON OF THE BODY TEMPERATURE READINGS OF THE CONTROL AND THE EXPERIMENTAL GROUP – RESISTANT ISOLATES

TABLE VI
COMPARISON OF THE MEAN BODY TEMPERATURE OF THE EXPERIMENTAL GROUPS – RESISTANT ISOLATES

Days	Control Group Mean ± S.E °F	Experimental Group sensitive isolates Mean ± S.E °F	Experimental Group Resistant isolates Mean ± S.E °F
1	98.5 ± 0.03	98.7 ± 0.01	98.8 ± 0.08
2	98.4 ± 0.04	98.6 ± 0.3	98.7 ± 0.04
3	98.4 ± 0.02	98.5 ± 0.02	98.6 ± 0.06

** - Significant

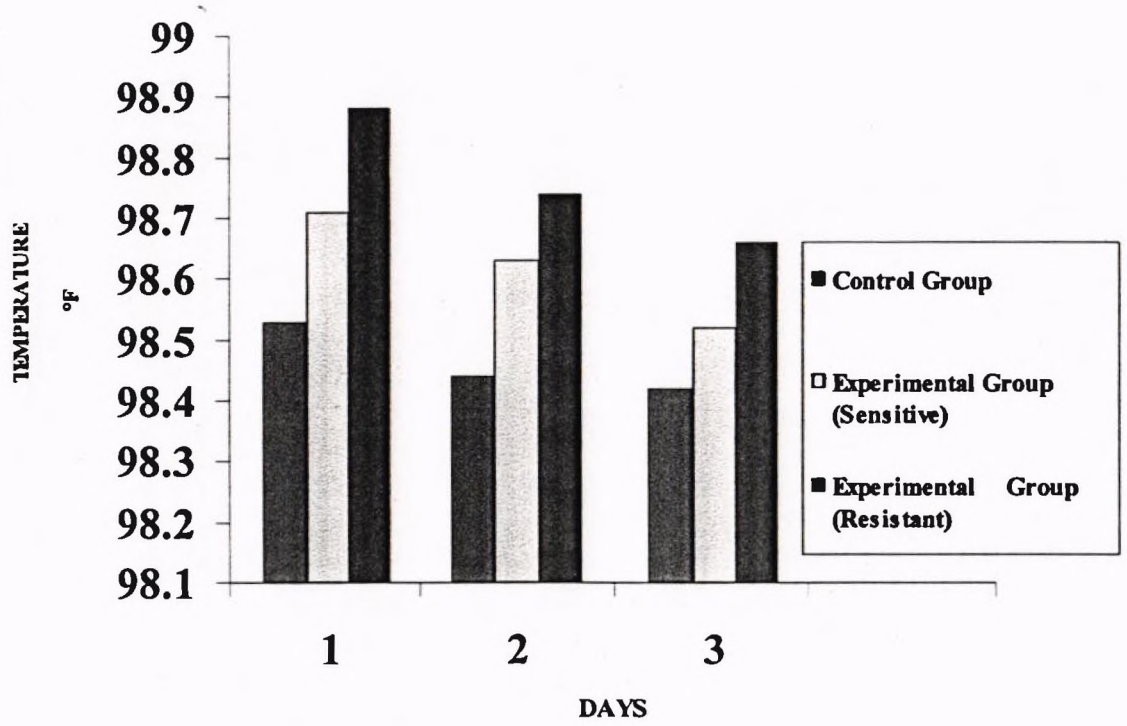


FIG. 17 COMPARISON OF THE BODY TEMPERATURE READINGS OF THE EXPERIMENTAL GROUPS SENSITIVE ISOLATES AND RESISTANT ISOLATES

III. BIOCHEMICAL TESTS

The biochemical tests were done using various parameters for the control as well as for the experimental group. However, there was no marked change observed between the groups (Table VII).

IV. BIOCHEMICAL CHARACTERIZATION OF *E. coli* ISOLATES

Biochemical reaction carried out confirmed the presence *E.coli* in all the 22 samples. Positive reaction was observed in indole test, methyl red test, catalase test and negative reaction in voges-proskauer test, citrate utilization test , hydrogen sulphide and urease test. The reaction towards triple sugar iron agar test was common A/AG (acid butt with gas) for all the isolates. Motility test revealed that isolates 2, 4, 5, 7, 8, 9, 10, 11, 15, 17, 18, 19, 20 & 22 were motile whereas isolates 1, 3, 6, 12, 13, 14, 16 & 21 were nonmotile. (Table VIII ; Fig. 18, 19, 20 , 21 & 22).

V. ISOLATION OF MULTIPLE ANTIBIOTIC RESISTANT AND / OR SENSITIVE ISOLATES

Out of the 22 isolates of *E.coli* tested, 16 were resistant and 6 were sensitive against the selected antibiotics. Growth of *E.coli* was observed around the antibiotic disc, in resistant isolates. In sensitive isolates a clear zone was visible around the antibiotic disc.

TABLE VII

TABULATION OF BIOCHEMICAL TESTS – OVERALL AVERAGE

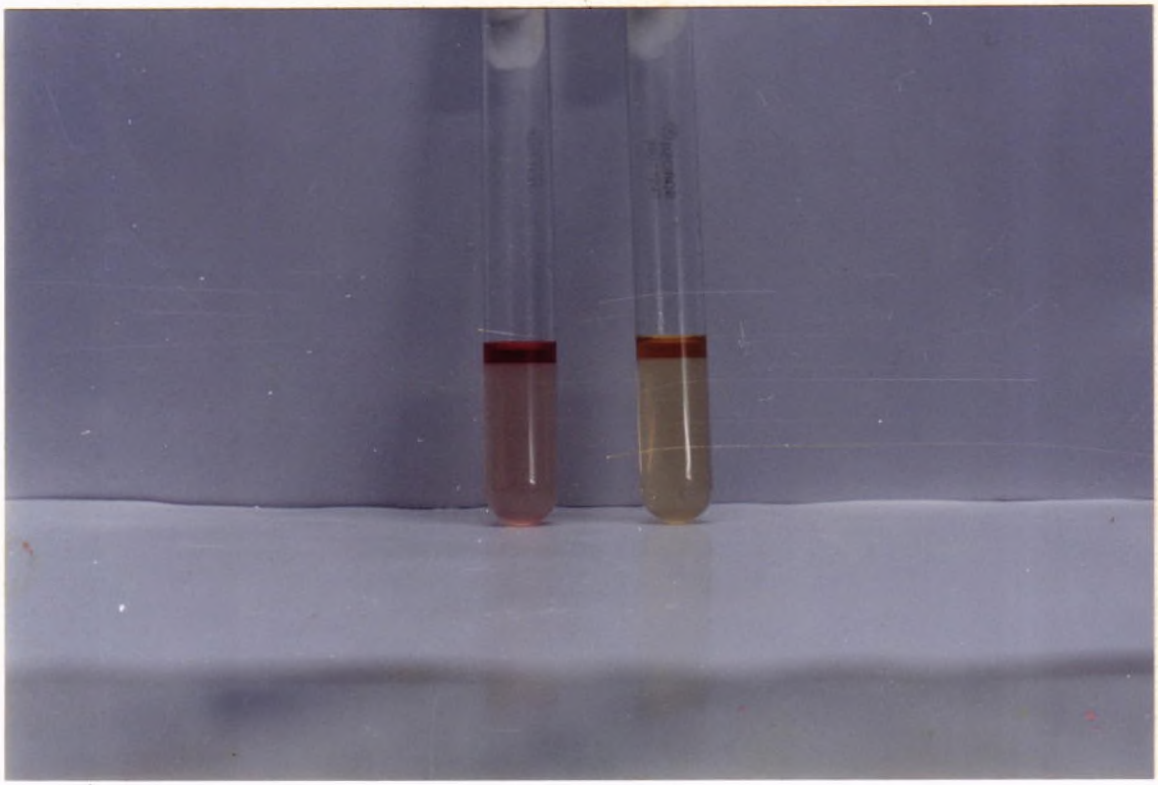
NATURE OF THE GROUP	Blood Pressure (,mmHg)	Blood sugar random (mgs %)	Blood Cholesterol (mgs %)	Haemoglobin (grams %)	Total Protein (grams %)	Albumin (gms %)	Globulin (grams %)	Liver function test				Urine Urea (mgs %)	Urine Creatinine (mgs %)
								Bilirubin (mgs %)	SGOT LU/L	SGPT LU/L	Serum alkline phosphates K.A./100		
I. CONTROL NON DIARRHOEAL	125.75	118	180	9.8	6.5	4.2	2.3	0.4	12.0	11.0	9.2	1350	115
II. EXPERIMENTAL SENSITIVE ISOLATES	125.80	130	185	11	6.8	4.8	2.6	0.6	10.2	8.6	7.8	1450	120
III. EXPERIMENTAL RESISTANT ISOLATES	110.75	126	200	9	7.5	3.5	2.8	0.5	12.5	11.7	9.1	1300	110

TABLE VIII
BIOCHEMICAL REACTION OF E. coli ISOLATES

ISOLATE NUMBER	IND	MR	VP	CIT	TSI	H_2S	UR	CAT	MOT
1.	+	+	-	-	A/AG	-	-	+	-
2.	+	+	-	-	A/AG	-	-	+	+
3.	+	+	-	-	A/AG	-	-	+	-
4.	+	+	-	-	A/AG	-	-	+	+
5.	+	+	-	-	A/AG	-	-	+	+
6.	+	+	-	-	A/AG	-	-	+	-
7.	+	+	-	-	A/AG	-	-	+	+
8.	+	+	-	-	A/AG	-	-	+	+
9.	+	+	-	-	A/AG	-	-	+	+
10.	+	+	-	-	A/AG	-	-	+	+
11.	+	+	-	-	A/AG	-	-	+	+
12.	+	+	-	-	A/AG	-	-	+	-
13.	+	+	-	-	A/AG	-	-	+	-
14.	+	+	-	-	A/AG	-	-	+	-
15.	+	+	-	-	A/AG	-	-	+	+
16.	+	+	-	-	A/AG	-	-	+	-
17.	+	+	-	-	A/AG	-	-	+	+
18.	+	+	-	-	A/AG	-	-	+	+
19.	+	+	-	-	A/AG	-	-	+	+
20.	+	+	-	-	A/AG	-	-	+	+
21.	+	+	-	-	A/AG	-	-	+	-
22.	+	+	-	-	A/AG	-	-	+	+

IND - INDOLE TEST
MR - METHYL RED TEST
VP - VOGES-PROSKAUER TEST
CIT - CITRATE UTILIZATION TEST
TSI - TRIPLE SUGAR IRON AGAR TEST
H_2S - HYDROGEN SULPHIDE TEST
UR - UREASE TEST
CAT - CATALASE TEST
MOT - MOTILITY TEST

\+ : POSITIVE
\- : NEGATIVE
A : ACID
A/AG : ACID BUTT WITH GAS



A B

Fig.18 Photographic Plate shows Indole (A) positive and Unsown control (B)

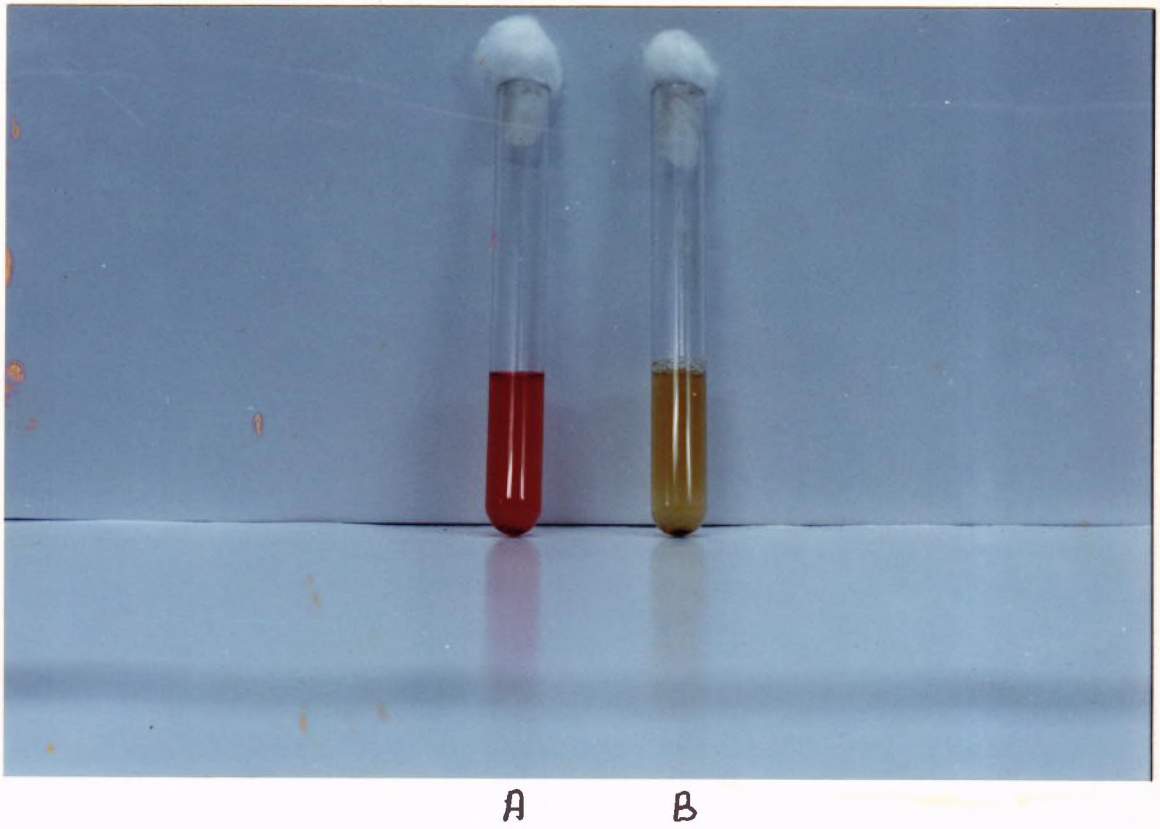
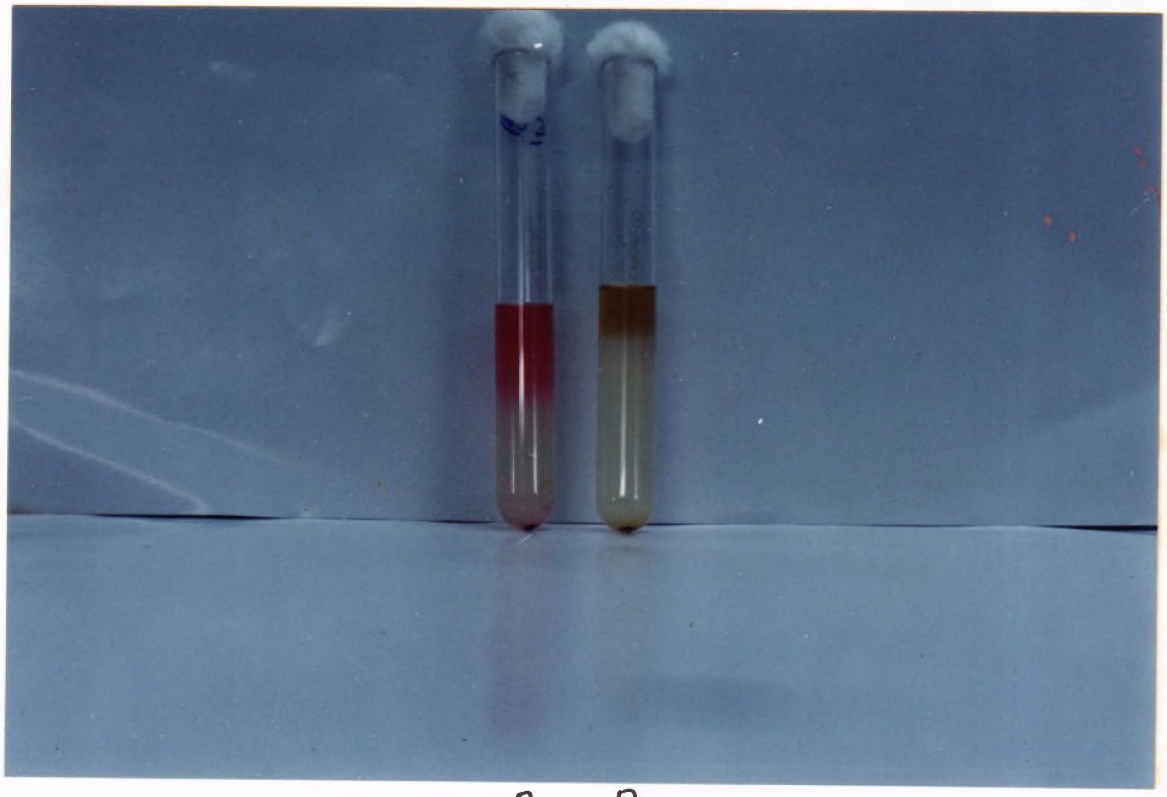
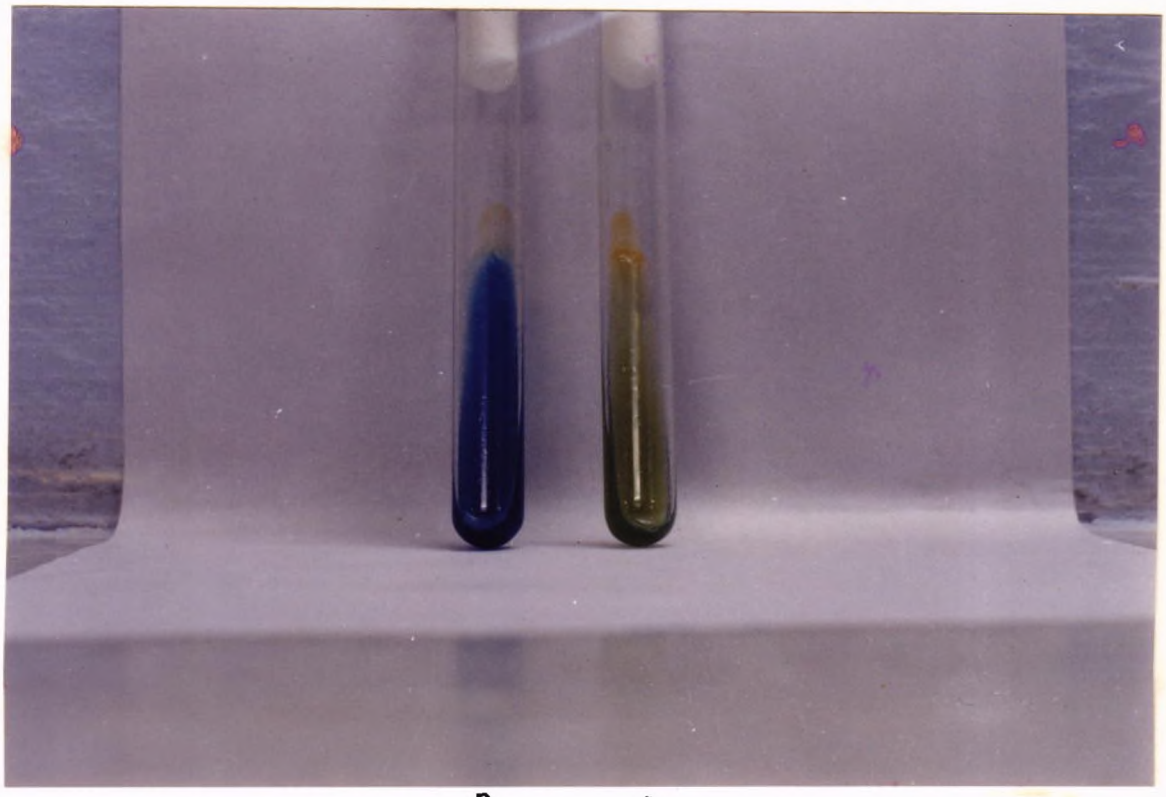


Fig.19 Photographic Plate shows Methyl Red (A) positive and Unsown control (B)



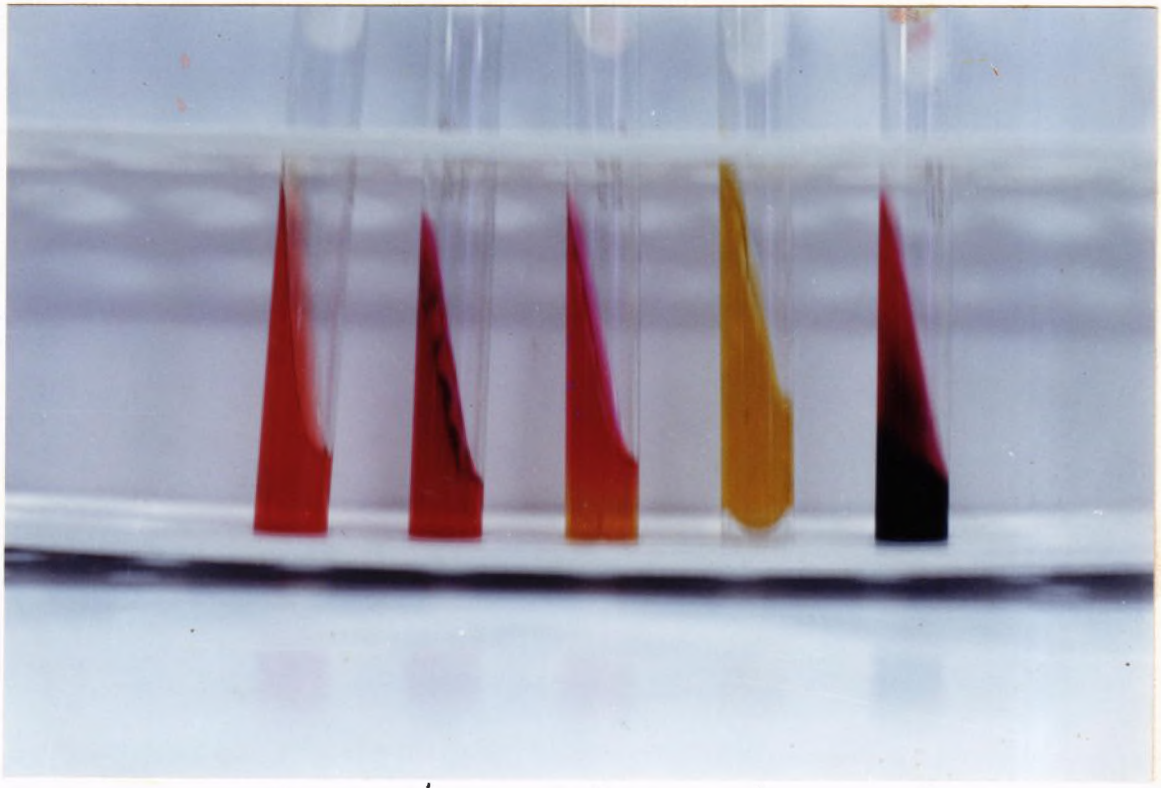
A B

Fig.20 Photographic Plate shows Voges (A) Proskauer positive and Unsown control (B)



A B

Fig.21 Photographic Plate shows Citrate (A) positive and Unsown control (B)



a b c d e

Fig.22 Photographic Plate shows Triple Sugar Iron Agar with

a) Unsown Control
b) Negative Control
c) +/-
d) $+/+$ ⟶ E.coli
e) $-/+$ ⟶ H_2S

RESISTANCE PATTERN OF *E.coli* ISOLATES TREATED WITH TEN ANTIBIOTICS

ISOLATE -1

Isolate 1 showed the highest rate of inhibition annule, 14 at 250 μg^{ml-1} , only with nalidixic acid (Table IX).

ISOLATE -2

Isolate 2 exhibited the highest rate of inhibition annules,30 each at 250 μg^{ml-1} , only with erythromycin, gentamicin and streptomycin (Table IX).

ISOLATE -3

Isolate 3 displayed the highest rate of inhibition annules, 13 each at 250 μg^{ml-1} , only with ciprofloxacin and nalidixic acid (Table X).

ISOLATE - 4

Isolate 4 showed the highest rate of inhibition annules, 13each at 250 μg^{ml-1} , only with four antibiotics namely, chlormaphenical, gentamicin, nalidixic acid and nitrofurantoin (Table X).

TABLE - IX

RESISTANCE PATTERN OF E. coli ISOLATE - 1 AND ISOLATE -2 TOWARDS SELECTED ANTIBIOTICS

ISOLATE NUMBER AND SOURCE	CONCENT-RATIONS μg^{ml-1}	NAME OF THE ANTIBIOTICS									
		AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE 1	10	3^+	4^+	3^+	4^+	6^+	7^+	3^+	2^+	#	#
	20	6^+	6^+	6^+	5^+	7^+	9^+	5^+	6^+	#	6^+
	50	8^+	8^+	7^+	7^+	9^+	11^+	7^+	8^+	6^+	8^+
	100	9^+	10^+	9^+	8^+	11^+	12^+	9^+	12^+	9^+	10^+
	250	11^+	12^+	10^+	11^+	13^+	14^+	10^+	13^+	11^+	12^+
ISOLATE 2	10	4^+	#	4^+	5^+	7^+	5^+	3^+	#	#	#
	20	6^+	5^+	6^+	7^+	8^+	6^+	6^+	#	5^+	4^+
	50	8^+	7^+	8^+	9^+	10^+	8^+	7^+	6^+	9^+	7^+
	100	10^+	9^+	10^+	11^+	12^+	10^+	9^+	9^+	11^+	9^+
	250	12^+	11^+	12^+	13^+	13^+	11^+	12^+	11^+	13^+	11^+

: RESISTANCE

+ : INHIBITION ANNULES (SENSITIVITY)

TABLE - X

RESISTANCE PATTERN OF E. coli ISOLATE - 3 AND ISOLATE - 4 TOWARDS SELECTED ANTIBIOTICS

ISOLATE NUMBER AND SOURCE	CONCENT-RATIONS μg^{ml-1}	NAME OF THE ANTIBIOTICS									
		AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE 3	10	3^+	#	#	#	#	#	#	#	#	#
	20	6^+	5^+	+	4^+	3^+	#	#	3^+	#	#
	50	7^+	8^+	9^+	7^+	6^+	9^+	7^+	5^+	#	7^+
	100	8^+	9^+	11^+	9^+	9^+	11^+	9^+	7^+	9^+	8^+
	250	10^+	11^+	13^+	11^+	12^+	13^+	11^+	9^+	12^+	10^+
ISOLATE 4	10	3^+	3^+	#	2^+	#	#	#	#	#	#
	20	5^+	8^+	5^+	6^+	5^+	9^+	10^+	#	#	#
	50	7^+	10^+	7^+	8^+	7^+	11^+	11^+	7^+	5^+	9^+
	100	9^+	12^+	10^+	10^+	10^+	12^+	12^+	10^+	7^+	10^+
	250	12^+	13^+	12^+	12^+	13^+	13^+	13^+	11^+	10^+	12^+

: RESISTANCE

+ : INHIBITION ANNULES (SENSITIVITY)

ISOLATE - 9

Isolate 9 recorded the highest rate of inhibition annules, 12 each at 250 μg^{ml-1} , with only three antibiotics namely, erythromycin, nalidixic acid and norfloxacin and (Table XI).

ISOLATE - 10

This isolate evidenced its highest rate of inhibition annule, 13 at 250μg^{ml-1} , with only one antibiotic namely trimethoprim / sulphamethoxazole (Table XI).

ISOLATE - 11

The highest rate of inhibition annule, 14 at 250μg^{ml-1} , was recorded by this isolates only with one antibiotic namely, nalidixic acid (Table XII).

ISOLATE - 12

This isolate recorded its highest inhibition annule, 14 at 250 μg^{ml-1} , with only one antibiotic namely, chloramphenical, (Table XII).

TABLE XI

RESISTANCE PATTERN OF E.coli ISOLATE - 9 AND ISOLATE - 10 TOWARDS SELECTED ANTIBIOTICS

ISOLATE NUMBER AND SOURCE	CONCENT-RATIONS μg^{ml-1}	NAME OF THE ANTIBIOTICS									
		AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE 9	10	6^+	3^+	2^-	6^+	5^+	4^+	3^+	4^+	5^+	6^+
	20	7^+	4^+	6^-	7^+	6^+	6^+	5^+	6^+	7^+	8^+
	50	8^+	6^+	8^+	8^+	8^+	9^+	7^+	7^+	9^+	9^+
	100	9^+	8^+	10^+	10^+	9^+	11^+	9^+	9^+	10^+	10^+
	250	10^-	9^+	11^-	12^+	11^+	12^+	11^+	12^+	11^+	11^+
ISOLATE 10	10	4^+	3^+	5^-	5^+	4^+	5^+	4^+	5^+	4^+	5^+
	20	6^+	5^+	6^+	8^+	6^+	7^+	5^+	7^+	6^+	7^+
	50	7^+	7^+	8^-	9^+	8^+	9^+	7^-	9^-	8^+	9^+
	100	9^+	9^+	9^-	10^+	10^+	10^+	9^-	11^-	9^+	11^+
	250	11^+	11^+	12^-	12^+	11^+	12^+	12^+	12^+	12^+	13^+

+ : INHIBITION ANNULES (RESISTANCE)

TABLE XII

RESISTANCE PATTERN OF E.coli ISOLATE -11 AND ISOLATE -12 TOWARDS SELECTED ANTIBIOTICS

ISOLATE NUMBER AND SOURCE	CONCENT-RATIONS $\mu g^{ml^{-1}}$	NAME OF THE ANTIBIOTICS									
		AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE	10	3^+	#	#	#	#	#	#	#	2^+	#
11	20	6^+	#	#	#	#	#	#	#	4^+	#
	50	8^+	9^+	#	3^+	7^+	#	9^+	#	7^+	#
	100	10^+	11^+	10^+	5^+	9^+	12^+	10^+	11^+	9^+	10^+
	250	12^+	13^+	12^+	9^+	11^+	14^+	12^+	12^+	11^+	13^+
ISOLATE	10	#	#	#	#	#	#	#	#	#	#
12	20	#	#	7^+	#	#	#	#	#	#	#
	50	11^+	#	9^+	6^+	#	#	#	9^+	#	#
	100	12^+	12^+	10^+	8^+	12^+	10^+	12^+	11^+	10^+	7^+
	250	13^+	14^+	12^+	10^+	13^+	12^+	13^+	12^+	12^+	9^+

: RESISTANCE

+ : INHIBITION ANNULES (RESISTANCE)

ISOLATE - 13

Isolate 13 had its highest rate of inhibiton annule, 12 at 250μg^{ml-1}, with three antibiotics namely, gentamicin, norfloxacin and streptomycin (Table XIII).

ISOLATE - 14 AND 15

Exhibited their highest inhibition annule, 13 at 250 μg^{ml-1}, only with one antibiotic namely, gentamicin (TableXIII &Table XIV).

ISOLATE - 16

This isolate showed its highest rate of inhibition annule, 12 at 250 μg^{ml-1} , with only one antibiotic namely, chloramphenical (Table XIV).

ISOLATE - 17

The highest rate of inhibition annule,12 at 250 μg^{ml-1} , was observable with only erythromycin (Table XV).

ISOLATE - 19

This isolate recorded its highest inhibition annule, 13 at 250 μg^{ml-1} , with only erythromycin (Table XV).

TABLE XIII

RESISTANCE PATTERN OF E.coli ISOLATE - 13 AND ISOLATE - 14 TOWARDS SELECTED ANTIBIOTICS

ISOLATE NUMBER AND SOURCE	CONCENT-RATIONS μg^{ml-1}	NAME OF THE ANTIBIOTICS									
		AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE	10	#	#	#	#	#	#	#	#	#	#
13	20	#	#	#	#	#	#	#	#	#	#
	50	#	#	5^{+}	7^{+}	#	6^{+}	4^{+}	#	#	4^{-}
	100	7^{+}	6^{+}	7^{+}	9^{+}	10^{+}	8^{+}	7^{+}	10^{+}	10^{+}	6^{-}
	250	9^{+}	8^{-}	10^{+}	11^{-}	12^{+}	11^{+}	10^{+}	12^{+}	12^{+}	9^{-}
ISOLATE	10	#	#	#	#	#	#	#	#	#	#
14	20	#	#	#	#	#	#	#	#	#	#
	50	6^{+}	#	7^{+}	7^{-}	9^{+}	7^{+}	4^{-}	3^{+}	3^{+}	4^{-}
	100	8^{+}	7^{+}	9^{+}	9^{-}	11^{+}	9^{+}	6^{+}	5^{+}	5^{+}	6^{-}
	250	10^{+}	9^{+}	11^{+}	12^{+}	13^{+}	11^{+}	8^{+}	7^{+}	9^{+}	7^{+}

: RESISTANCE

+ : INHIBITION ANNULES (RESISTANCE)

TABLE XIV

RESISTANCE PATTERN OF E.coli ISOLATE - 15 AND ISOLATE - 16 TOWARDS SELECTED ANTIBIOTICS

ISOLATE NUMBER AND SOURCE	CONCENT-RATIONS μg^{ml-1}	NAME OF THE ANTIBIOTICS									
		AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE	10	#	#	#	#	#	#	#	#	#	#
15	20	3^+	#	#	#	4^+	#	#	#	#	#
	50	5^+	7^+	6^+	9^+	7^+	3^+	4^+	#	6^+	#
	100	7^+	9^+	9^+	11^+	9^+	6^+	8^+	2^+	7^+	3^+
	250	9^+	11^+	10^+	12^+	13^+	8^+	9^+	4^+	9^+	8^+
ISOLATE	10	#	#	#	#	#	#	#	#	#	#
16	20	4^+	#	#	#	#	#	#	#	#	#
	50	5^+	9^+	#	#	6^+	#	3^+	1^+	2^+	6^+
	100	7^+	10^+	10^+	7^+	9^+	7^+	5^+	3^+	5^+	7^+
	250	9^+	12^+	11^+	10^+	11^+	10^+	8^+	5^+	7^+	9^+

: RESISTANCE

+ : INHIBITION ANNULES (RESISTANCE)

TABLE XV

RESISTANCE PATTERN OF E.coli ISOLATE -17 AND ISOLATE -19 TOWARDS SELECTED ANTIBIOTICS

ISOLATE NUMBER AND SOURCE	CONCENT-RATIONS μg^{ml-1}	NAME OF THE ANTIBIOTICS									
		AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE	10	#	#	#	#	#	#	#	2^{+}	#	#
17	20	3^{+}	#	#	#	#	#	#	4^{+}	#	#
	50	5^{+}	6^{+}	3^{+}	#	4^{+}	#	#	7^{+}	#	2+
	100	6^{+}	8^{+}	7^{+}	10^{-}	7^{+}	6^{-}	3^{-}	8^{+}	4^{+}	5+
	250	8^{+}	10^{+}	11^{+}	12^{-}	8^{+}	9^{-}	7^{-}	10^{+}	7^{+}	6+
ISOLATE	10	#	#	#	#	#	#	#	#	#	#
19	20	#	#	5^{+}	#	#	#	#	#	#	#
	50	4^{+}	#	7^{-}	10^{-}	5^{+}	7^{-}	6^{+}	5^{+}	#	3^{+}
	100	7^{+}	4^{+}	9^{+}	12^{-}	7^{+}	9^{+}	8^{+}	7^{+}	3^{+}	4^{+}
	250	11^{+}	8^{+}	10^{+}	13^{+}	9^{+}	11^{+}	10^{+}	9^{+}	5^{+}	7^{+}

: RESISTANCE

+ : INHIBITION ANNULES (RESISTANCE)

ISOLATE - 20

The highest rate of inhibition annule, 13 at 250 μg^{ml-1}, was recorded only with streptomycin (Table XVI).

ISOLATE - 21

This isolate exhibited its highest rate of inhibition annules, 13 each at 250 μg^{ml-1}, with two antibiotic namely gentamycin and norfloxacin (Table XVI).

SENSITIVITY PATTERN OF E.coli ISOLATES TREATED WITH TEN ANTIBIOTICS

ISOLATE - 5

The highest rate of inhibition annule, 27 at 250 μg^{ml-1}, was found with erythromycin as well as with norfloxacin (Table XVII ; Fig.23).

ISOLATE - 6

This isolate recorded its highest rate of inhibition annule, 26 at 250 μg^{ml-1}, with three antibiotics namely, ciprofloxacin, nalidixic acid, and trimethoprim / sulphamethoxazole (Table XVII ; Fig.24).

TABLE XVI

RESISTANCE PATTERN OF E.coli ISOLATE - 20 AND ISOLATE - 21 TOWARDS SELECTED ANTIBIOTICS

SOLATE NUMBER AND SOURCE	CONCENT-RATIONS μg^{ml-1}	NAME OF THE ANTIBIOTICS									
		AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE	10	#	#	#	#	#	#	#	#	#	#
20	20	3^+	#	#	#	#	#	2^+	#	#	#
	50	5^+	4^+	#	3^+	#	5^+	5^+	4^+	#	3^+
891	100	8^+	5^+	7^+	6^+	4^+	7^+	6^+	7^+	10^+	5^+
	280	10^+	9^+	9^+	8^+	9^+	9^+	9^+	10^+	13^+	7^+
ISOLATE	10	#	#	#	#	#	#	#	#	#	#
21	20	#	#	#	#	#	#	#	#	#	#
	50	2^+	3^+	#	2^+	10^+	5^+	3^+	9^+	8^+	6^+
	100	3^+	6^+	5^+	3^+	11^+	7^+	5^+	11^+	10^+	8^+
	280	6^+	7^+	8^+	6^+	13^+	8^+	6^+	13^+	12^+	9^+

: RESISTANCE

+ : INHIBITION ANNULES (RESISTANCE)

TABLE - XVII

SENSITIVITY PATTERN OF E. coli ISOLATE - 5 AND ISOLATE - 6 TOWARDS SELECTED ANTIBIOTICS

ISOLATE NUMBER AND SOURCE	CONCENT-RATIONS μg^{ml-1}	NAME OF THE ANTIBIOTICS									
		AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE	10	@	@	@	@	@	@	@	@	@	@
5	20	@	@	@	19⁻	@	@	@	16⁻	@	@
	50	12⁻	@	21⁻	20⁻	17⁻	21⁻	@	19⁻	17⁻	@
	100	15⁻	17⁻	22⁻	23⁻	20⁻	23⁻	19⁻	23⁻	19⁻	19⁻
101	250	17⁻	19⁻	24⁻	27⁻	23⁻	25⁻	21⁻	27⁻	21⁻	22⁻
	10	@	@	@	@	@	@	@	@	@	@
ISOLATE	20	@	@	@	@	@	@	@	@	@	@
6	50	17⁻	@	21⁻	@	18⁻	21⁻	@	20⁻	@	20⁻
	100	18⁻	19⁻	23⁻	15⁻	20⁻	23⁻	20⁻	21⁻	18⁻	23⁻
	250	21⁻	20⁻	26⁻	19⁻	21⁻	26⁻	24⁻	23⁻	23⁻	26⁻

@ : RESISTANCE

- : INHIBITION ANNULES (SENSITIVITY)

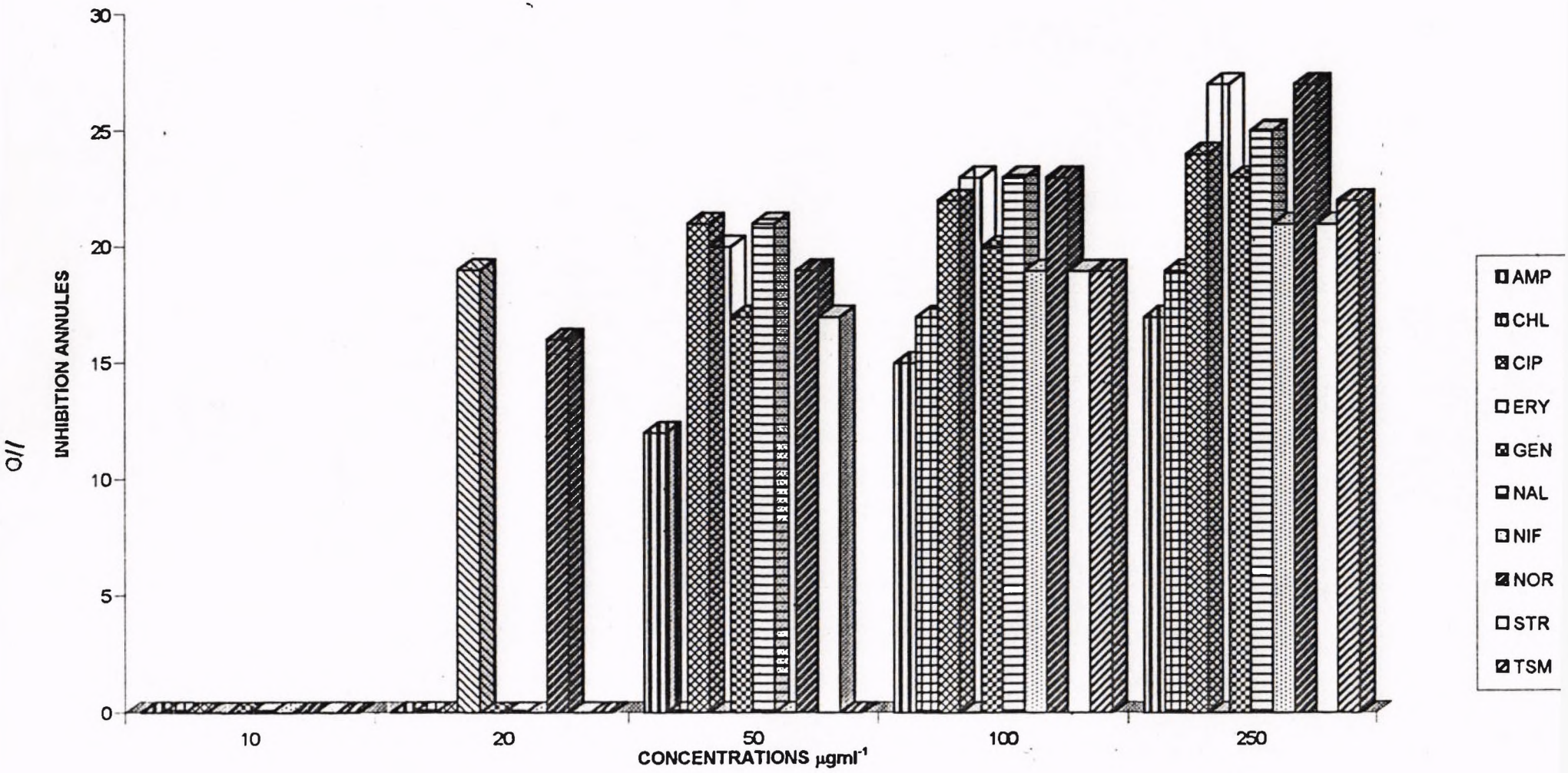


Fig 23 SENSITIVITY PATTERN OF E.coli ISOLATE-5 TOWARDS SELECTED ANTIBIOTICS

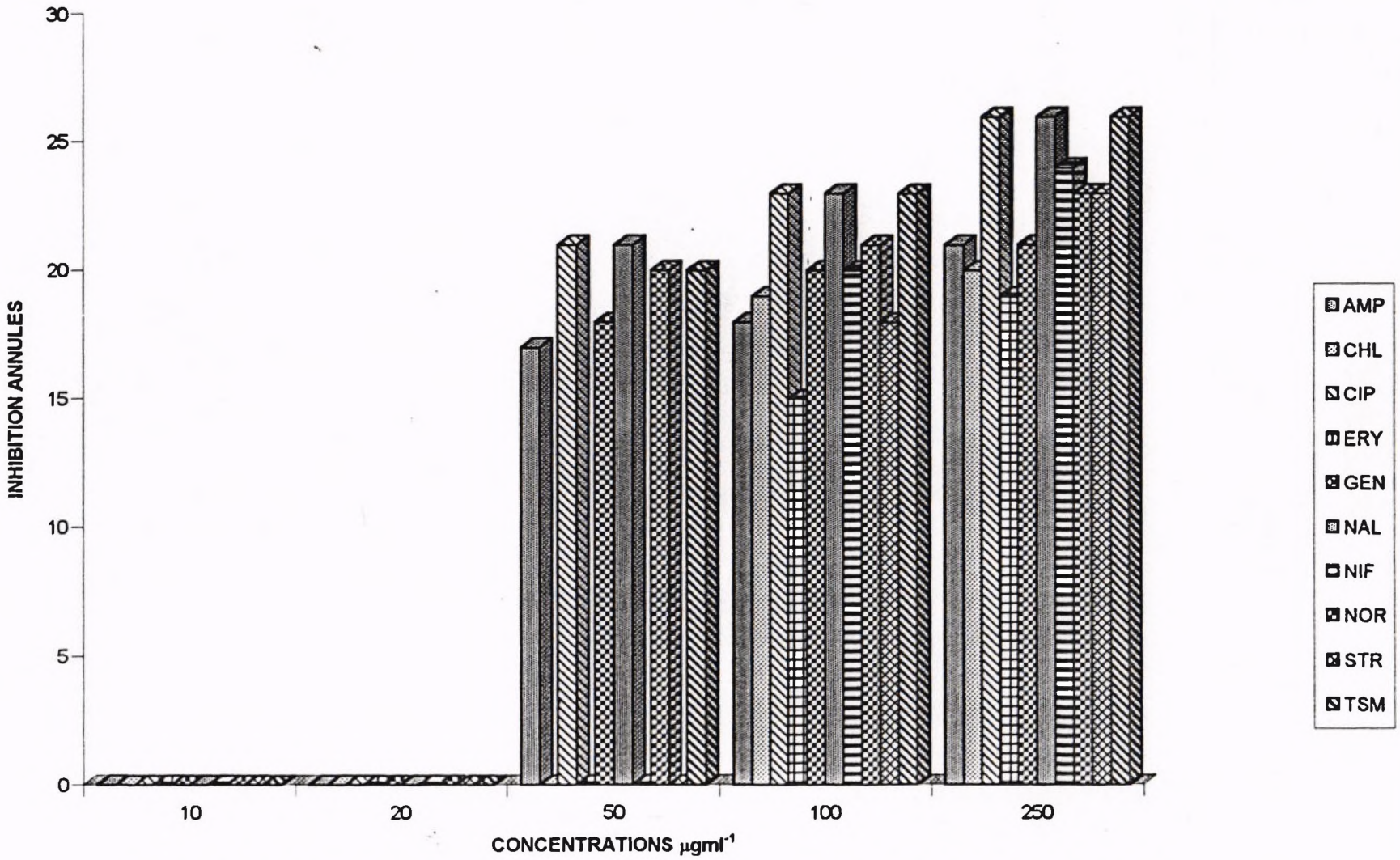


Fig.2 SENSITIVITY PATTERN OF E.coli ISOLATE- 6 TOWARDS SELECTED ANTIBIOTICS

ISOLATE - 7

This isolate showed its highest rate of inhibition annule, 24 at 250 μg^{ml-1} , with three antibiotics namely, erythromycin, nalidixic acid and streptomycin (Table XVIII ; Fig.25).

ISOLATE - 8

The highest rate of inhibition annule shown by this isolate with erythromycin was 28 at 250 μg^{ml-1} (Table XVIII ; Fig.26).

ISOLATE - 18

With ciprofloxacin, gentamicin and nitrofurantoin, this isolate recorded its highest rate of inhibition annule , 23 at 250 μg^{ml-1} (Table XIX ; Fig.27)

ISOLATE - 22

With chloramphenical, ciprofloxacin and norfloxacin, this isolate showed its highest inhibition annule 23 at 250 μg^{ml-1} (Table XIX; Fig.28).

TABLE -XVIII

SENSITIVITY PATTERN OF E. coli ISOLATE - 7 AND ISOLATE - 8 TOWARDS SELECTED ANTIBIOTICS

ISOLATE NUMBER AND SOURCE	CONCENT-RATIONS μg^{ml-1}	NAME OF THE ANTIBIOTICS									
		AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE 7	10	@	@	@	@	@	@	@	@	@	@
	20	16-	@	@	19-	@	@	@	@	@	17-
	50	18-	19-	@	21-	17-	@	19-	@	19-	18-
	100	20-	21-	17-	23-	19-	22-	20-	18-	22-	20-
	250	21-	23-	21-	24-	20-	24-	23-	20-	24-	21-
ISOLATE 8	10	@	@	@	@	@	@	@	@	@	@
	20	14-	@	19-	@	18-	@	@	@	@	@
	50	17-	19-	21-	19-	23-	20-	18-	21-	17-	20-
	100	19-	23-	23-	26-	25-	21-	21-	23-	19-	21-
	250	23-	25-	24-	28-	26-	23-	23-	25-	21-	22-

@ : RESISTANCE

- : INHIBITION ANNULES (SENSITIVITY)

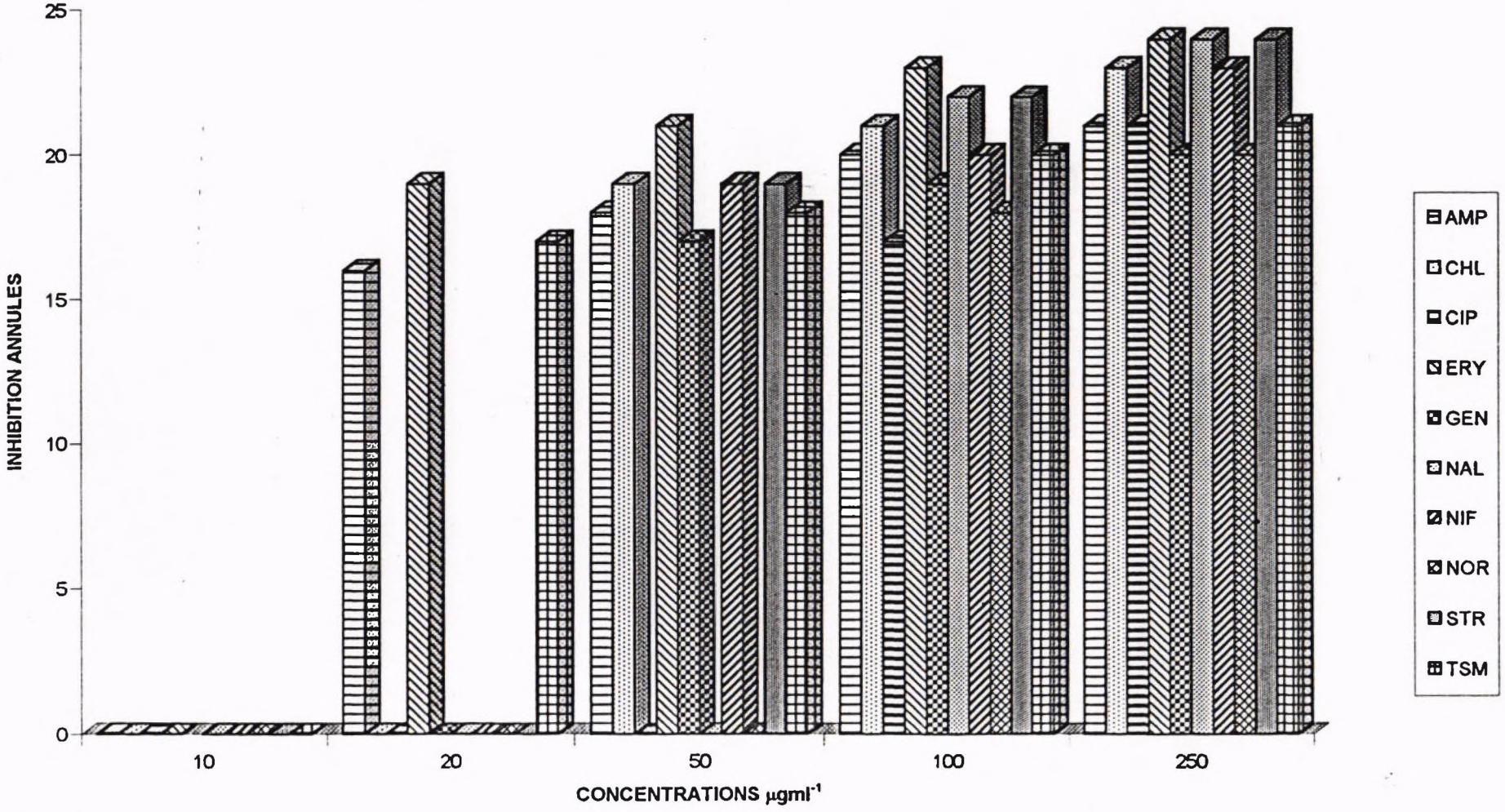


Fig 25 SENSITIVITY PATTERN OF E.coli ISOLATE- 7 TOWARDS SELECTED ANTIBIOTICS

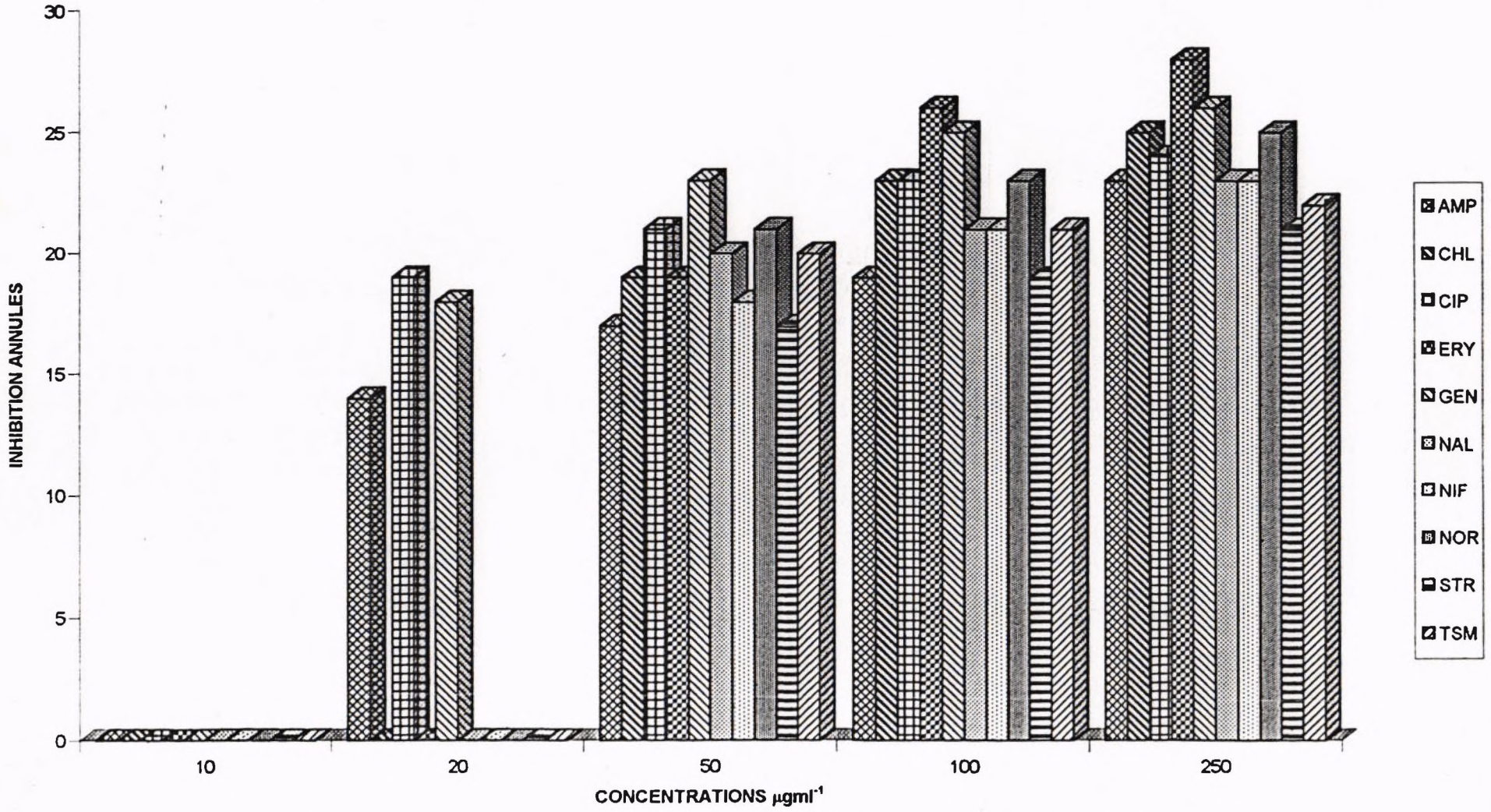


Fig 26 SENSITIVITY PATTERN OF E.coli ISOLATE- 8 TOWARDS SELECTED ANTIBIOTICS

TABLE -XIX

SENSITIVITY PATTERN OF E. coli ISOLATE - 18 AND ISOLATE - 22 TOWARDS SELECTED ANTIBIOTICS

ISOLATE NUMBER AND SOURCE	CONCEN-TRATIONS μg^{ml-1}	NAME OF THE ANTIBIOTICS									
		AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE 18	10	@	@	14^-	@	@	@	@	@	@	@
	20	@	15^-	17^-	@	@	@	@	@!	@	@
	50	14^-	17^-	19^-	19^-	18^-	@	19^-	17^-	16^-	17^-
	100	17^-	19^-	21^-	20^-	21^-	20^-	21^-	18^-	17^-	19^-
	250	20^-	22^-	23^-	22^-	23^-	21^-	23^-	21^-	20^-	21^-
ISOLATE 22	10	@	@	@	@	@	@	@	@	@	@
	20	@	@	@	@	@	@	@	@	@	@
	50	15^-	17^-	21^-	17^-	@	@	19^-	@	14^-	13^-
	100	17^-	19^-	22^-	19^-	17^-	21^-	21^-	20^-	16^-	17^-
	250	21^-	23^-	23^-	21^-	19^-	22^-	22^-	23^-	18^-	19^-

@ : RESISTANCE

- : INHIBITION ANNULES (SENSITIVITY)

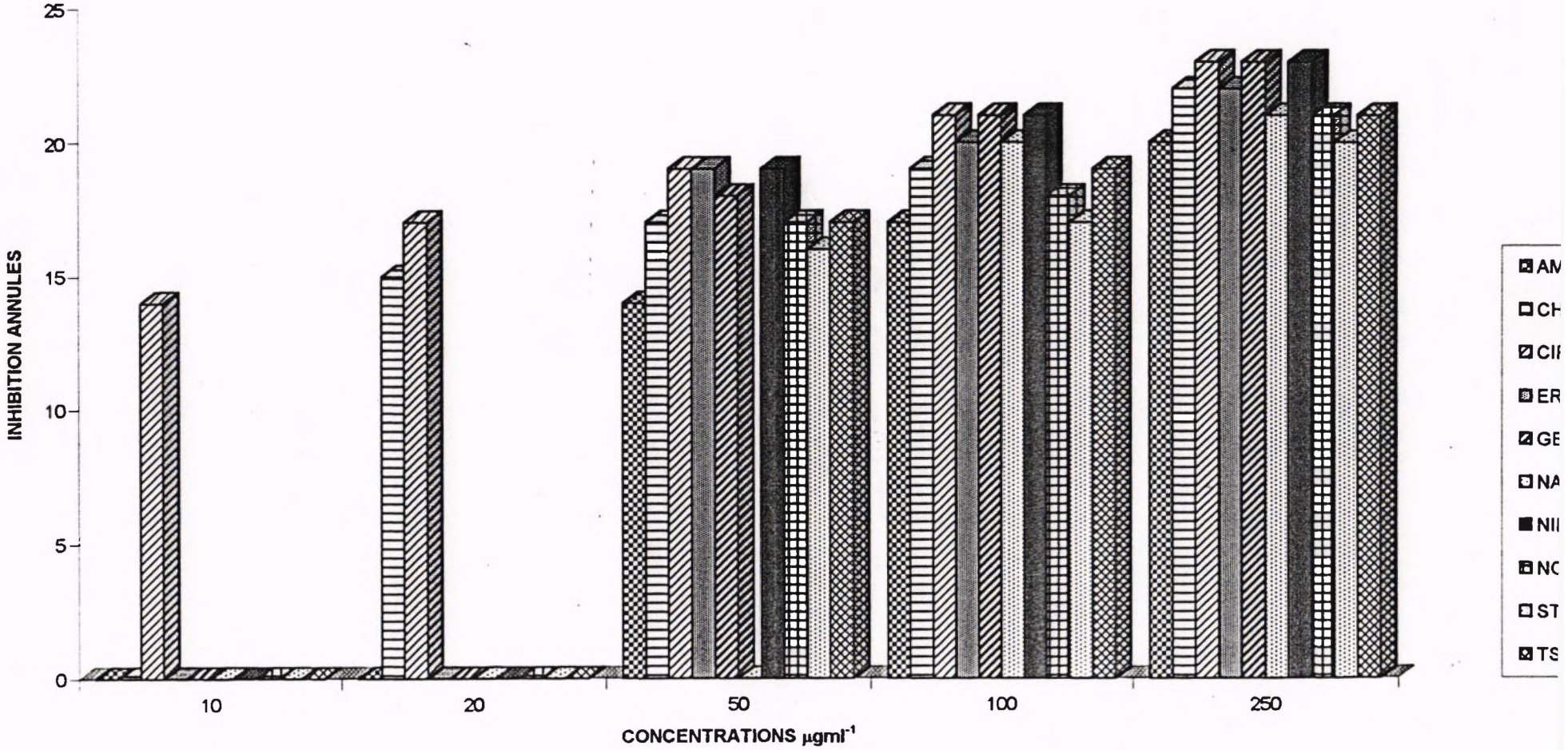


Fig 27 SENSITIVITY PATTERN OF E.coli ISOLATE - 18 TOWARDS SELECTED ANTIBIOTICS

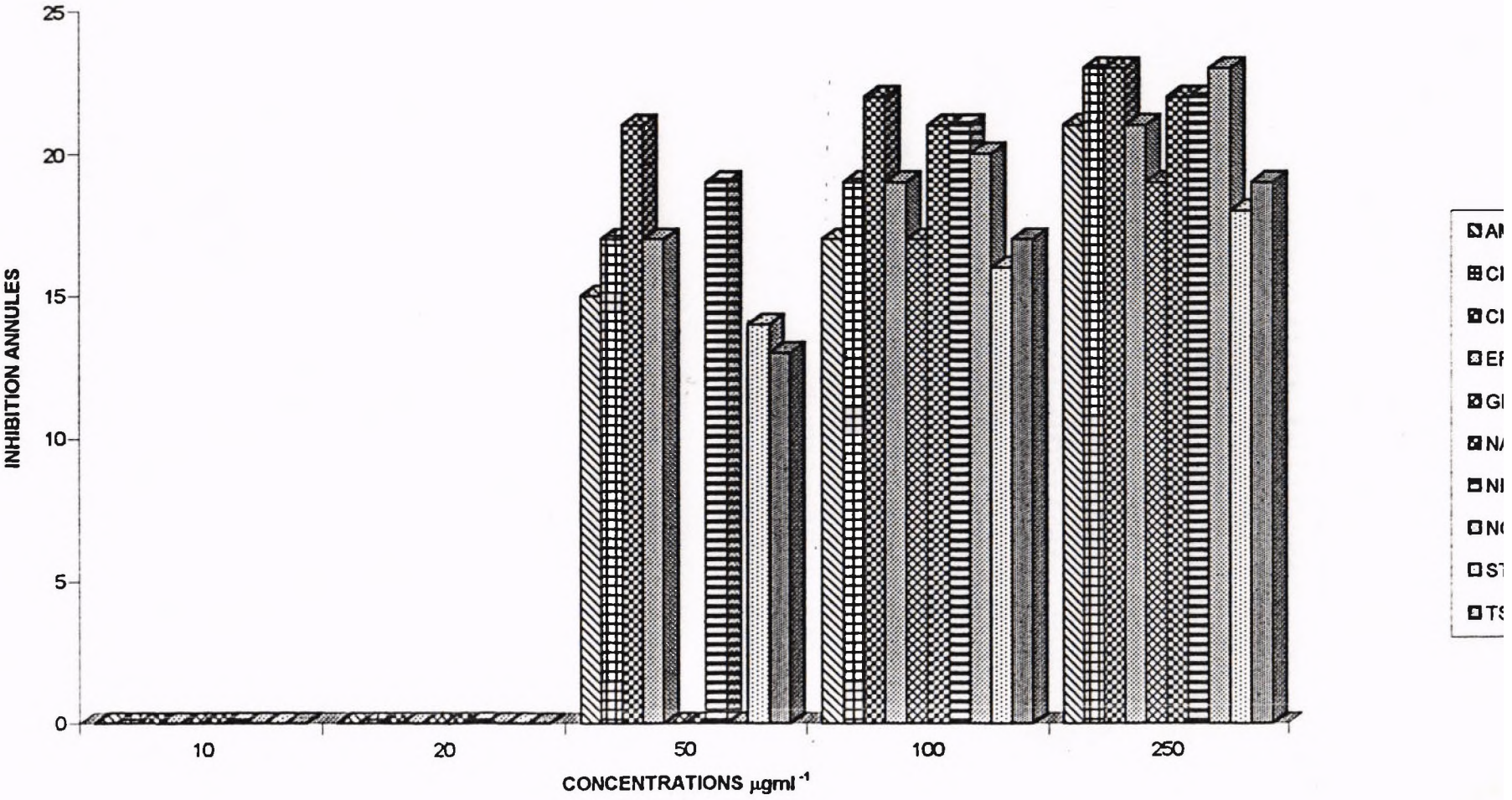


Fig.28 SENSITIVITY PATTERN OF E.coli ISOLATE - 22 TOWARDS SELECTED ANTIBIOTICS

VI. MECHANISM OF RESISTANCE PATTERN (MOLECULAR CHARACTERIZATION) IN *E.coli* ISOLATES

a) PLASMID PROFILE OF SENSITIVE ISOLATES

Observation by electrophonogram technique revealed the absence of plasmids in sensitive isolates as there were no bands to be seen (Fig.29).

b) PLASMID PROFILE OF RESISTANT ISOLATES

16 out of 22 isolates were identified to possesss Multiple Antibiotic Resistance. Through the electrophonogram technique, bands were observed in the 16 isolates. Thus, the presence of plasmids carrying R-factor was confirmed. Although bands were seen in all the 16 isolates, they were distinct in only ten isolates (1,2,3,4,10,16,17,19,20&21) (Figs 30 & 31). These ten isolates therefore, were selected for Restriction Fragment Length Polymorphism study (RFLP) and Polymerase Chain Reaction study (PCR).

c) RESTRICTION FRAGMENT LENGTH POLYMORPHISM STUDY (RFLP)

The RFLP study in the 10 isolates with distinct plasmid bands showed a difference among the restricted fragments. 3 isolates among the 10 exhibited similar

Fig.24 PLASMID PROFILE OF SENSITIVE ISOLATES

LANE 1-6 INDICATES THE ABSENCE OF PLASMIDS IN SENSITIVE ISOLATES

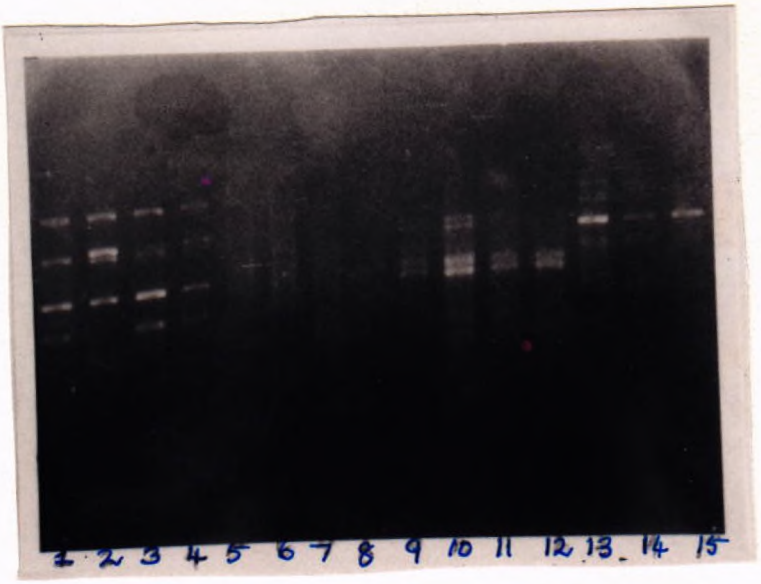


Fig. 30 PLASMID PROFILE OF RESISTANT/SENSITIVE ISOLATES

LANE - 1, 2, 3, 4, 9, 10, 11, 12, 13, 14, 15 WHICH CONTAINS

PLASMIDS (RESISTANT ISOLATES) – BANDS

LANE - 5, 6, 7, 8 ABSENCE OF PLASMIDS (SENSITIVE ISOLATES)

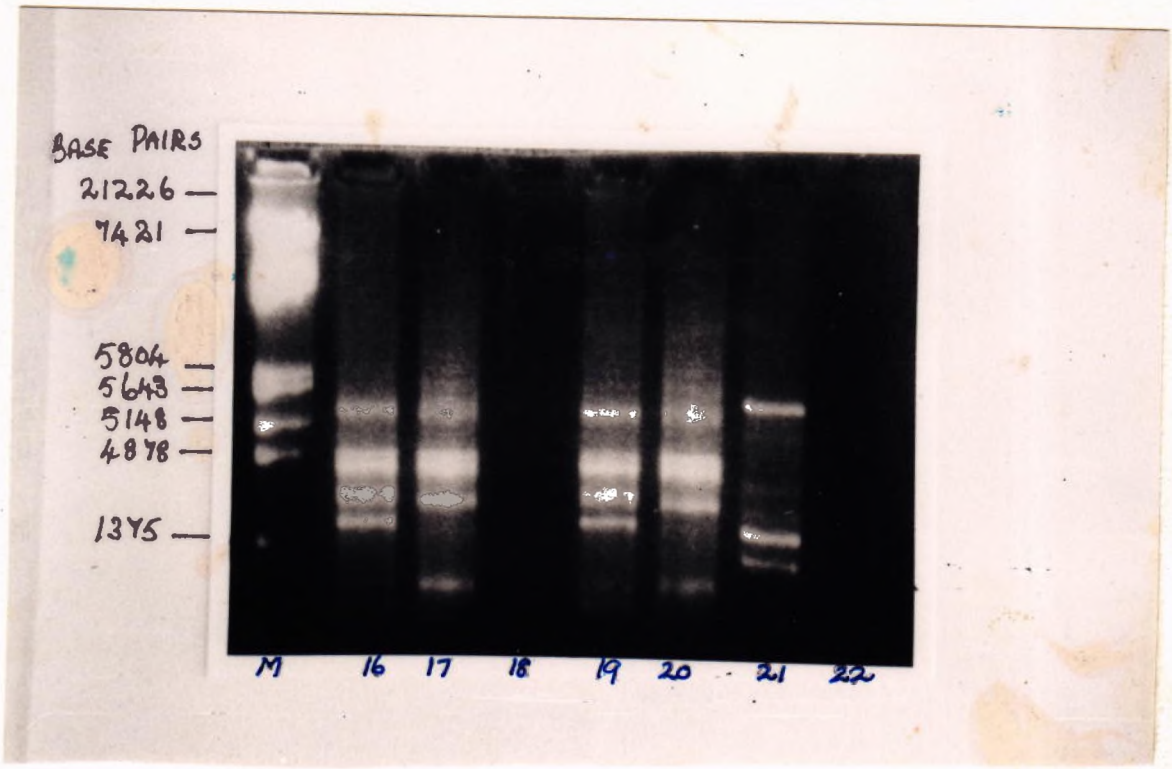


Fig. 3) PLASMID PROFILE OF RESISTANT/SENSITIVE ISOLATES

LANE - 16,17,19, 20,21 WHICH CONTAINS PLASMIDS

(RESISTANT ISOLATES) -- BANDS

LANE - 18,22 ABSENCE OF PLASM IDS (SENSITIVE ISOLATES)

banding pattern reflecting the same characteristics of the plasmids. 2 other isolates(4 and 16) resembled each other in the banding pattern. Isolates 20 and 21 had similar banding pattern. The banding pattern of isolates 10,17 and 19 differed not only from one another but also from that of the other isolates (Fig.32).

d) POLYMERASE CHAIN REACTION STUDY (PCR)

The presence of plasmids in the 10 resistant isolates were further confirmed by the amplification of restricted fragments through PCR study (Fig.33).

VII. R-PLASMIDS CURING OFF AND THEIR SENSITIVITY TO ANTIBIOTICS

Curing off method facilitated the removal of plasmids from the 10 resistant isolates (having distinct plasmid bands) and made them sensitive to the selected antibiotics. To free the isolates from plasmids, acridine orange treatment was adopted and this was repeated many times. After this, the cured off isolates were tested for sensitivity to the antibiotics.

After curing off treatment, isolates 1 & 2 have shown the highest rate of inhibition annules,24and 25 at 250 μg^{ml-1}, respectively, towards trimethoprim / sulphamethoxazole (Table XX ; Fig.34 & 35). Isolate 3 showed the highest rate of inhibition annules, 25 at 250 μg^{ml-1} , with gentamicin, nitrofurantoin and norfloxacin (Table XXI ; Fig.36). Isolate 4 exhibited the highest rate of inhibition annule, 26 at 250 μg^{ml-1}, with only chloramphenical (Table XXI ; Fig.37). The highest rate of Inhibition annule, 25 at 250 μg^{ml-1} , was exhibited by isolate 10 with ampicillin and streptomycin. Isolate 16 with trimethoprim/sulphamethoxazole showed 25 at 250 μg^{ml-1} (Table XXII ; Figs.38 & 39). Isolate 17 had the highest inhibition annule, 24 at 250 μg^{ml-1} , with nalidixicacid (Table XXIII ; Fig.40). Similarly, treatment of isolate 19 with norfloxacin showed the highest value of 27 at 250 μg^{ml-1} (Table XXIII ; Fig.41). Isolates 20 & 21, on treatment with ciprofloxacin, exhibited the highest rate of inhibition value, 24 and 25 respectively, at 250 μg^{ml-1} while isolate 20, on treatment with trimethoprim/sulphamethoxazole showed the highest inhibition value, 24 at 250 μg^{ml-1} (Table XXIV ; Fig.42 & 43).

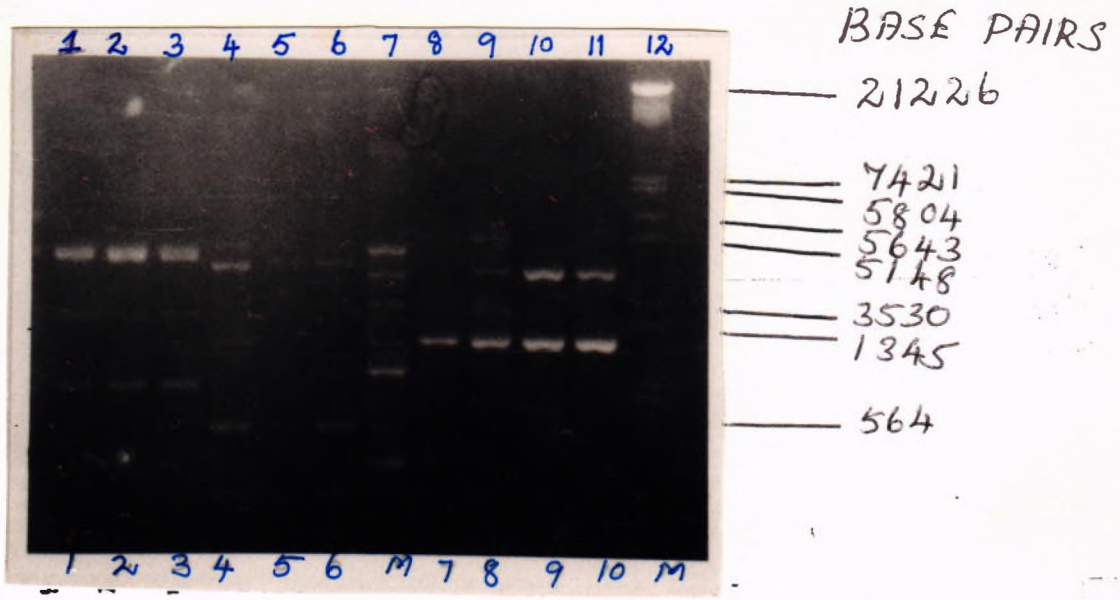


Fig. 32 RFLP PATTERN OF RESISTANT ISOLATES

LANE NO.	ISOLATE NO
1	1
2	2
3	3
4	4
5	10
6	16
7	MARKER
8	17
9	19
10	20
11	21
12	MARKER

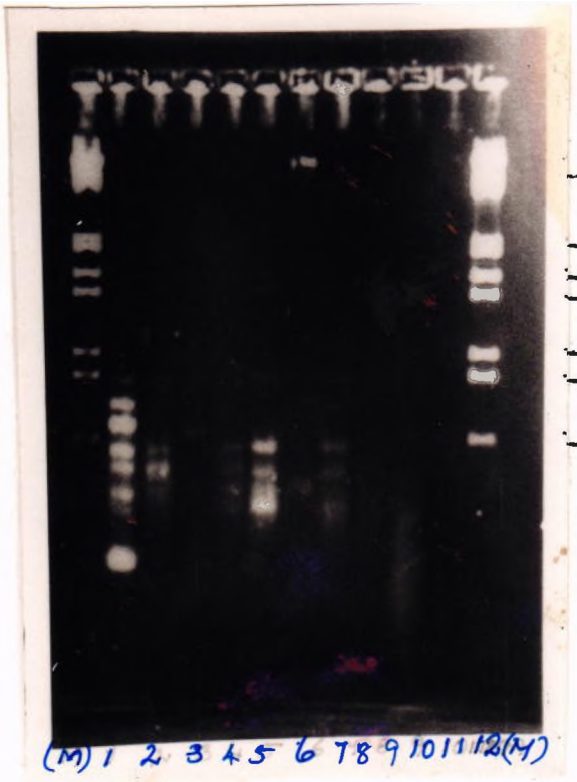


Fig.33 RFLP PCR AMPLIFICATION OF RESISTANT ISOLATES

LANE NO.	ISOLATE NO
1	MARKER
2	1
3	2
4	3
5	4
6	10
7	16
8	17
9	19
10	20
11	21
12	MARKER

TABLE - XX

SENSITIVITY PATTERN OF E. Coli ISOLATE - 1 AND ISOLATE - 2 TOWARDS SELECTED ANTIBIOTICS AFTER PLASMID CURING OFF TREATMENT

ISOLATE NUMBER AND SOURCE	CONCEN-TRATIONS	NAME OF THE ANTIBIOTICS									
	Mg^{ml-1}	AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE 1	10	@	@	@	@	@	@	@	@	@	@
	20	@	@	@	@	@	@	@	@	@	18^{-}
	50	13^{-}	@	17^{-}	15^{-}	@	16^{-}	@	17^{-}	@	19^{-}
	100	14^{-}	16^{-}	18^{-}	17^{-}	19^{-}	18^{-}	19^{-}	20^{-}	21^{-}	21^{-}
	250	17^{-}	18^{-}	20^{-}	19^{-}	21^{-}	20^{-}	21^{-}	22^{-}	23^{-}	24^{-}
ISOLATE 2	10	@	@	@	@	@	@	@	@	@	@
	20	@	17^{-}	@	@	@	@	18^{-}	@	21^{-}	@
	50	14^{-}	19^{-}	19^{-}	18^{-}	17^{-}	17^{-}	19^{-}	20^{-}	22^{-}	21^{-}
	100	18^{-}	20^{-}	21^{-}	19^{-}	19^{-}	19^{-}	21^{-}	21^{-}	23^{-}	24^{-}
	250	20^{-}	21^{-}	22^{-}	20^{-}	21^{-}	23^{-}	22^{-}	23^{-}	24^{-}	25^{-}

@ : RESISTANCE

- : INHIBITION ANNULES (SENSITIVITY)

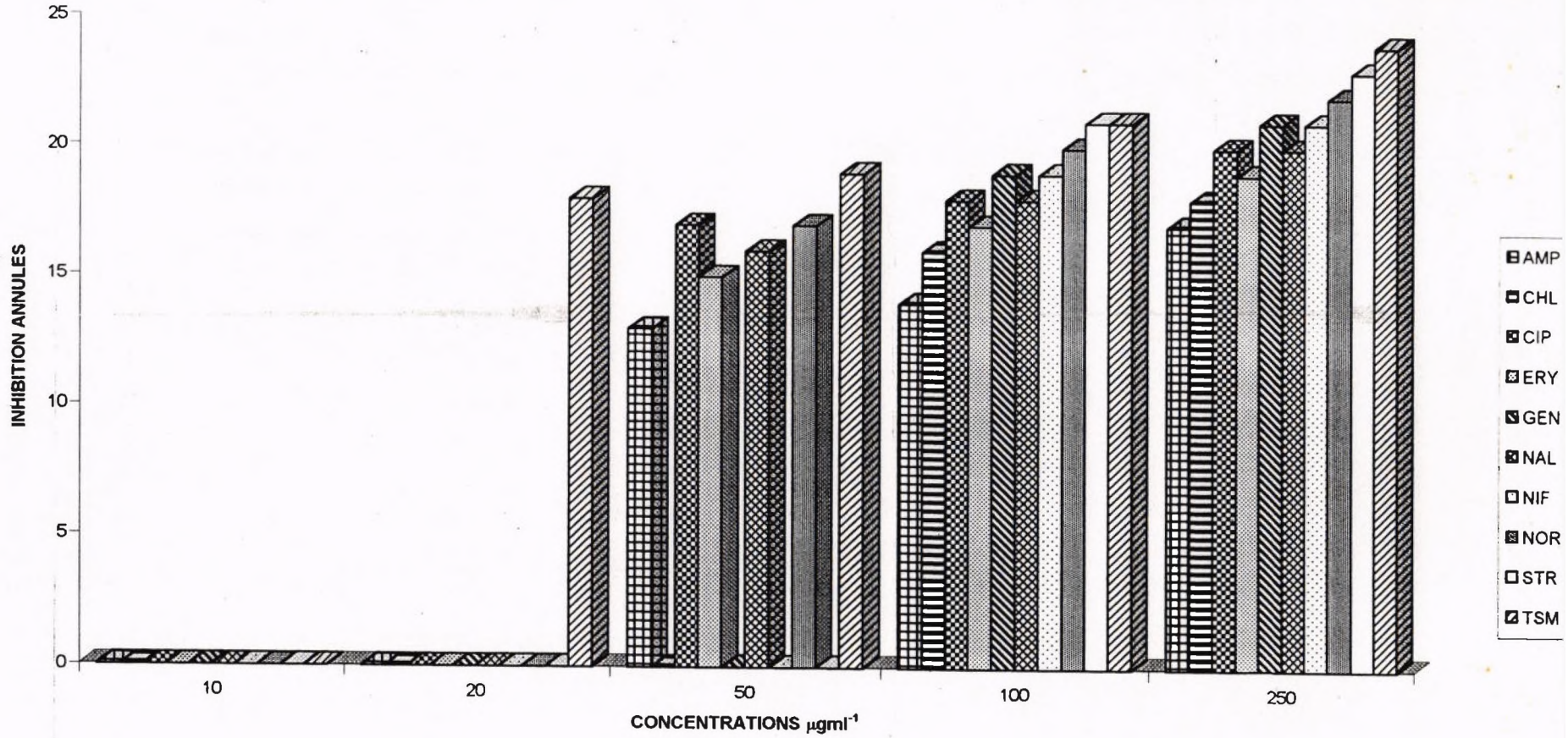


Fig.34 SENSITIVITY PATTERN OF E.coli ISOLATE - 1 TOWARDS SELECTED ANTIBIOTICS AFTER PLASMID CURING OFF TREATMENT

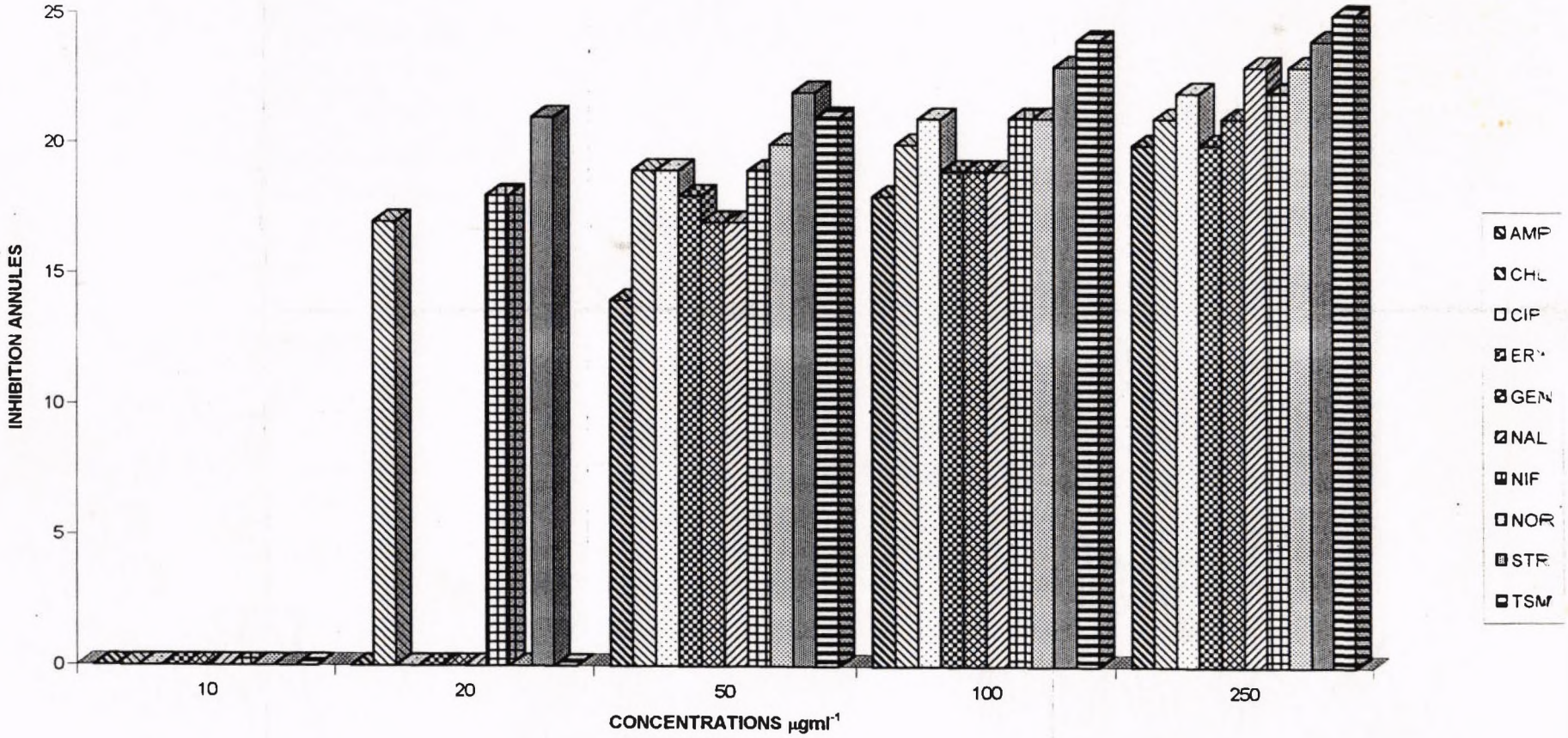


Fig.35 SENSITIVITY PATTERN OF E.coli ISOLATE - 2 TOWARDS SELECTED ANTIBIOTICS AFTER PLASMID CURING OFF TREATMENT

TABLE XXI

SENSITIVITY PATTERN OF E.coli ISOLATE - 3 AND ISOLATE - 4 TOWARDS SELECTED ANTIBIOTICS AFTER PLASMID CURING OFF TREATMENT

ISOLATE NUMBER AND SOURCE	CONCENT-RATIONS μg^{ml-1}	NAME OF THE ANTIBIOTICS									
		AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE	10	@	@	@	@	@	@	@	@	@	@
	20	@	@	16-	@	@	@	@	@	@	@
3	50	15-	@	19-	17-	21-	@	21-	20-	@	17-
	100	17-	19-	22-	18-	23-	19-	23-	22-	19-	21-
	250	21-	21-	23-	19-	25-	23-	25-	25-	21-	23-
ISOLATE	10	@	@	@	@	@	@	@	@	@	@
	20	@	@	@	16-	@	@	@	19-	@	@
4	50	21-	23-	19-	18-	@	20-	19-	21-	17-	19-
	100	23-	25-	22-	20-	22-	21-	23-	23-	21-	22-
	250	24-	26-	25-	21-	23-	23-	25-	25-	23-	23-

@: RESISTANCE

- : INHIBITION ANNULES (RESISTANCE)

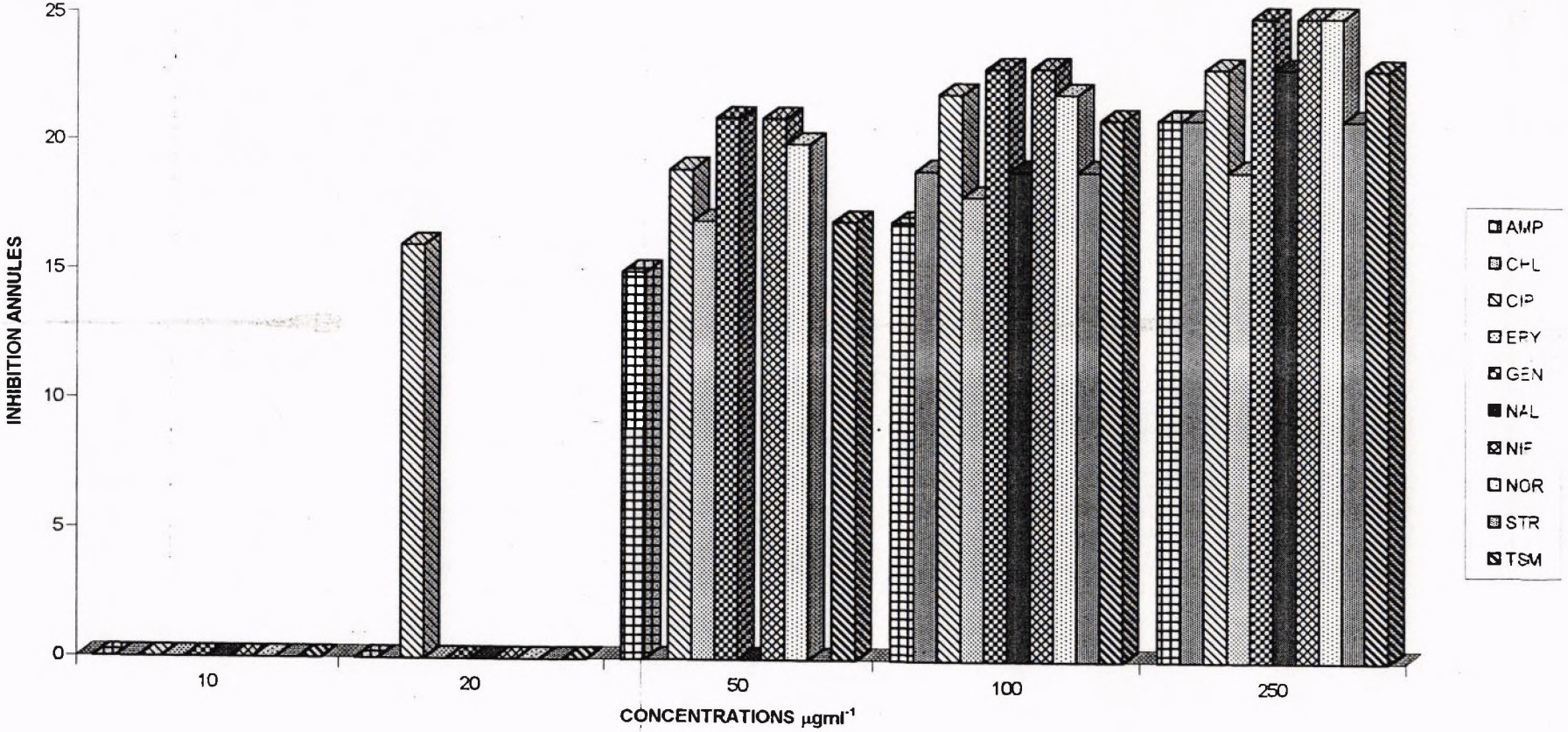


Fig. 36 SENSITIVITY PATTERN OF E.coli ISOLATE - 3 TOWARDS SELECTED ANTIBIOTICS AFTER PLASMID CURING OFF TREATMENT

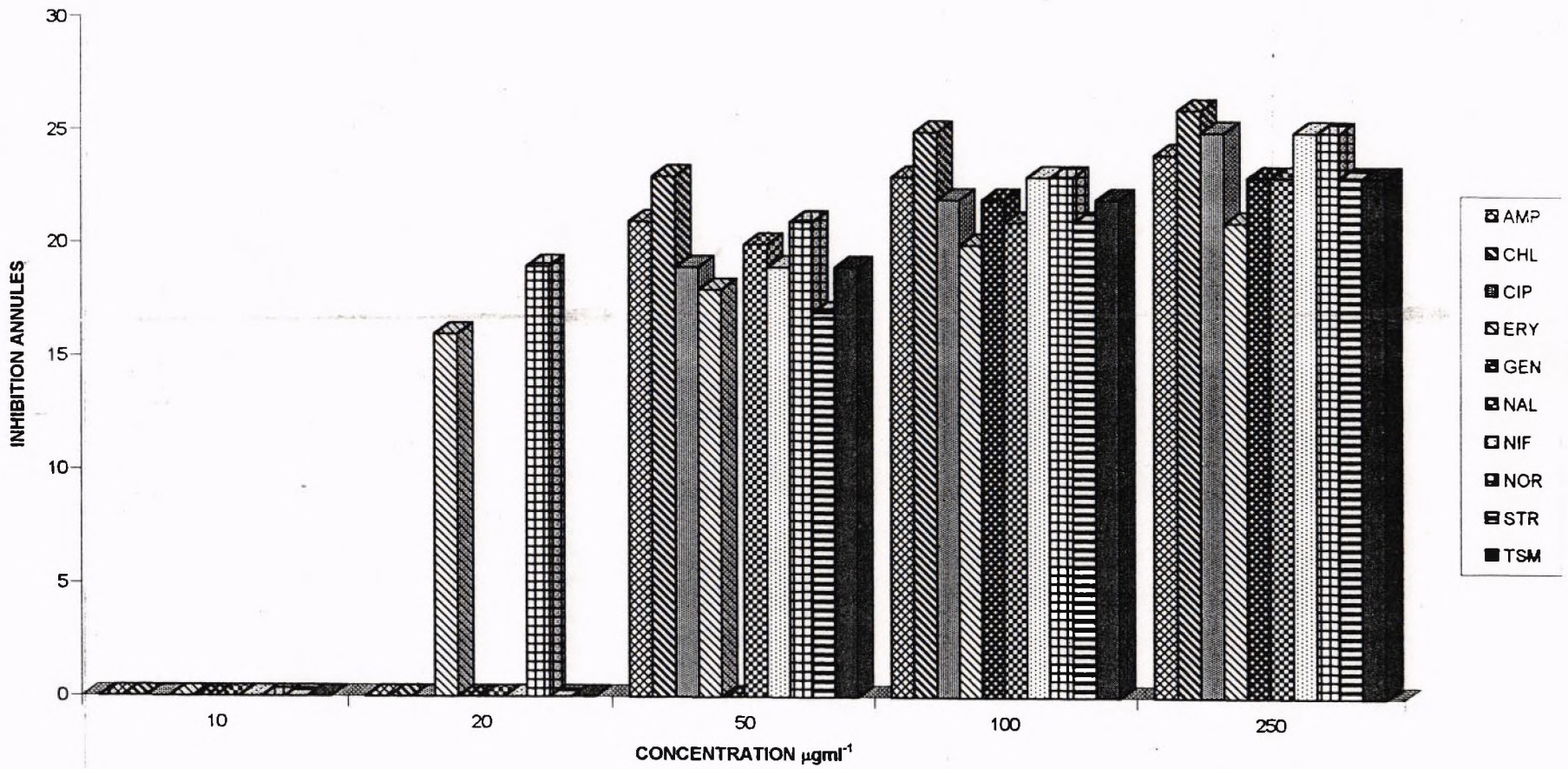


Fig.37 SENSITIVITY PATTERN OF E.coli ISOLATE - 4 TOWARDS SELECTED ANTIBIOTICS AFTER PLASMID CURING OFF TREATMENT

TABLE XXII

SENSITIVITY PATTERN OF E.coli ISOLATE - 10 AND ISOLATE - 16 TOWARDS SELECTED ANTIBIOTICS AFTER PLASMID CURING OFF TREATMENT

ISOLATE NUMBER AND SOURCE	CONCENT-RATIONS μg^{ml-1}	NAME OF THE ANTIBIOTICS									
		AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE 10	10	@	@	@	@	@	@	@	@	@	@
	20	@	16-	@	17-	@	@	19-	17-	19-	17-
	50	19-	17-	21-	19-	18-	21-	20-	20-	20-	19-
	100	21-	21-	23-	20-	19-	23-	22-	21-	21-	20-
	250	25-	22-	24-	21-	20-	24-	23-	24-	25-	21-
ISOLATE 16	10	@	@	@	@	@	@	@	@	@	@
	20	@	@	@	16-	@	15-	@	@	@	21-
	50	17-	19-	20-	19-	17-	19-	19-	18-	21-	23-
	100	19-	20-	21-	21-	21-	21-	21-	19-	22-	24-
	250	23-	21-	23-	23-	23-	24-	23-	20-	23-	25-

@ : RESISTANCE

- : INHIBITION ANNULES (SENSITIVITY)

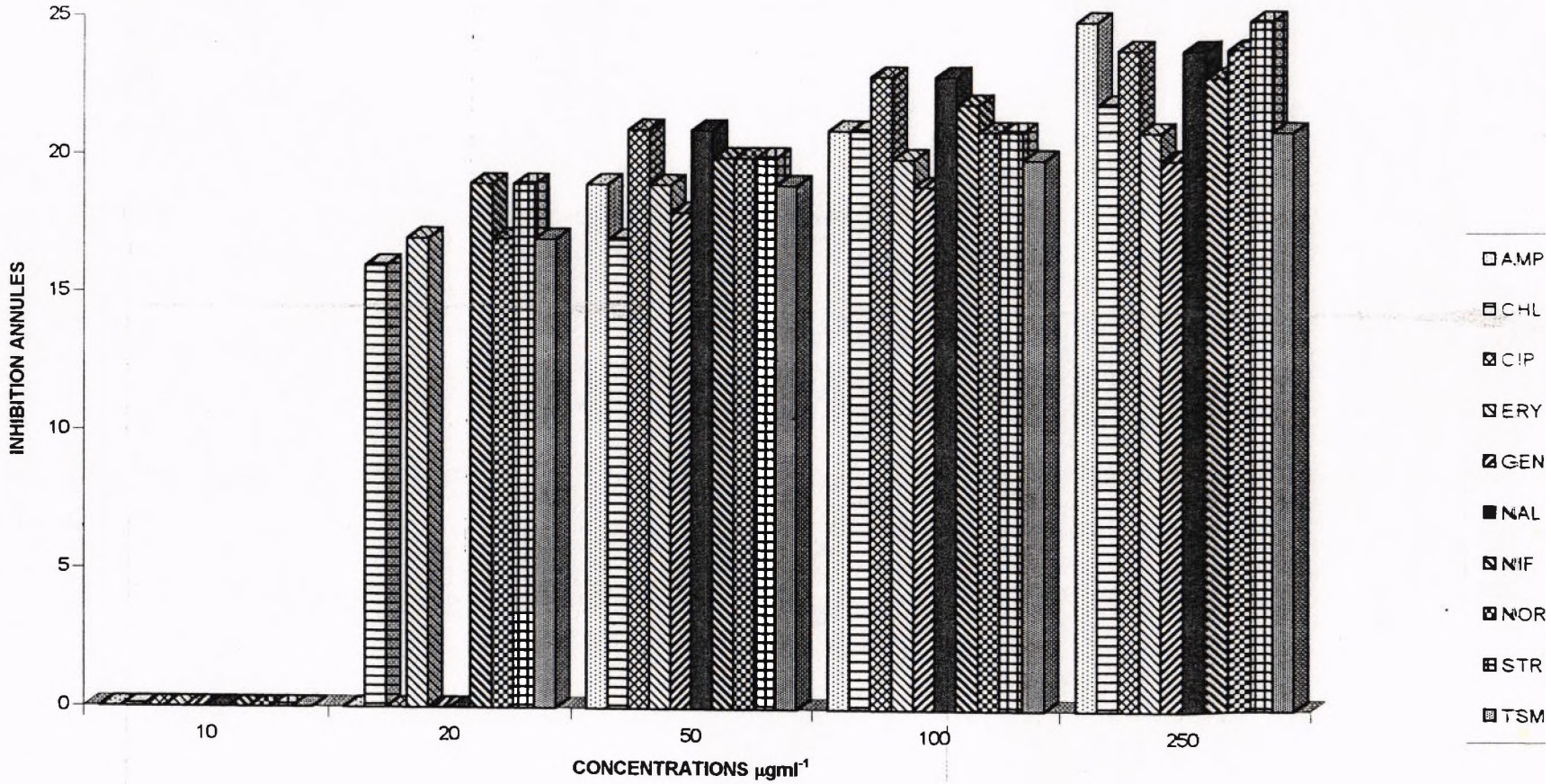


Fig 26 SENSITIVITY PATTERN OF E.coli ISOLATE- 10 TOWARDS SELECTED ANTIBIOTICS AFTER PLASMID CURING OFF TREATMENT

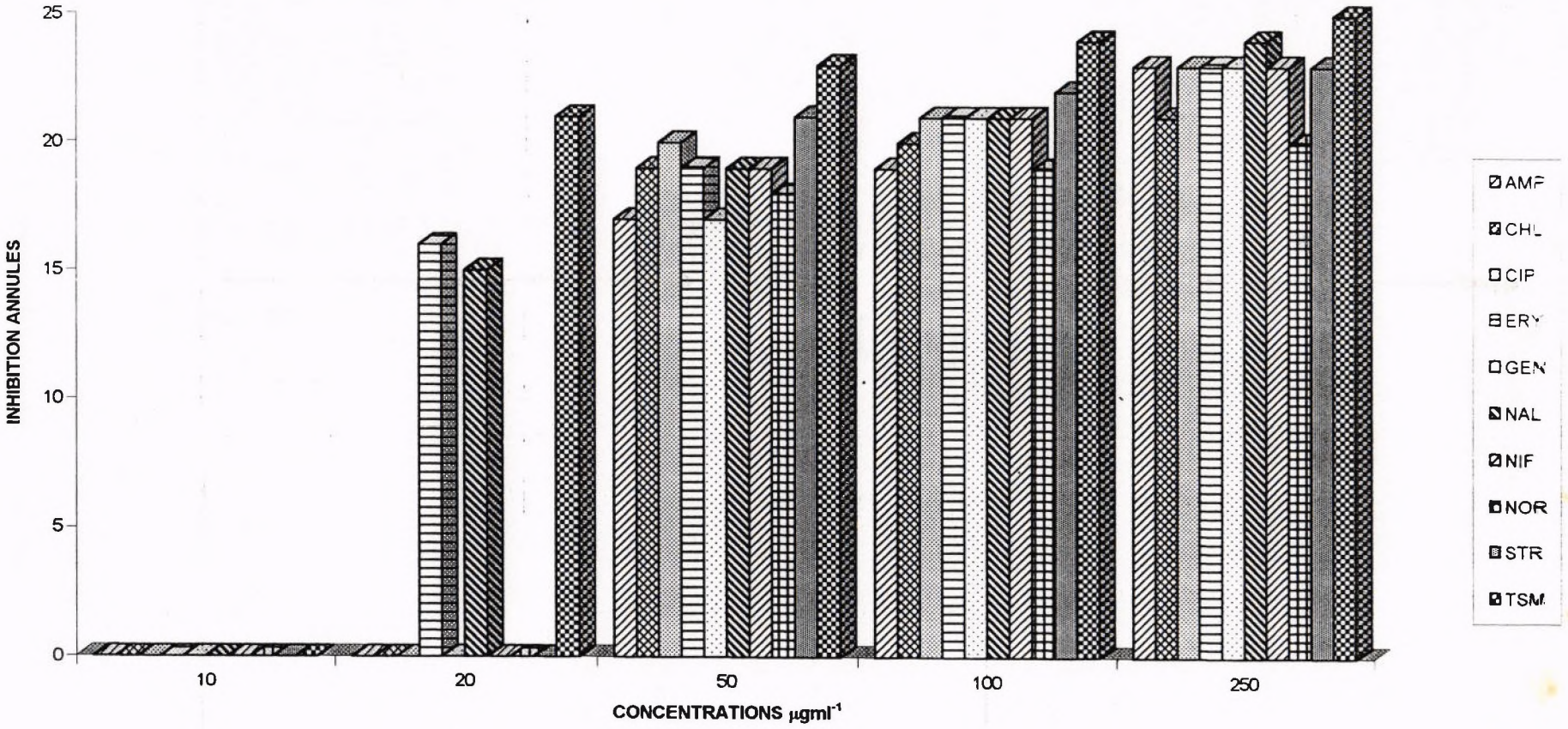


Fig.37 SENSITIVITY PATTERN OF E.coli ISOLATE - 16 TOWARDS SELECTED ANTIBIOTICS AFTER PLASMID CURING OFF TREATMENT

TABLE XXIII

SENSITIVITY PATTERN OF E.coli ISOLATE - 17 AND ISOLATE - 19 TOWARDS SELECTED ANTIBIOTICS AFTER PLASMID CURING OFF TREATMENT

ISOLATE NUMBER AND SOURCE	CONCENT-RATIONS μg^{ml-1}	NAME OF THE ANTIBIOTICS									
		AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE	10	@	@	@	@	@	@	@	@	@	@
	20	@	@	@	@	@	@	@	@	@	@
17	50	18-	@	17-	16-	@	17-	@	19-	18-	@
	100	19-	19-	19-	18-	19-	18-	18-	22-	21-	19-
	250	21-	22-	23-	21-	22-	24-	20-	23-	22-	23-
ISOLATE	10	@	@	@	@	@	@	@	16-	@	@
	20	17-	@	@	@	18-	@	@	18-	@	@
19	50	19-	21-	20-	21-	19-	17-	19-	20-	@	18-
	100	21-	23-	21-	23-	21-	19-	23-	25-	20-	19-
	250	23-	25-	24-	24-	23-	21-	25-	27-	21-	21-

@ RESISTANCE

- : INHIBITION ANNULES (SENSITIVITY)

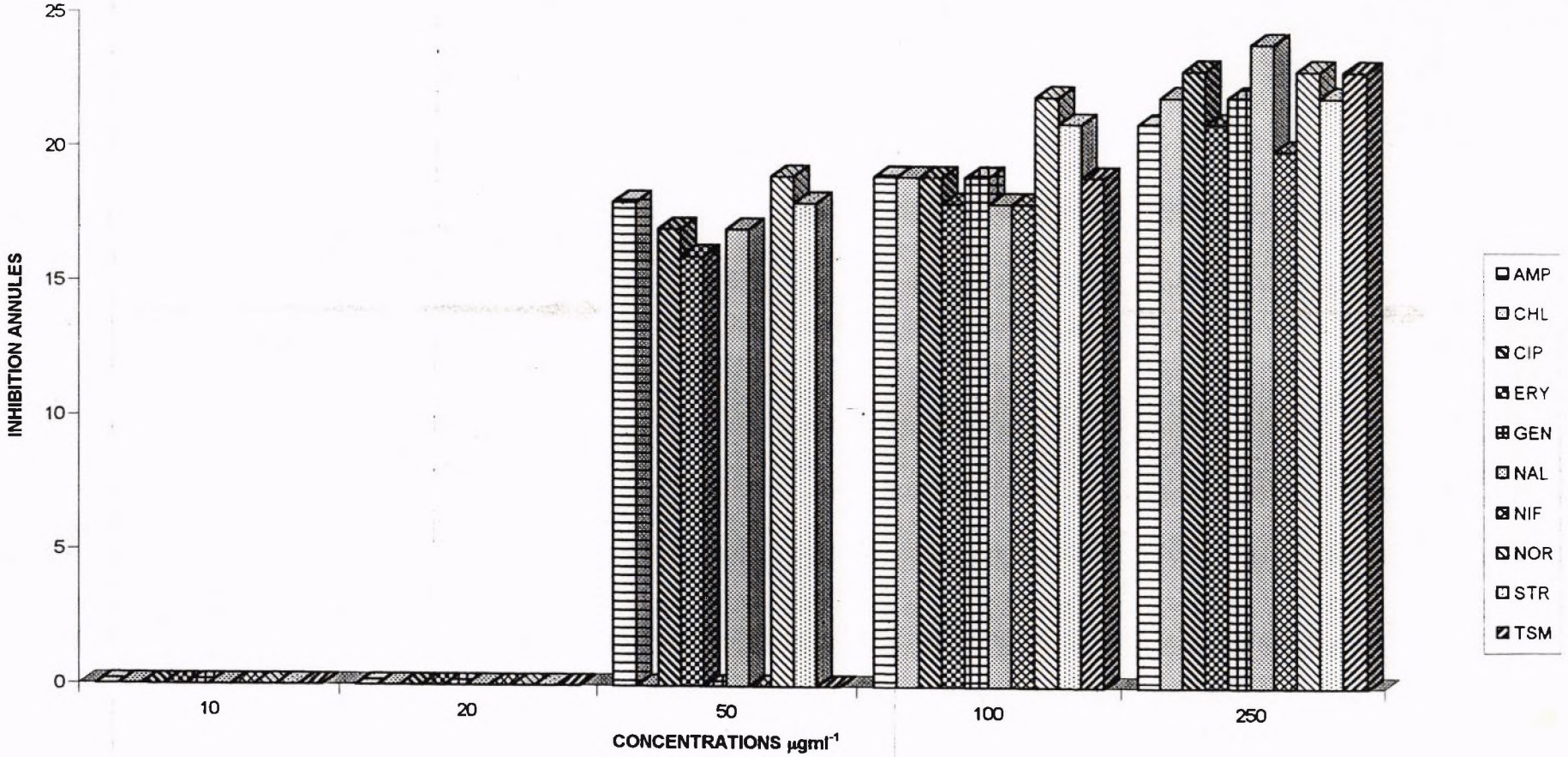


Fig. 40 SENSITIVITY PATTERN OF E.coli ISOLATE- 17 TOWARDS SELECTED ANTIBIOTICS AFTER PLASMID CURING OFF TREATMENT

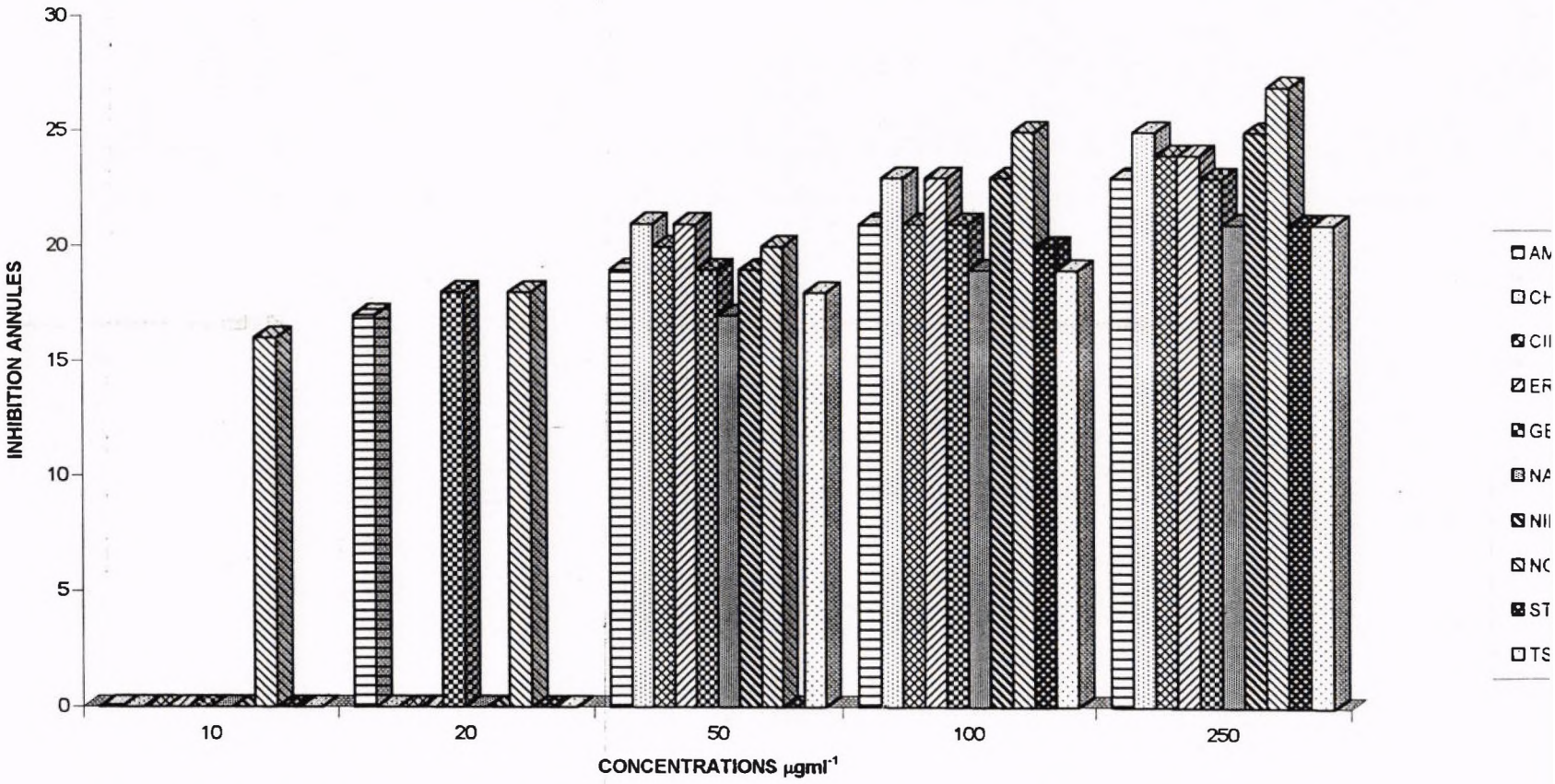


Fig. 41 SENSITIVITY PATTERN OF E.coli ISOLATE - 19 TOWARDS SELECTED ANTIBIOTICS AFTER PLASMID CURING OFF TREATMENT

TABLE XXIV

SENSITIVITY PATTERN OF E.coli ISOLATE - 20 AND ISOLATE - 21 TOWARDS SELECTED ANTIBIOTICS AFTER PLASMID CURING OFF TREATMENT

ISOLATE NUMBER AND SOURCE	CONCENT-RATIONS μg^{ml-1}	NAME OF THE ANTIBIOTICS									
		AMP	CHL	CIP	ERY	GEN	NAL	NIF	NOR	STR	TSM
ISOLATE	10	@	@	19-	@	@	@	@	@	@	19-
	20	14-	15-	20-	17-	@	@	16-	@	@	20-
20	50	17-	19-	21-	19-	17-	20-	18-	19-	17-	21-
	100	19-	21-	22-	20-	20-	21-	21-	21-	19-	23-
	250	22-	23-	24-	22-	22-	23-	22-	22-	23-	24-
ISOLATE	10	@	@	@	@	@	@	@	@	@	@
	20	@	@	20-	@	@	@	@	@	@	@
21	50	15-	19-	22-	16-	21-	20-	15-	19-	18-	17-
	100	17-	20-	23-	18-	23-	22-	18-	20-	21-	20-
	250	20-	22-	25-	19-	24-	23-	19-	22-	23-	21-

@ RESISTANCE

- : INHIBITION ANNULES (SENSITIVITY)

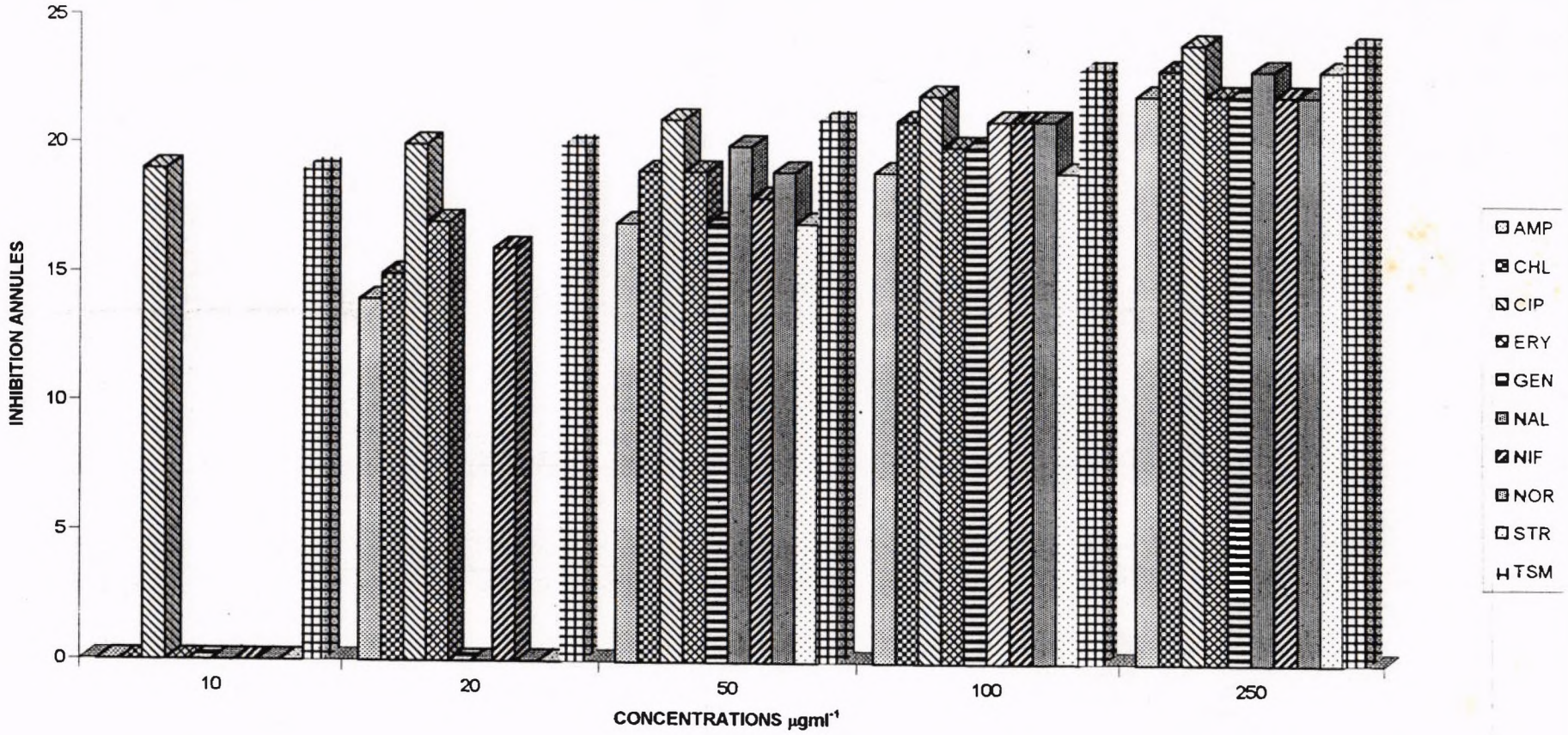


Fig. 42 SENSITIVITY PATTERN OF E.coli ISOLATE - 20 TOWARDS SELECTED ANTIBIOTICS AFTER PLASMID CURING OFF TREATMENT

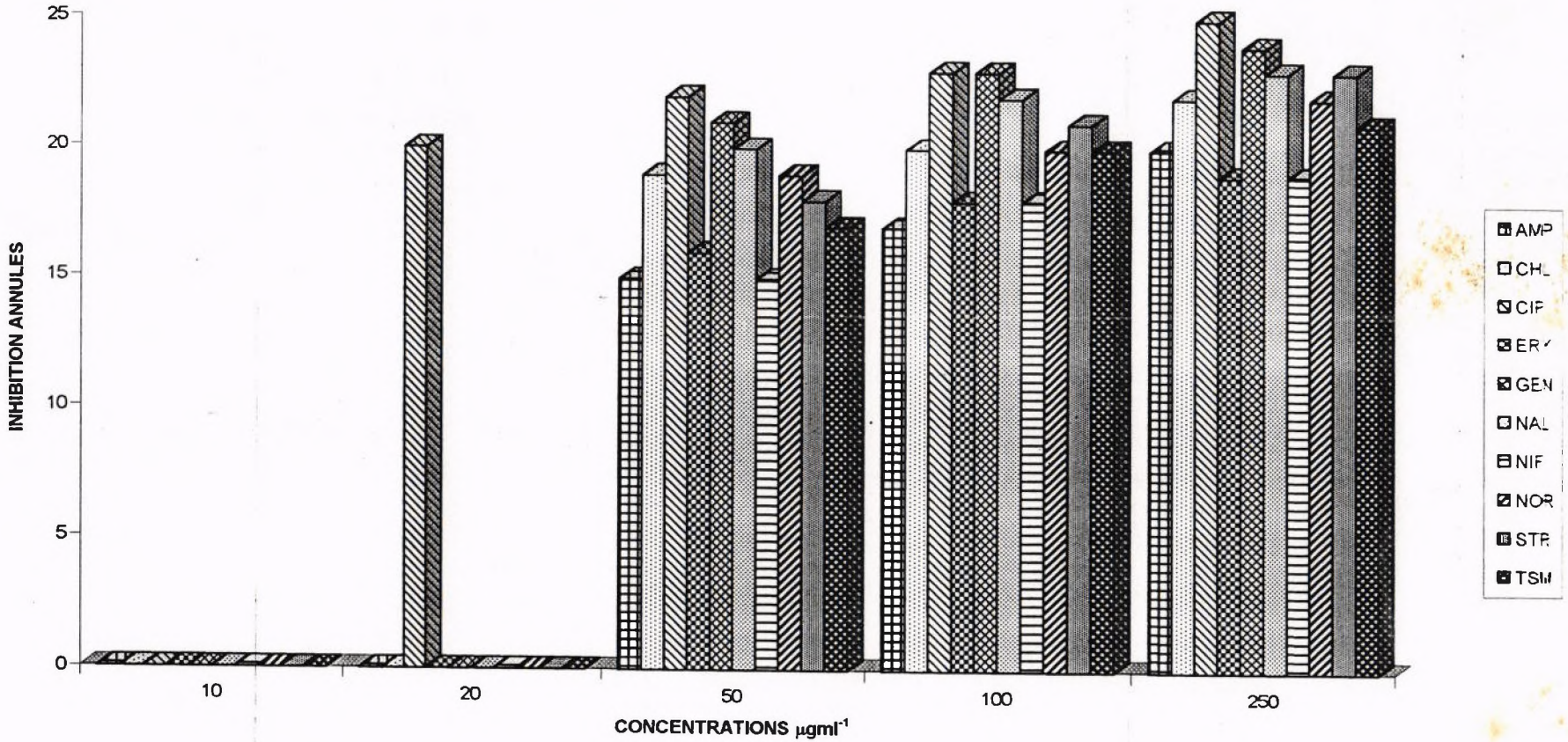


Fig.43 SENSITIVITY PATTERN OF E.coli ISOLATE - 21 TOWARDS SELECTED ANTIBIOTICS AFTER PLASMID CURING OFF TREATMENT

VIII. PYROGEN CONFIRMATION BY ANIMAL INOCULATION

Interpretation of the variations in body temperature of mice before and after pathogenic *E.coli* inoculation confirms that *E.coli* acts as a pyrogen in the human host. The pyrogenic activity of the drug resistant isolate *E.coli* was confirmed by animal inoculation, following observations of rhythmic changes in the experimental human group. Thus by the data in TableXXV & XXVI the pyrogenic activity of *E.coli* was confirmed. A steady increase in the body temperature of the pyrogen inoculated mice was observed. In general, the individual temperature of all the mice increased up to 1.4°F(Fig.44).

Generally, human beings suffering from diarrhoeal infection were found to have an increase in their body temperature i.e., from 98.2°F to100.1°F. This was confirmed by pyrogenic activity in animals.

TABLE XXV
BODY TEMPERATURE READINGS °F OF THE MICE AFTER PYROGEN (E.coli) INOCULATION

Animal No / Time in minutes	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}	A_{11}	A_{12}	A_{13}	A_{14}	A_{15}	A_{16}	A_{17}	A_{18}	A_{19}	A_{20}
0	98.6	98.6	98.6	98.5	98.4	98.3	98.2	98.6	98.5	98.4	98.3	98.2	98.3	98.4	98.5	98.6	98.5	98.4	98.3	98.2
30	99	99	99	98.9	98.8	98.7	98.6	99	98.9	98.8	98.7	98.6	98.7	98.8	98.9	99	98.8	98.8	98.7	98.6
60	99.2	99.2	99.2	99.1	99	98.9	98.8	99.2	99.1	98.9	98.8	98.7	98.8	98.9	99	99.1	99	98.9	98.8	98.7
90	99.6	99.6	99.6	99.5	99.4	99.3	99.2	99.6	99.5	99.4	99.3	99.2	99.3	99.4	99.5	99.6	99.5	99.4	99.3	99.2
120	99.6	99.6	99.6	99.5	99.4	99.3	99.2	99.6	99.5	99.4	99.3	99.2	99.3	99.4	99.5	99.6	99.5	99.4	99.3	99.2
150	99.8	99.8	99.8	99.7	99.6	99.5	99.4	99.8	99.7	99.6	99.5	99.4	99.5	99.6	99.7	99.8	99.7	99.6	99.5	99.4
180	100	100	100	99.9	99.8	99.7	99.6	100	99.9	99.8	99.7	99.6	99.7	99.8	99.9	100	99.9	99.8	99.7	99.6

TABLE XXVI

TABLE SHOWING THE DIFFERENCE BETWEEN THE INITIAL AND FINAL BODY TEMPERATURE READINGS OF THE MICE (CONFIRMING THE PYROGENIC EFFECT OF *E.coli*)

Animal Number	Initial Reading (°F)	Final reading (°F)	Difference (°F)
1	98.6	100	1.4
2	98.6	100	1.4
3	98.6	100	1.4
4	98.5	99.9	1.4
5	98.4	99.8	1.4
6	98.3	99.7	1.4
7	98.2	99.6	1.4
8	98.6	100	1.4
9	98.5	99.9	1.4
10	98.4	99.8	1.4
11	98.3	99.7	1.4
12	98.2	99.6	1.4
13	98.3	99.7	1.4
14	98.4	99.8	1.4
15	98.5	99.9	1.4
16	98.6	100	1.4
17	98.5	99.9	1.4
18	98.4	99.8	1.4
19	98.3	99.7	1.4
20	98.2	99.6	1.4

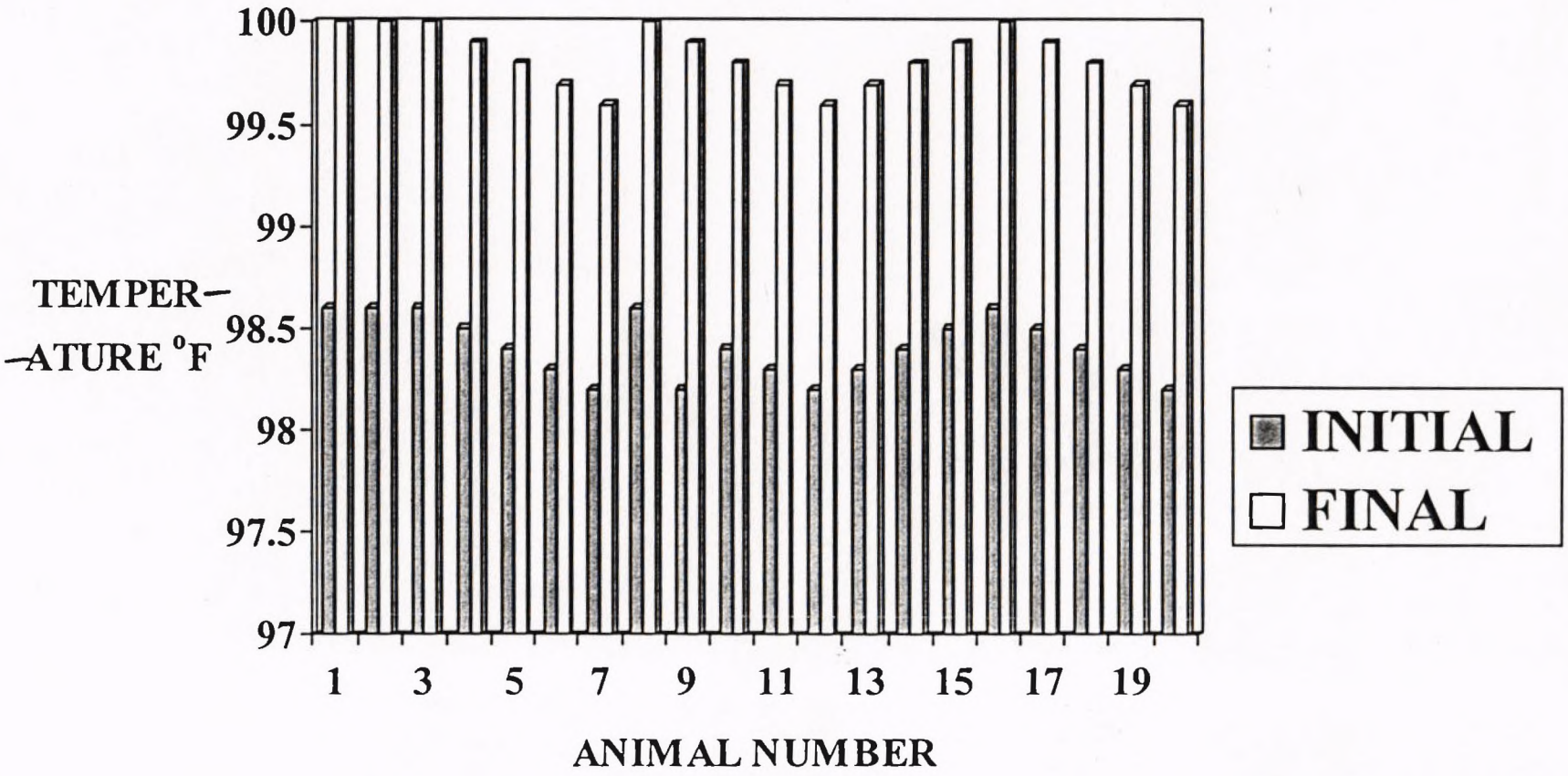


Fig. 44 DIFFERENCE BETWEEN THE INITIAL AND FINAL BODY TEMPERATURE READINGS OF THE MICE AFTER PYROGEN INOCULATION

DISCUSSION

CHAPTER - V

DISCUSSION

Microorganisms like bacteria and viruses prevalent in the environment are of an invasive nature. They make an opportune entry into the host human body through oral, nasal and gastrointestinal tracts. They are potential guests and may even impair the patterned rhythmic functioning of the host human organism including body temperature rhythmicity. Human body exhibits rhythmicity in the functioning of its various parts. e.g. Rhythmicity is observable in the rapid oscillations in the muscles of the heart, the lungs, the diurnal rhythm of sleep activity and in body temperature of human beings (Aschoff, 1965). Again, the Oesinophil content of blood, the electrolyte content, the melotonin content and the excretion vary in a rhythmic pattern . Rhythmicity is a feature of many biochemical process, physiological functions and behavioural aspects of living organisms. Microorganisms cause injury to the body cells by bringing infection and disease.

E.coli , among the pathogenic bacteria, causes gastroenteritis , diarrhoea and urinary tract infection in infants, children and adults. *E.coli* organisms, reportedly found mainly in the intestinal tract, are recognised as the causative agent of acute diarrhoeal diseases (Pushpha and Mahabirsingh, 1987). Transmission of *E.coli* from man to man, from animal to man and from animal to animal have been reported by Linton (1986).

A wide variety of aerobic and anaerobic bacteria form a stable and complex ecosystem in the human gastrointestinal tract (Moore and Holdeman, 1974; Simon and Gorbach, 1986). In the present study, the *E.coil* isolates (clinical) were collected from diarrhoeal patients.

Out of the 22 isolates, 16 were found to be resistant and 6, sensitive isolates. Among the 16 resistant isolates only 10 were used for the RFLP, PCR and Antibiotic Sensitivity studies. The diarrhoeal patients were observed for twenty four hours variations in their body temperature. The pyrogenic activity of *E.coli* was further confirmed by mice inoculation.

This study is similar to two earlier studies on *E.coli* isolates from diseased chickens and on *E.coli* isolates from diarroheal faecal of cow and buffalow calves done by Raheem et al.(1986) and Gulati et al. (1993), respectively. In the former study, the isolates have shown to be sensitive to streptromycin, erythromycin and to trimethoprim/sulphamethoxazole. In the latter study, the isolates were resistant to ampicillin, chloramphenical, co-trimaxazole, gentamicin, kanamicin, nalidixic acid, neomycin, tetracycline and streptomycin.

Gopal Reddy et al .(1990) had reported that Klebsiella species and *E.coli* were more drug resistant than other bacteria. These two organisms were found to be resistant to ampicillin, streptomycin, tetracycline and chloramphenicol. These drug resistant bacteria were also examined for their susceptibility to

curing agents. It was to find out if the plasmids (drug resistant markers) could be eliminated by the agents. Likewise, in the present study, multiple drug resistant *E.coli* pathogens were examined for their susceptibility to curing agents. The plasmids (drug resistant markers) were eliminated by acridine orange treatment. This suggests that *E.coli* isolates, are resistant to selected antibiotics and that the mediating plasmids could be eliminated through curing off process to deprive resistance.

E.coli has been employed, extensively, to study not only microbial genetics but also the genetical basis of drug resistance. Earlier studies have proved that ,genes for resistance were frequently carried by transmissible plasmids although plasmids without such genes may be carried by sensitive strains (platt et al., 1984). Transmissible plasmids may carry genes for resistance (R) to antibiotics (walton, 1977). Similar earlier studies substantiate and support the current work wherein the prevalence of resistance in 1-16 isolates could be attributed to these Resistance factors (R-factors) which are carried by mediating plasmids. Tests such as Restriction Fragment Length Polymorphism (RFLP) and Polymerase Chain Reaction (PCR) confirm mediation. The present study highlights that the difference between the restricted fragments and the products of Polymerase Chain Reaction (PCR) could give the amplification of these restricted fragments.

Plasmid analysis is an important tool in epidemiological studies (Tietze and Tschape, 1983) . *E.coli* isolates, collected from farm animals suffering from diarrhoea, have been characterized (Wise et al., 1985). Earlier, Hartley et al. (1977) had reported that isolating resistant strains of *E.coli* from adult humans without the aid of selective media (plasmid), was rare. There are examples of antimicrobial agents that can eliminate plasmids. Acridines are more effective against Gram positive than against Gram negative bacteria (Foster and Russell, 1971). Acridines have also been found to eliminate some, and not all plasmids (Howe, 1982). This agrees with the observations made in the present study during the electrophonogram technique when out of the 16 resistant isolates, 10 isolates plasmids remained distinct (seen as bands). Plasmid mediation, in the present study, confirms that plasmids are the carriers of R-factors. Plasmid curing and exposure of the cured, sensitive isolates to selected antibiotics further confirmed that plasmids do mediate resistance.

A variety of effective antibiotics are now available. But a particular antibiotic may become ineffective by prolonged or improper or insufficient intake. Indiscriminate use of antibiotic drug (s) is also responsible for the high incidence of resistance exerted by bacteria. Much of the bacterial resistance is mediated by R-factor plasmids. This results in the rapid spread of resistance, among various bacteria, in the environment. It has been observed that resistance, generally, to antibiotics is common in Gram negative bacteria (Gopal Reddy et al., 1990).

High frequency of antibiotic resistance was found in environments where the antibiotic concentrations were high e.g. hospitals and farms rearing livestock intensively (Bale et al., 1987). Plasmid mediated sulphonamide - resistant dihydropteroate synthase and trimethoprim - resistant dihydrofolate reductase enzymes can cause sulphonamide and trimethoprim resistance (Ubukata et al., 1985). This implicates that only certain isolates (as in the present study) of *E.coli* , could be resistant to trimethoprim / sulphamethoxazole and to other selected antibiotics. In the present study only 16 out of the 22 isolates are found to be resistant. They resist only specific antibiotics (e.g. Chloramphenicol, Gentamicin, Nalidixic acid and Nitrofurantoin).

Most antibiotic resistance genes that can be identified, either as DNA sequences or as their protein products, are found on a wide variety of unrelated plasmids. These sequences are, often, components of bacterial transposons (Wiedemann et al., 1986; Levy and Novick, 1986).

It is stated that R plasmids consist of a Resistance Transfer Factor (RTF) linked to a resistance determinant (r-det). The reason is historical (Novick and Ribbons, 1969; Helinski, 1973). The earliest studies on R plasmids had involved members of one incompatibility group. In the light of more diverse studies, it could be seen that the description is more specific than general. But many R plasmids have structures that do not conform to this simple compartmentalized scheme (Gorai et al., 1979; Villarroet et al., 1983).

E.coli bacteria with antibiotic resistance factors have an advantage over antibiotic sensitive forms, in the natural environment. When sufficient number of such resistant bacteria are injested in the gut they can transfer their resistance factor to the intestinal flora (Hatha et al., 1993). The resistance may persist for a variable duration of time, after exposure to the antimicrobial agent (Hinton et al., 1986).

Multidrug resistance may result from selection of resistant mutants, through the indiscriminate and widespread use of antibiotics (Onyemelukwe and Njoku - Obi, 1992). Multiple drug resistance of *E.coli* , in Bhavani river region, was studied by Hatha et al. (1993). 107 *E.coli* isolates were obtained from the water sediment and the fish from the Bhavani river (all of which were considered to be the potential cause of human enteric disease). 62% of the isolates were resistant to more than 4 antibiotics. The levels of resistance to bacitracin, penicillin and novobiocin were generally high whereas resistance to polymyxin B and to chloramphenical was low. A high incidence of multiple antibiotic resistance or multiple drug resistance index of the strains showed that 95% of the strains had originated either from man or from cattle (Hatha et al., 1993). From the above findings and from the observations in the present study, it is clear that if the resistant bacteria survive in the intestinal environment then, they can also transfer their resistance factor to the non- resistant and non-sensitive isolates.

The foregoing discussion makes clear that the multiple drug resistance pattern, observable in the isolates, was mediated by plasmids. The curing off treatment and the antibiotic sensitivity test carried out confirmed that plasmids mediate resistance. Restriction fragment length polymorphism and Polymerase chain reaction tests, conducted in the present study, are an added confirmation of mediation of plasmids in the resistant isolates.

During this investigation, fluctuations in body temperature of the patients was also noticed. It was found that in diarrhoeal human patients, the temperature increased from 98.2^0 F to 100.1^0 F. This increase could be attributed to *E.coli* since when pyrogen was administerated to mice, then there was an increase in their body temperature. Small doses of pyrogen, usually when administer to human beings develops fever within 15 minutes. Pyrogen acts on granulocytes, monocytes and the tissue cells which release small molecular protein. This passes along the blood stream and acts upon the thermoregulatory center in the hypothalamus and raises the temperature base line. This is further supported by the current observations, that in diarrhoeal human beings (experimental group) the rhythmicity of body temperature was disturbed. They showed a higher body temperature than non diarrhoeal patients, proving the impact of *E.coli* (the guest organism) in the body. Thus temperature which affects the chemical reaction rates profoundly gets affected upon the impact of the *E.coli* organisms. And most organic responses involve chemical transactions . Evidently, temperature is a major parameter in virtually all biological activities of the human sub systems. Further, there exists between the self

sustaining chemical systems and the environment an elaborately and precisely regulated exchanges of energy and materials.

Unlike other Coliforms, pathogenic *E.coli* bacteria habitate and thrive only in the intestinal environment of human beings and animals. Discharged in motion and in urine, they remain viable for only some days but cause infectious diseases by polluting water and food stuffs. Therefore, mediating plasmids in isolates may be acquired from drinking water (natural environment), and food stuffs. However, these *E.coli* bacteria, transferred by plasmid mediation in the selected *E.coli* isolates, could be destroyed i.e., killed by certain antibiotics. Of course, the present study establishes that the selection of the antibiotic to be administered depends on the mediating plasmids' resistance or sensitivity.

SUMMARY
&
CONCLUSION

CHAPTER - VI

SUMMARY AND CONCLUSION

The current work entitled "studies on the antibiotic esistance pattern and the impact on human body temperature of *E.coli* " is based on the following premises:

1. Possibility of isolating the resistant and the sensitive isolates among the disease causing *E.coli* bacteria.
2. Identification of the pattern of resistance or of sensitivity in the pathogenic bacteria.
3. Analysing factors that enable the survival of the resistant isolates in the intestinal environment.

The researcher's concern was to study if plasmids mediate resistance and to identify *E.coli* bacteria with multiple antibiotic resistance.

The following steps have been adopted:

1. Observations on body temperature and certain biochemical tests for the diarrhoeal patients; finding out the impact of *E.coli* on the host body temperature
2. Collection of pathogenic *E.coli* clinical isolates from various diarrhoeal samples (Human host)
3. Confirming the pyrogenic activity of *E.coli* in the human host by animal inoculation

4. Characterization of the collected isolates by observing their biochemical reactions
5. Testing the pathogenic isolates for the prevalence of multiple antibiotic resistance or antibiotic sensitivity
6. Subjecting the sensitive isolates to plasmid analysis
7. Subjecting the resistant isolates to plasmid analysis
8. Analysing plasmids for RFLP and PCR studies
9. Removing and testing the plasmids for sensitivity to selected antibiotics

This study also highlights the disadvantages of the improper and inadequate use of antibiotics and how it increases the chances for the survival of resistant strains of *E.coli* pathogens. However, it could be inferred that the resistant strains can be destroyed by the simultaneous administeration of two or more antibiotic drugs. The conclusion of the study is that if the strain is resistant to one of the antibiotics given it could be destroyed by the other. The significance of this study is thus attesting the probability that the pathogenic organism will acquire resistance to two drugs which is roughly the product of the two individual probabilities.

Use of one and the same antibiotic for both human beings and farm animals is declared illegal in European countries. This is a sensible policy for the following reasons:

1. There are known instances in which R-factor-carrying pathogenic bacteria, spread from farm animals to human beings.
2. There is correlation between the widespread use of an antibiotic and the prevalence of R factors mediating the

resistance to that antibiotic. The fact that bacterial infections could be counteracted by antibiotics remains undisputed. Antibiotic drugs have changed drastically, the ecological balance between human beings and the various pathogenic microorganisms. Yet, the introduction of every new antibiotic into clinical practice has been challenged by the emergence of a newer resistant organism. The organisms often harboured R - plasmids that mediated resistance. Sometimes, the resistant strains were also opportunistic. Plasmid mediated drug resistance plays an important part in the acquired resistance of Gram negative organisms like *E.coli* pathogenic bacteria.

OUTCOME OF THIS WORK

1. *E.coli* is a pyrogen capable of increasing the body temperature.

2. By removal of R-plasmids, the pathogenic *E.coli* which are surviving in the environment could be destroyed. These plasmids often confer simultaneous resistance to several antibiotics. They carry genes that encode catalytic enzymes, effecting chemical modifications of antibiotics and converting them to derivatives without antibiotic actions. All these chemical modifications result in the loss of antibiotic activity.

3. Antibiotic resistance studies will give more information about the usage of drugs and their antimicrobial activity. Through this study, one could gain a clear idea about the

resistance or sensitivity pattern of pathogens prevalent in the environment.

4. The current study with an added dimension of social relevance will help in monitoring the resistant genes, the potential source for all the plasmid borne resistance in the environment.

RECOMMENDATIONS

1. Human health and environmental condition are two important aspects, requiring much attention in this millennium. Prevalence of R- plasmids is a widespread phenomenon.
2. Resistant organisms are increasingly responsible for human diseases of the intestinal tract, lungs, skin and urinary tract.
3. Microorganisms may acquire resistance to antibiotics in a number of ways. In some cases resistance arises from the ability of the microorganisms to destroy the antibiotic. Moreover, drug resistance may be due to an altered metabolic pathway in the microorganisms, a pathway that bypasses the reaction normally inhibited by the drug.

4. Antibiotic abuse favours the emergence of resistant forms.
   a. Pharmaceutical companies promote antibiotics heavily in commercial interest.
   b. Patients pressurise doctors for quick recovery.
   c. Improper diagnostic procedures followed by medical professionals. Poor diagnosis in clinics .

d. Growing trend, among educated people, of self – medication.

e. People may diagnose their own illness and take left over antibiotics from their medicine chests for ailments where antibiotics are useless.

5. Hospitals are another forcing ground for the emergence of resistant bacteria – lack of cleanliness owing to poor maintenance; compelling patients to buy prescriptions only from the drug store within the hospital campus etc.,

   a. In many cases, physicians use unnecessarily large doses of antibiotics to prevent infection during and following surgery. It is not uncommon to see persons in queue holding a long prescription list before the dispensary.

   b. This increases the possibility of resistant strains over susceptible strains and subsequently spreading contamination.

   c. Moreover, the problem of antibiotic abuse is widespread in livestock feeds. In poultry, dairy farms and in cattle breeding and rearing centres.

6. A major problem is the development of antibiotic resistant strains of microorganisms arising from any one of several sources including changes in microbial biochemistry. Antibiotic resistance threatens to put an end to the cures available for infectious disease.

7. Multiple resistant bacterial strains almost ,always ,owe their resistance property to the presence of an R factor. Plasmids have a wide range of hosts that are readily transferable between different bacterial genera.

8. R factor increasing dissemination in natural bacterial populations is the most serious aspect of the problem of microbial resistance to antibiotics.

9. Unless some solution to this problem is found, the future therapeutic effectiveness of antibiotics is in jeopardy.

10. A chemotherapeutic substance should :
    (i). Ideally be inhibitory to the microorganisms in very low doses.
    (ii). Compatible with mammalian tissues (of humans and animals) producing least side effects.
    (iii). when administered orally (tablets, capsules, liquids) or parentarally (injections, drips) should reach the sites (receptor sites) where the microorganisms are lodged, and leave the system slowly (24 – 48 hours) through the excretory systems.

11. It should also be economical for both the product company and the consumer.

12. Because of these limitations, only a small proportion of the thousands of antimicrobial substances discovered have come into clinical use.

SCOPE AND SUGGESTIONS FOR FURTHER RESEARCH

1. Research on sequencing the drug resistant genes could be undertaken for more information regarding drug resistance.

2. Research may be undertaken to evaluate the exact concentration of the divalent cations that enhance recombination in natural media. If this could be studied, the treatment of patient with pain killers will become easy preventing the pathogen to undergo genetic recombination.

3. Research may be carried out in gene mapping.

4. Research may be undertaken in protein engineering.

5. Intensive research may be carried out in the inhibition of transposon action by requinomycin. By finding out the chemical agents that inhibit the recombination may lead to novel advances in chemotherapy.

6. Progressive research on the possibility of terminating the drug resistant pathogens, in the human host environment and thereby preventing disease transmission through gene transfer or otherwise would be an applauding service to human beings in the new millennium years ahead.

7. **It would be a dream coming true, the richest and the best contribution to UNESCO'S concern over World Health, if the field scientists bestow maximum attention in this area of Microbiological studies involving Environmental factors.**

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APPENDIX

APPENDIX

1. NUTRIENT AGAR

Peptone	-	5.0 gm
Sodium chloride	-	5.0 gm
Beef extract	-	3.0 gm
Yeast extract	-	3.0 gm
Agar	-	15.0 gm
Distilled water	-	1000.0 ml
pH	-	7.0

2. BLOOD AGAR

Infusion from beef heart	-	500.0 gm
Tryptose	-	10.0 gm
Sodium chloride	-	5.0 gm
Agar	-	15.0 gm
Distilled water	-	1000.0 ml
pH	-	7.3

Dissolved the above ingredients in distilled water and autoclaved at 121° for 15 minutes. Cooled to 45°C to 60°C and 50 ml of sterile blood was added aseptically Rotated to mix thoroughly avoiding accumulation of airbubbles and poured immediately into sterile plates and the plates were allowed to solidify.

3. MACCONKEY AGAR

Peptone	-	20.0 gm
Sodium chloride	-	5.0 gm
Bile salts	-	1.5 gm
Neutral red solution (0.1 % aqueous)	-	10.0 ml
Crystal violet	-	0.001 gm
Agar	-	13.5 gm
Distilled water	-	1000.0 ml

pH - 7.1

4. INDOLE TEST

This test was done to determine the ability of microorganisms to degrade the amino acid tryptophan. Tryptophan is an essential amino acid that can undergo oxidation by way of the enzymatic activities of some bacteria. Conversion of tryptophan into metobolic products is mediated by the enzyme tryptophanase. In this test, tryptone broth was used which contains the substrate tryptophan. The presence of indole was detected by adding kovac's reagent, which gives a red ring at top layer. ***E.coli*** and Proteus gives positive result.

TRYPTONE BROTH

Tryptone		-	10.0 gm
Sodium chloride		-	5.0 gm
Calcium chloride	(1M)	-	1.0 ml
Distilled water		-	1000.0 ml

Previously sterilized Calcium Chloride is to be added after autoclaving the medium.

KOVAC'S REAGENT

p-Dimethyl amino benzaldehyde	-	5.0 gm
Amylalcohol	-	75.0 ml
Hydrochloric acid (Concentrated)	-	25.0 ml

NOTE : p-Dimethyl amino benzaldehyde was dissolved in amylalcohol and then hydrochloric acid was added.

5. METHYL RED TEST

The Hexose monosaccharide Glucose is the major substrate oxidized by all enteric organisms for energy production. The end products of this process will vary depending on the specific enzymatic pathway present in the bacteria. In this test the P^H indicator was methyl red. This indicator in the P^H range of 4 will turn red. The MR-VP broth was inoculated with suspected culture. After incubation at 37°C for 24 to 48 hours, methyl red indicator was added. Positive reaction was indicated by persistant red colour. This test gives positive to ***E.coli*** , Proteus and Staphylococcus aureus.

MR-VP MEDIUM

Peptone	-	7.0 gm
Dextrose	-	5.0 gm
Potassium phosphate	-	5.0 gm
Distilled water	-	1000.0 ml
pH	-	6.9

METHYL RED INDICATOR

Methyl red	-	0.1 gm
Ethyl alcohol	-	300.0 ml
Distilled water	-	200.0 ml

NOTE : Methyl red was dissolved in 95 % ethyl alcohol and was furthe diluted to 500 ml with distilled water.

6. VOGES-PROSKAUER TEST

The Voges-Proskauer test was used to determine the capability of organism to produce non-acidic or neutral end products, such as acetyl methyl carbinol, from the organic acids that result from glucose metabolism.

Development of a deep rose colour in the culture, following the addition o Barrit's reagent A and B is indicated by the presence of acetyl methyl carbinol and represent a positive result. The absence of rose coloration is a negative result. I gives positive to Klebsiella & Staphylococcus aureus.

BARRIT'S REAGENT - For detection of acetyl methyl carbinol.

SOLUTION A

Alpha napthol	-	5.0 gm
Ethanol, Absolute	-	95.0 ml

NOTE : Alpha napthol was dissolved in the ethanol with constant stirring.

SOLUTION B

Potassium hydroxide	-	40.0 gm
Creatin	-	0.3 gm
Distilled water	-	100.0 ml

NOTE : Potassium hydroxide was dissolved in 75 ml of distilled water. Th resultant warm solution was allowed to cool at room temperature. Creatin was adde and stirred to dissolve. Remaining water was added and stored in a refrigerator.

7. CITRATE UTILIZATION TEST

This test was performed to differentiate among enteric organisms on th basis of the ability to ferment citrate as a sole carbon source.

In the absence of fermentable glucose or lactose, some microorganisms a capable of using citrate as a carbon source for their energy. While utilization, th citrate is enzymatically converted to pyruvic acid and carbon-dioxide that was generate combines with sodium and water to form sodium carbonate, an alkaline product. Th presence of sodium carbonate changes the bromothymol blue indicator incorporate into the medium from green to deep prussian blue.

Following incubation, citrate positive culture was identified by the presenc of growth on the surface of the slant, which was accompanied by blue coloration. Citra negative culture will show no growth and the medium will remain green. The citra tubes, inoculated with suspected culture gives positive for Klebsiella, Proteus ar Pseudomonas.

SIMMON'S CITRATE AGAR

Ammonium dihydrogen phosphate	-	1.0 gm
Sodium chloride	-	1.0 gm
Sodium Citrate	-	2.0 gm
Magnesium sulphate	-	0.2 gm
Bromothymol blue	-	0.08 gm
Agar	-	15.0 gm
Distilled water	-	1000.0 ml
pH	-	6.9

8. TRIPLE SUGAR IRON AGAR TEST

Triple sugar iron (TSI) agar test is designed to differentiate among the diffe ent groups or genera of the Enterobacteriaceae, which are all Gram negative baci capable of fermenting glucose with the production of acid, and to distinguish th Enterobacteriaceae, from other Gram negative intestinal bacilli. This differentiation made on the basis of differences in carbohydrate fermentation patterns and hydroge sulphide production by the various groups of intestinal organisms.

TSI slants contain glucose, lactose and sucrose . The acid-base indicato phenol red is also incorporated to detect carbohydrate fermentation that is indicated b a change in the color of the medium from orange red to yellow in the presence of acids The slant is inoculated by means of a stab and streak procedure. This requires th insertion of a sterile, straight needle from the base of the slant into butt. Upon with drawing of the needle, the slanted surface of the medium is streaked. Following incu bation results are determined by the fermentative activities of the organisms.

a). Alkaline slant(red) and acid butt (yellow) with or without gas production (breaks in the sugar butt).

Only glucose fermentation has occured. The organisms preferentially degrade glucose first. Since this substrate is present in minimal concentration, the small amount of acid produced on the slant surface is oxidized rapidly. The peptone in the medium is also used in the production of alkali. In the butt, the acid reaction is maintained because of reduced oxygen tension and slower growth of the organisms.

b). Acid slant (yellow) and Acid butt (yellow) with or without gas production.

Lactose and/or Sucrose fermentation has occured. Since these substances are present in higher concentrations, they serve as substrates for continued fermentative activities with maintainence of an acid reaction in both slant and butt.

c). Alkaline slant (red) and Alkaline butt(red) or No change (Orange red) butt.

No carbohydrate fermentation has occured. Instead peptones are catabolized and anaerobic and/or aerobic conditions, resulting in an alkaline PH due to production of ammonia. If only aerobic degradation of peptones occurs, the alkaline reaction is evidenced only on the slant surface. If there is aerobic and anaerobic utilization of peptone, the alkaline reaction is evidenced only on the slant surface. If there is aerobic and anaerobic utilization of peptone, the alkaline reaction is present on the slant and the TSI agar medium contains sodium thiosulphate, a substrate for hydrogen sulfide (H_2S) production, and ferrous sulphate for detection of this colorless end product. Following incubation only, cultures of organisms capable of producing hydrogen sulfide will show an extensive blackening in the butt because of the precipitation of the insoluble ferrous sulfide.

TRIPLE SUGAR IRON AGAR

Peptone	-	15.0 gm
Protease Peptone	-	5.0 gm
Beef extract	-	3.0 gm
Yeast extract	-	3.0 gm
Lactose	-	10.0 gm
Sucrose	-	10.0 gm
Dextrose	-	1.0 gm
Sodium chloride	-	5.0 gm
Ferrous sulphate	-	0.2 gm
Sodium thiosulphate	-	0.3 gm
Phenol red	-	0.024 gm
Agar	-	10.0 gm
Distilled water	-	1000.0 ml

9. CATALASE TEST

During aerobic respiration, microorganisms produce hydrogen peroxide and in some cases, an extremely toxic superoxide. Accumulation of these substances will result in death of the organism unless they can be enzymatically degraded. These substances are produced when aerobes, facultative anaerobes, and microaerophiles use the aerobic respiratory pathway in which oxygen is the final electron acceptor, during degradation of carbohydrates for energy production. Organisms capable of producing catalase or peroxidase rapidly degrade hydrogen peroxide.

Superoxide dismutase is the enzyme responsible for degradation of the especially toxic superoxide in catalase-negative aerobic organisms.

Catalase production can be determined by adding hydrogen peroxide to an appropriately inoculated nutrient broth cultures. If catalase present the chemical reaction is indicated by bubbles of free oxygen gas, this is positive for catalase test. The absence of bubble formation is a negative test.

NUTRIENT BROTH

Peptone	-	5.0 gm
Sodium chloride	-	5.0 gm
Beef extract	-	3.0 gm
Yeast extract	-	3.0 gm
pH	-	7.0

HYDROGEN PEROXIDE 3%

For detection of catalase activity.

NOTE : Refrigerate when not in use.

10. UREASE TEST

Urea is a major organic waste product of protein digestion in most vertebrates and is excreted in the urine. Some microorganisms have the ability to produce the enzyme urease. The urease is a hydrolytic enzyme which attacks the carbon and nitrogen bond amide compounds (example : urea) with the liberation of ammonia.

It is a useful diagnostic test for identifying bacteria, especially to distinguish members of the genus proteus from other Non Lactose Fermenting (NLF) enteric microorganisms. Proteus vulgaris is an important and fast producer of urease.

Urease test is performed by growing the test organisms on urea agar medium containing the pH indicator phenol red (pH 6.9). During incubation, microorganisms possessing urease will produce ammonia that raises the pH of the medium (pH 8.4) changing the color of phenol red indicator, brown red to deep ink.

Failure of the development of a deep pink color due to no ammonia production is evidence of lack of urease production by the microorganisms. This test shows positive for Klebsiella, Proteus and Staphylococcus aureus.

11. UREA AGAR MEDIUM

Peptone	-	1.0 gm
Sodium chloride	-	5.0 gm
Potassium monohydrogen Or Dihydrogen phosphate	-	2.0 gm
Glucose	-	1.0 gm
Phenol red (o.o2% solution)	-	6.0 ml
Urea 20% (aqueous solution)	-	100.0 ml
Distilled water	-	1000.0 ml
pH	-	6.8

All the ingredients except glucose, Phenol red and urea was dissolved in distilled water. Autoclaved at 15 lb for 15 minutes and allowed to cool to 50° C.

Glucose and Phenol red was added to the molten base and steamed for 1 hour and cooled to 50° C. 100 ml Urea (filter sterilized solution) was added to the basal medium.

All the constituents were mixed well and dispensed into sterile culture tubes and allowed to solidify in a slanting position to form slants.

12. MOTILITY TEST

The gram negative cultures were inoculated in the motility medium which contains0.5% agar. The motile positive cultures had turbid growth in the medium. While non motile culture was grown along the line of inoculation.

MOTILITY MEDIUM

Peptone	-	10.0 gm
Sodium chloride	-	5.0 gm
Agar	-	5.0 gm
Distilled water	-	100.0 ml

13. MULLER HINTON AGAR

Beef infusion	-	300.0 gm
Casaminoacids	-	17.5 gm
Starch	-	1.5 gm
Agar	-	17.0 gm
Distilled water	-	1000.0 ml
p^H	-	7.4

14. EOSIN METHYLENE BLUE AGAR (EMB)

To isolate and differentiate the Gram negative enteric bacteria from clinical and non clinical specimens.

Principle

EMB media contains peptic digest of animal tissue and phosphate, methylene blue and eosin –Y inhibit Gram positive bacteria to a limited degree. These dyes serve as differential indicators in response to the fermentation of carbohydrates. Sucrose was added in the medium as an alternate carbohydrate source for lactose fermenting, Gram negative bacilli. The Coliforms produce purplish black colonies due to taking up of methylene blue – eosin dye complex, when the pH drops.

EMB Media

Peptone	-	10g
Dipotassium phosphate	-	2g
Lactose	-	5g
Sucrose	-	5g
Eosin – Y	-	0.4g
Methylene blue	-	0.065g
Agar	-	13.5g

15. STAINS AND REAGENTS FOR GRAM STAINING

Crystal violet (Huchlers)

Solution – A

1. Crystal violet - 2g
2. 95 % ethanol - 20ml

Solution – B

1. Ammonium oxalate - 0.8g
2. Distilled water - 80ml

Mix solution A & B

Gram's Iodine

1. Iodine - 1g
2. Potassium Iodide - 2g
3. Distilled water - 300ml

95 % ethanol

16. BIOCHEMICAL TESTS

a. Blood Pressure

Blood pressure was always measured in (mm Hg)
because the mercury manometer has been used as the standard reference for measuring blood pressure throughout the history of physiology and as the systolic and diastolic fluctuations occur too
rapidly to be registered accurately by a mercury manometer.

b. Estimation of Glucose
(GOD/POD method)

Principle

Glucose is oxidised by the enzyme glucose oxidase (GOD) to give D-gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of enzyme peroxidase (POD) oxidises phenol which combines with 4-aminoantipyrine to produce a red colored quinoneimine dye. The intensity of the color developed is proportional to glucose concentration in the sample.

$$\text{D - Glucose} + H_2O + O_2 \xrightarrow{\text{GOD}} \text{D - gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{4 - Aminoantiphrine} + \text{phenol} \xrightarrow{\text{POD}} \text{Red Quinoneiminedye} + H_2O$$

C. Estimation of serum cholesterol Total
(Wybenga Pileggi Method)

Principle

Both free and esterefied cholesterol reacts with hot ethyl acetate and sulphuric acid in the presence of ferric iron to give a purple coloured complex.This is measured at 520 - 540 nm (Green filter).

d. Estimation of Haemoglobin
(cyahmethemoglobin method)

Principle

The blood haemoglobin is treated with Drabkin's solution. Ferricyanide from Drabkin's solution converts haemoglobin to methamoglobin which is further converted to cyanmethemoglobin by the action of cyanide. The cyanmethemoglobin has an absorbance which is proportional to the haemoglobin concentration.

e. Estimation of Total Protein and Albumin
(Biuret and BCGDYE binding method)

Principle

Proteins bound with copper inos in an alkaline medium of the Biruret Reagent and produce a purple coloured complex; it's absorbance is proportional to the protein concentration. Albumin in buffered medium binds with Bromoresol green (BCG) and produces a green color whose absorbance is proportional to the Albumin concentration.

Globulins gm% was estimated by
Total Proteins in gm% - Albumin in gm%

f. Estimation of Bilirubin
(Modified Jendrassik & Grof Method)

Principle

Bilirubin reacts with diazotized sulfanilic acid in acidic medium to form azobilirubin, a purple coloured complex substance whose absorbance is proportional to bilirubin concentration. Direct bilirubin being water soluble is allowed to react with diazotized sulfanilic acid in the absence of an activator, while for total bilirubin, (Direct&indirect) the diazotization is carried out in the presence of an activator.

g. Estimation of (ASAT) Aspartate Amino Transferase (SGOT) (DNPH method)

Principle

Aspartate aminotransferase catalyses the following reaction.

L - Aspartate + OXO- 2. glutarate $\xrightarrow{\text{GOT}}$ Oxaloacetate + L-glutamate

GOT activity is shown by the quantity of oxaloacetate formed. This keto acid may be assayedby the formation of 2, 4 - dinitrophenyl hydrazone which yields a brown color in alkaline medium. The indensity of this color is a function of the quantity of oxalo acetate formed, and thus the catalytic activity of aspatate aminotransferase.

h. Estimation of (ALAT) Alanine Amino Transferase (SGPT) (DNPH Method)

Principle

Alanine aminotransferase catalyses the following reaction.

$$\text{L-alanine + OXO - 2- glutarate} \xrightarrow{\text{GPT}} \text{Pyruvate + L- glutamate}$$

The GPT activity controls the quantity of pyruvate formed. This keto acid may be assayed by the formation of 2, 4 dinitrophenyl hydroazone which yields a brown colour in alkaline medium. The intensity of that colour is a function of the quantity of pyruvate formed and thus the catalytic activity of alanine aminotransferase.

i. Estimation of Alkaline Phosphatase (ALP)
(kind and kings Method)

Serum ALP hydrolyzes phenyl phosphate into phenol and disodium hydrogen phosphate at p^H 10.0. The phenol so formed reacts with 4 - aminoantipyrine is alkaline medium in presence of oxidising agent potassium ferricyanide to form red coloured complex whose absorbance is proportional to the enzyme activity.

ALP

Disodium phenyl + H_2O -----> Phenol + Disodium
phosphate p^H 10.0 hydrogen
phenol phosphate
+ Alkaline Medium
Amino- ----------> Red coloured antipyrine
Potassium ferricyanide complex

j. Estimation of Urea in Urine
(DAM Method)

Principle

Urea reacts with DAM in an actidic medium to produce a colored complex. The color is intesified by using thiosemicarbazide and a cadmium salt. The intensity of the colour produced is proportional to urea concentration.

k. Estimation of Creatinine in Urine
(Alkaline Picrate Method)

Principle

In alkaline medium picric acid reacts with creatinine and produces a red colored complex, whose absorbance is proportional to creatinine concentration. Pricric acid reagent has dual role, as a deproteinizing agent and as a reactant.