SEWAGE ANALYSES FOR ANTIBIOTIC RESISTANCE WITHIN FECAL *E. COLI* ISOLATES

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ABSTRACT

This investigation was undertaken to explore possible surveillance methods which might be applied in surveys of the incidence of acquired antibiotic resistance in fecal bacteria being shed by an urban population; the Palmerston North City sewage system served as a sampling device. Fecal *E. coli* was used as an indicator organism by virtue of its inherent sensitivity to several relevant antibiotics and, further, by virtue of the fact that antibiotic resistance in this microorganism can, in general, be attributed to plasmids coding for the resistance character(s).

In the course of these exploratory studies it was observed that fecal *E. coli* accounted for 6 to 14% of the total coliforms present in sewage samples; the number of fecal *E. coli* in any given sewage sample was affected by the flow rate of the sewage and the rainfall.

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INTRODUCTION

Microbial resistance has been described as the ability of a microbial cell and its progeny to survive and multiply under environmental conditions that would inhibit or destroy other organisms (Tepper, 1969). Recognised to be present in nature long before the introduction of antibiotics in chemotherapy, bacterial resistance to drugs have received increasing attention as the list of resistant organisms has continued to expand ahead of the rapid development of newer antibiotics (Takafuji, 1977).

1.1 DEVELOPMENT OF DRUG RESISTANCE

Resistance to drugs e.g. to arsenicals by trypanosomes, was known for many years, but it was not until 1935 that bacterial resistance was recognised as a growing problem. In the 1930s, *Neisseria gonorrhoea* infections were adequately treated with sulphonamides, but by the late 1940s greater than 80% of the strains were resistant to 100 mg% or more (Takafuji, 1977). Other organisms such as *Strep pyogenes*, *N. meningitidis* and members of the *Enterobacteriaceae* also developed similar resistance to sulphonamides, rendering a once very satisfactory antibacterial drug ineffective in the treatment of serious infections.

Presently, the factors contributing to the emergence and spread of antibiotic resistance include the following: -

a) Antibiotic use in man

The appearance of antibiotic-resistant strains of bacteria is closely linked to antibiotic use in the treatment of human infections (Mouton et al, 1976). Resistance may appear rapidly or slowly, depending on the organism concerned, the volume and type of antibiotic used, and the method of application.

The most clear-cut evidence relating antibiotic use and resistance has come from hospital studies in which outbreaks of resistant nosocomial infections were related to the extensive use of antibiotics, e.g. infections with *Klebsiella* related to the use of ampicillin in a neurosurgical unit (Price and Sleigh, 1970), infections with pseudomonads resistant to carbenicillin in a burns unit (Lowbury et al, 1972), and resistant infections with *Serratia* associated with the use of gentamicin in an intensive care unit.

In community infections, data from Japan showed that a rise in tetracycline resistance in *pneumococci* was closely associated with an increase in tetracycline use. Again, there was a sharp increase in macrolide use in Japan from 1967 onwards, the quantities used had risen from 50,000 kgs/year to almost 200,000 kgs/year by 1973. Macrolide-resistant strains of group A hemolytic *streptococci* were first recognised in Japan during the early 1970s and, by 1974, 75% of the strains isolated were resistant to erythromycin and linconcycin. Resistance to the tetracyclines and that to chloramphenicol were at a level of 90% and 75% respectively (WHO Technical Report, 1978).

b) Antibiotic use in animals

The use of antibiotics as feed additives for growth promotion is widespread (Linton, 1977; Hartley & Richmond, 1975). The commonest drugs currently used for this purpose are the tetracyclines - one of the most potent agents for provoking the emergence and selection of resistance plasmids and at the same time a very useful therapeutic agent.

The rapid emergence and spread of drug-resistant *Salmonellae*, during the 1960s resulted from antibiotic use in animals (Anderson, 1968). Transmission of these *Salmonellae* to man resulted in many human infections, and

the resistances of such strains, plasmid borne and chromosomal, were acquired in the animal host.

c) The role of food in the spread of antibiotic-resistant bacteria

The presence of antibiotic-resistant nonpathogenic enterobacteria in food is of public health significance. Although some food containing resistant bacteria is decontaminated during cooking, before being cooked it may contaminate other cooked or uncooked food in the kitchen and so transmit drug-resistant entero bacteria to man. Multiresistant nonpathogenic bacteria may add to the number of drug-resistant bacteria, with their plasmids, in the human intestine, but when the organisms concerned are pathogenic they may also produce foodborne diseases (WHO Technical Report, 1976).

d) The role of sewage and surface waters

Sewage and surface waters contribute to the distribution and circulation of resistant organisms. They represent a natural medium in which R-plasmid transfer can occur under certain physical, chemical or biological conditions. Sewage and surface waters contain resistant bacteria from human and animal wastes and can be regarded as a source of all plasmid types, which circulate and are selected under appropriate environmental conditions. These resistant bacteria from sewage and surface waters can be transferred under some circumstances to food and drinking water, which leads to a recycling to man and animals (Smith, 1970; Richmond, 1972).

1.2 DISCOVERY OF R FACTORS

At the end of World War II, various derivatives of sulphonamides were introduced in Japan for the treatment of dysentery and proved to be very effective for the first several years. After about 1949, the incidence of dysentery again increased, despite extensive use of sulphonamide, and sulphonamide-resistant *Shigella* strains appeared rapidly and reached a maximum of 80-90% (Mitsuhashi et al, 1969, 1976; Watanabe, 1963). Subsequently, newer antibiotics such as streptomycin, chloramphenicol and tetracycline were employed for the treatment of sulphonamide-resistant *Shigella*, with initially excellent therapeutic effects. With the increase of antibiotic usage, antibiotic resistant *Shigella* strains appeared, but the isolation frequency was still low and singly chloramphenicol-resistant strains were never isolated. The first isolation of a multiple-resistant *Shigella* strain from a dysenteric patient occurred in 1952, this multiple resistance involving tetracycline, streptomycin and sulphonamide (Mitsuhashi, 1971). In 1955, a quadruple-resistant *Shigella* strain, was isolated by Kitamoto and coworkers, from a woman afflected with bacillary dysentery. This strain was resistant to sulphonamides, streptomycin, chloramphenicol and tetracycline. Since 1956, more and more multi-resistant *Shigella* strains have been isolated in Japan and the frequency of such strains among the total isolates of *Shigella* in Japan in 1967 was estimated to be higher than 70% (Watanabe, 1969).

The familiar process of mutation and selection could not explain either this rapid increase in multiple resistance or a number of other findings concerning the dysentery epidemics. For one thing, during a single outbreak of the disease, resistant *Shigellae* were isolated from some patients and sensitive *Shigellae* of exactly the same type from other patients (Akiba & Kimura, 1959; Matsuyama et al, 1959; Ochiai, 1959). Even the same patient might yield both sensitive and resistant bacteria of the same type.

Moreover, the administration of a single drug, say chloramphenicol, to patients harbouring a sensitive organism could cause them to excrete bacteria that were resistant to all four drugs (Kagiwada et al, 1960; Kobari & Tajiri, 1959; Ochiai et al, 1959). Then it was found that many of the patients who harboured drug-resistant *Shigellae* also harboured strains of the relatively harmless colon bacillus *E. coli* that were resistant to the four drugs. It was impossible, on the other hand, to obtain multiple resistance in the laboratory by exposing sensitive *Shigellae* or *E. coli* to any single drug; multiply resistant mutants could be obtained only after serial selections with each drug in turn, and these mutants, unlike the ones taken from sick patients, multiplied very slowly. No acceptable explanation of these curious findings was forthcoming until Akiba (1959) suggested that multiple drug resistance might be transferred from multiply-resistant *E. coli* to sensitive *Shigellae* within a patient's digestive tract. This transfer was demonstrated independently by Ochiai et al, 1959 and Akiba et al, 1960 in vitro, and thereupon confirmed the possibility by transferring resistance from resistant *E. coli* to sensitive *Shigellae* - and from resistant *Shigellae* to sensitive *E. coli* - in liquid cultures. Other investigators demonstrated the same kind of transfer in laboratory animals (Akiba et al, 1961; Mitsuhashi et al, 1960; Walton, 1966; Guinee, 1968) and eventually in human volunteers (Akiba et al, 1961; Kagiwada et al, 1960) under experimental conditions. It was finally concluded that the factor responsible for infectious drug resistance was an extrachromosomal element, which was called the R-factor (resistance factor). These R-factors are obtained by bacteria only by infection from other R-factor-carrying cells, never by spontaneous mutation. Transfer occurred not only among *Shigella* organisms, but among members of the family *Enterobacteriacaeae* as a whole, as well as to such unrelated gram-ve bacteria as *Pseudomonas*,, *Vibrio cholera* and *Pasteurella pestis* (Watanabe, 1963).

1.3 GENERAL PROPERTIES OF R FACTORS

The R factors are extrachromosomal genetic elements many of which are conjugationally transmissible and capable of conferring resistance to various chemotherapeutic agents and heavy metal ions on their host bacteria (Mitsuhashi, 1976, 1971; Watanabe, 1963; Anderson & Lewis, 1965). The R factors can also mediate genetic properties other than those responsible for drug resistance: -

a) conjugational transferability
b) autonomous replication
c) stable residence within the host
d) maleness of the host (R mating), and
e) interference with plasmids and bacteriophages.

All genetic characters of the R factor are conjugationally transmissible as a whole or are jointly transduced by some bacteriophages when the phage has the same (or larger) size of DNA as that of R factor.

An important point in the spread of R factors to pathogenic bacteria is that they are transferred to these bacteria by way of usually non pathogenic bacteria, such as *E. coli* (Watanabe, 1966; Holloway & Asche, 1977). If an R factor carrying nonpathogenic bacterium enters the intestinal flora, other Gram-ve enteric bacteria in the intestinal tract may receive the R factor. Thus, the factor may spread within the intestinal Gram-ve bacterial flora through successive transfer. If the patient happens to become infected with drug-sensitive pathogens, such as *Shigella*, *Salmonella* and *Vibrio*, these pathogens may become infected with R factors from the intestinal flora. It is now found that R factors were transferable not only among all species of the family *Enterobacteriaceae*, but also to the *Vibrio* group and *Pasteurella* and *Serratia*.

The frequency of transfer of R factor is governed by: -

a) the recipient strain (Smith & Linggood, 1970); Anderson & Lewis, 1965) - some accepting R factors more efficiently than others. For example, *Klebsiella-Enterobacter* species, *E. coli*, *Shigella* species, *Citrobacter* species and *Pseudomonas aeruginosa* are considered as 'good' recipients (Falkow, 1975; Datta and Hedges, 1972).

b) the donor strain; some are more efficient donors than others. For example, 'good' R factor donors are, in order of their quality: *E. coli*, *Shigella* species, *Citrobacter* and *Klebsiella-Enterobacter* species. Many laboratories employ the *E. coli* K12 strain as a standard donor and recipient of R factors as it is excellent in both respects.

c) the physiological state of the donor and recipient cells. Whole living cells of both donor and recipient are required for resistance (Mitsuhashi et al, 1960). No transfer could be demonstrated using cell-free medium from a resistant culture; neither phage nor F factor is required. Watanabe (1966) observed that the highest frequencies of transfer of R factors ranged between 10^{-1} and 10^{-2} infected cells per donor cell in mixed cultures at 37^{o}C for 60 minutes.

It is generally found that R factor-mediated resistance is more powerful than that mediated by chromosomal resistance (Anderson, 1968; Davies & Rownd, 1972). Although resistance may apply to a single drug, multiple drug resistance is the rule - resistance to as many as none antibacterials being transferred by a single R factor (Van Hest & Hofmeyr, 1974). Various R factors resistant against the vast majority of antibacterial drugs used today have been found. These include ampicillin (Datta & Richmond, 1966); chloramphenicol (Smith, 1973; Anderson, 1973); carbenicillin (Sykes, 1971); gentamicin (Datta and Hedges, 1973); kanamycin (Benveniste & Davies, 1971); streptomycin (Grinsted & Lacey, 1973); sulphonamide (Watanabe, 1963) and tetracycline (Mitsuhashi,

1969; Watanabe, 1963).

The levels of resistance conferred by R factors vary from drug to drug, from factor to factor, and also the same factor in different hosts gives different levels of resistance (Watanabe & Fukasawa, 1961a). For example, one multiple-drug-resistant R factor causes *E. coli* to be resistant to only 10ug of Sm/ml but the same R factor confers on *Shigella flexneri* resistance to more than 1000 ug/ml. The tetracycline resistance of *Salmonella typhimurium* with this R factor is about 50 ug/ml, although resistance of *E. coli* and *Shigella* strains with the same R factor is as high as 200 ug/ml (Watanabe, 1966).

The fact that the transfer of R factors usually occurs without the transfer of the bacterial chromosome, indicates that the R factors usually exist in the bacterial cytoplasm (Watanabe & Fukasawa, 1961a). Other evidence supporting the cytoplasmic nature of transferable drug resistance factors comes from the 'curing' of the drug resistance with acridine dyes, which are known to eliminate cytoplasmic agents (Watanabe & Fukasawa, 1961b).

R factors are self-replicating genetic elements that carry the genetic information of drug resistances and their conjugal transferability (Watanabe, 1966, 1969, 1971). They are inactivated by the decay of incorporated ^{32}P (Watanabe & Takano, 1963). They can be transduced by phages, and the transducing particles of R factors are inactivated by UV irradiation. These facts suggested that R factors were composed of DNA. This was later confirmed by Falkow et al (1966) and Rownd et al (1966). Using *Proteus mirabilis*, they isolated and purified satellite DNA, which was identified as R factor DNA. Electron microscopic examination of multiply resistant bacteria confirmed that R factors exist in the cytoplasm as circular, double-stranded DNA entities (Clowes, 1973).

1.4 EVOLUTION OF R FACTORS

R factors mediating resistance to antibiotics are not considered a new phenomenon. It has been shown that R factors must have existed in the pre-antibiotic era. The best evidence for an early existence of R factors was the discovery by David Smith (1967) of a tetracycline-streptomycin plasmid in a strain of *E. coli* isolated before 1937 and freeze dried in 1946 - before the clinical introduction of these drugs. Indirect evidence of a similar nature came from the observation by Smith (1967) and Evans and coworkers (1968) that R factors mediating resistance to semisynthetic penicillins and several aminoglycoside antibiotics were present in enteric bacteria isolated years before these agents were available. Their studies revealed that strains of *E. coli*, *Shigella flexneri* and *Salmonella typhimurium* isolated in Mexico in 1956 contained R factors mediating O type β-lactamase ampicillin-resistance. The finding that a β-lactamase with a high degree of affinity for semi-synthetic penicillins was already present in 1956, suggested that it was previously evolved to fit a naturally occurring substrate. Similarly, the R factor in the 1946 lyophil was streptomycin resistant by virtue of a streptomycin phosphotransferase that cannot be distinguished from present day examples. It can thus be concluded that R factors are not just a recent phenomenon, and that selective forces were operative in Nature before 'the antibiotic era' to confer sufficient evolutionary advantage for the association of a transfer factor with drug-resistance genes (Falkow, 1975).

In 1966, Watanabe suggested that if R factors had been evolving in natural populations of micro-organisms, then it would be useful to examine 'antibiotic-virgin' populations to determine their prevalance and characteristics under such conditions. Such a study was conducted by Mare (1968) among a group of 47 Kalahari bushmen and wild animals in 'drug-free' communities of Rhodesia. Although some antibiotic

resistant bacteria were found, in no case was this property auto transmissable, and no segregants were found, so that it was not certain that these resistances were plasmid-borne. In 1969, Gardner et al examined 21 stool specimens and 19 soil specimens taken from a 'drug-free' community in the Solomon Islands. R factors, R-Tc-Sm were recovered from two of the 40 specimens processed. One was present in an *E. coli* strain resident in the bowel of a native and the other was from an 'alcaligenes-like' species of the soil. Furthermore, the biochemical mechanism of the resistance mediated by these R factors was the same as that of R factors recovered in developed countries. Another successful study of an 'antibiotic-virgin' community was undertaken by Davis and Anandan (1970), who examined the enteric flora of 128 people who inhabited an isolated village in North Borneo. Of 1017 bacterial isolates, 50 were multiply resistant and 6 strains of *E. coli* from 4 different individuals could transmit their resistance by conjugation. Two of them had resistance markers to sulphonamide, streptomycin, chloramphenicol and tetracycline; three had resistance markers to streptomycin, chloramphenicol, sulphonamide, tetracycline and ampicillin, and one had sulphonamide, streptomycin, chloramphenicol and ampicillin markers. These studies of isolated communities reveal therefore that R factors can be found under natural conditions, without the selective force of deliberately administered antimicrobial drugs.

The discovery of an R factor from a soil organism by Gardner et al (1969) could suggest that the drug resistance genes may have originated from those organisms which actually produce antibiotics and whose natural habitat is the soil (Walker & Walker, 1970; Benveniste & Davies, 1973). This is because many such strains contain enzymes that resemble the R enzymes in the way that they inactivate the antibiotics. Since such organisms are themselves vulnerable to their own products, the enzymes might serve as detoxifiers, or play a role in their biosynthesis, or in their own transport out of the cell (Dowding & Davies, 1975).

R-factor-Cm-resistance is generally due to the production of chloramphenicol-acetylating enzymes (Gaffney et al, 1978). Many streptomycetes are able to acetylate chloramphenical but Shaw and Hopwood (1976) could not detect Cm acetylation in *Streptomyces venezuelae*, which is the organism that produces chloramphenicol, and which is highly resistant to it. It is possible that the drug is inactivated here by another mechanism. But, *Streptomyces acrimycini* (a non-producer of chloramphenicol) appears to owe some of its rather low resistance to the presence of acetylating enzyme (Wright & Hopwood, 1977). Further work on this was done by Benveniste and Davies (1973) who initiated a search in the actinomycetes for aminoglycoside-modifying enzymes like those that have been characterised in strains carrying R factors. Benveniste and Davies found that *Streptomyces kanamyceticus* (which produces kanamycin) contained an enzyme that acetylated the 6'-amino group of kanamycin A and B, gentamicin C_{1a}, and neomycin. This was the same reaction as that catalyzed by R^+ *E. coli* which elaborate kanamycin acetyltransferase. Similarly, *Streptomyces spectabilis* (producer of spectinonycin) was found to produce an enzyme that accetylated the 2'-amino groups of the hexose ring of gentamicin C_{1a}, which was identical to the reaction mediated by R^+ bacteria which produce gentamicin actyltransferase II. The metabolic rate of the aminoglycoside modifying enzyme is not known. They may be required for the synthesis of precursors of the finished antibiotic, for detoxification of antibiotics as is the case for R^+ bacteria, for transport of antibiotics in or out of the cell, or they may have nothing to do with antibiotic synthesis and may play a role in other biosynthetic processes.

For all of this speculation and experimental evidence, it seems that whatever their origin, R factors were present in free-living bacteria before the widespread use of antibiotics by man.

1.5 DISTRIBUTION OF TRANSFERABLE DRUG RESISTANCE

Antibiotic resistant *Enterobacteriaceae* appear to be widespread in the environment, and are isolated frequently where selective pressure favours their predominance. Resistant strains, many of which carry transferable resistance are encountered commonly in hospitals where they rapidly colonise new patients, especially those receiving antibiotic therapy (Datta, 1971), on farms and in slaughter houses where there are animals receiving feeds containing antibiotics (Mercer et al, 1971; Moorhouse, 1971; Wells & James, 1973; Wiedman & Knothe, 1971) and in cultured fish treated with antibiotics (Aoki et al, 1971a,b; Aoki et al, 1972, Watanabe et al, 1971). Resistant strains may also reach the environment with both raw and treated municipal and hospital wastes (Grabow & Prozesky, 1973; Linton et al, 1974; Smith, 1970; Sturtevant et al, 1971; Sturtevant and Feary, 1969). Resistant strains carrying transferable resistance have also been demonstrated to occur in receiving waters (Feary et al, 1972; Smith, 1970; 1971).

The importance of transferable drug resistance in human and animal medicine and its interest to the fields of microbial genetics and molecular biology have stimulated a wide search for it in both pathogenic and non-pathogenic bacteria, in many parts of the world; the phenomenon has been found wherever it has been sought (Anderson, 1968) (Refer Table 1). In Japan, the proportion of multiresistant *Shigella* (Sm, Cm, Tc, Su) found during epidemics rose from 10-20% in 1955-1956 to 80% in 1968 (Watanabe, 1963; Mitsuhashi, 1965). Multiresistant *Shigella* has also been found in Rumania by Sasarman (1964, 1965, 1966, 1967), in England by Lewis (1967), in Germany by Brandis and Morgenroth (1967) and in South Africa by Watson (1967).

R factors were first demonstrated in Europe in the *Salmonellae* in England by Datta (1962) and in Germany by Lebek (1963). Datta (1962) found infectious streptomycin, tetracycline

TABLE 1 - DISTRIBUTION OF TRANSFERABLE DRUG RESISTANCE IN THE ENTEROBACTERIA - PUBLISHED BY SOME OF THE INVESTIGATORS THROUGHOUT THE WORLD

(adapted from Anderson, 1968 and Falkow, 1975)

Year and location	Genera of Enterobacteria	Reference
1952 (Japan)	Shigella	Mitsuhashi, 1969
1962 (Great Britain)	Salmonella	Datta, 1962
1964 (Hungary)	Shigella	Ketyi & Vertenyi, 1965
(Great Britain)	Salmonella	Anderson, 1968
1965 (U.S.A.)	Salmonella	Gill & Hook, 1966
1966 (U.S.A.)	Salmonella	Smith, 1966
(U.S.A.)	Shigella) Salmonella) E. coli)	Kabins & Cohen, 1966
(U.S.A.)	E. coli) Klebsiella) Citrobacter)	Salzmann & Klemm, 1966
(Ethiopia)	E. coli	Mann & Gedeban, 1966
(Rumania)	Shigella	Sasarman et al, 1966
1967 (Britain)	Shigella	Lewis, 1967
(Germany)	Shigella	Brandis & Morgenroth, 1967
(Japan)	Shigella	Tanaka et al, 1967
(Japan)	E. coli) Citrobacter) Klebsiella) Cloaca) Proteus)	Naide et al, 1967
(Japan)	E. coli) Proteus) Klebsiella)	Mitsuhashi et al, 1967
(Rumania)	Salmonella	Sasu et al, 1967
(S. Africa)	Shigella	Watson, 1967
(Greece)	Shigella) Salmonella)	Kontomichalou, 1967
1969 (Great Britain)	Shigella	Davies et al, 1970
1970 (Switzerland)	Shigella) Salmonella)	Lebek, 1972

and sulphonamide resistance in 15 out of 306 cultures of *Salmonella typhimurium*, phage type 27, which had been isolated during and out-break of gastroenteritis in a London hospital in 1959. By 1963-1964, the proportion of multiresistant *Salmonella typhimurium* reached 21% (Anderson & Lewis, 1965; Datta, 1965). Lebak (1963) found an R factor conferring resistance to the six drugs streptomycin, tetracycline, chloramphenicol, sulphonamide, kanamycin and neomyin in *Salmonella typhimurium* from a young child in Munich. *E. coli* with the same resistance pattern was found in this child's faeces at a time when the *Salmonellae* was still drug sensitive. Lebek subsequently found R factors carrying various resistance patters in enteric bacteria, including entero-pathogenic types of *E. coli* in Germany (1963b,c) and Switzerland (1972). *Salmonella* carrying R factors were also found in the United States by Kabins and Cohen (1966), Smith (1966), Gill and Hook (1966) and in Rumania by Sasu, Candrea and Apostica (1967).

Mitsuhashi (1971) suggested that the worldwide distribution of R factors was not caused by travellers but arise independently in many places at almost the same time in response to the universal use of antibiotics.

1.6 THE BIOCHEMICAL BASIS OF R-FACTOR-MEDIATED DRUG RESISTANCE

The earliest ideas about the biochemical basis for R-factor drug resistance were influenced by the abrupt appearance of resistance to so many drugs at once (Falkow, 1975; Davies & Rownd, 1972). It was postulated that the multiple resistance was attributable to a single genetic determinant which conferred a non-specific permeability barrier on the host. This hypothesis had to be modified when R factor segregants were found which indicated that each drug resistance was specified by a separate gene. However, it is still considered likely that specific drug impermeability may account for resistance in some cases, e.g. tetracycline and sulphomamide

resistance. However, resistance to penicillins, chloramphemicol and the aminoglycoside antibiotics have been found to be associated with specific enzymes which modify or hydrolyze the drug to a more innocuous form.

a) Tetracycline Resistance

Tetracycline resistance is prbably the most common naturally occurring R factor resistance, perhaps because the gene that determines this trait is closely linked to the RTF in most R factors (Watanabe, 1963; Mitsuhashi, 1969). Furthermore, tetracycline is poorly absorbed in the small gut, so that much of what is ingested is carried into the lower intestine and feces, where selection and transfer probably occur (Broda, 1979; Linton et al, 1972). It appears that the resistance of R^+ strains is due to their ability to concentrate tetracycline (Izaki & Arima, 1963; Izaki et al, 1966). Whereas drug-sensitive bacteria actively accumulate tetracycline leading to inhibition of ribosome function, R^+ strains possess a constitutive low-level resistance to the drug, which prevents it from reaching the ribosome target (Franklin, 1967; Franklin & Higginson, 1970). The specific mechanism whereby tetracycline is inhibited from entering the cell is not known and studies or radioactivity-labelled tetracycline provide no evidence for enzymatic inactivation (Franklin, 1967).

b) Pencillin Resistance

In both Gram+ve and Gram-ve bacteria, the basis for penicillin resistance has been attributed to the production of inactivating enzymes - an amidase and a penicillinase (β lactamase). Enteric bacteria produce both enzymes but R-factor-mediated resistance to penicillin has been solely associated with B lactamase activity - an enzyme which breaks the β-lactam ring of the penicillin molecule (Anderson & Datta , 1965; Datta & Kontomichalou, 1965; Datta & Richmond, 1966).

The increased use of penicillin particularly ampicillin has been paralleled by the appearance of Ap genes in R factor chromosomes. This is because Ap is a popular drug because of its wide spectrum of antibacterial activity, ease of administration and little side effects. For instance, in 1966 (Falkow) no instance of Ap genes in R factors was found in *Shigella* organisms isolated at the Children's Hospital in Washington, D.C. However, in 1970, after extensive use of the drug, 85% of the R factors found among the *Shigella* isolates in the hospital carried an Ap gene. In each of the isolate examined, the R factor-mediated resistance to Ap was correlated with the production of a β lactamase - a phenomenan generally observed since the first description of R-factor-mediated β-lactamase by Datta and Richmond, 1966.

Datta and Richmond, 1966 showed that the penicillinase produced by *E. coli* strain TEM having an R factor differs from the penicillinases produced by *Bacillus* or *Staphylococcus* - perhaps the most striking is the markedly lower molecular weight ($\triangleq$ 16,700 as against $\triangleq$30,000 for the penicillinase from the Gram+ve species). In addition, enzyme synthesis in *E. coli* strain TEM is constitutive, unlike the situation found with all strains of Gram+ve bacteria that synthesize penicillinase. Among Gram-ve rods several β-lactamases may be distinguished by their effects on various substracts (penicillins and cephalosporins). Some are governed by chromosomal genes, others by extrachromosomal elements, and both maybe present in the same bacterial strain. In the main, the β-lactamases elaborated by Ap resident clinical isolates which do not carry an R factor or by Ap resistant mutants selected in the laboratory, possess a high activity for cephalosporins than for Ap. These enzymes are often inducible. However, the β-lactamases elaborated by the Ap gene of R factors displays high activity on both ampicillin and the cephalosporins (Datta & Richmond, 1966).

c) Aminoglycoside Antibiotics Resistance

Resistance to aminoglycoside antibiotics was considered to be due to chromosomal mutations, associated with specific alterations of the bacterial ribosome. The addition of an aminoglycoside to a sensitive cell is associated with an inhibition of protein synthesis because of the misreading of the genetic code. Mutation to resistance alters the ribosome in a way that prevents or inhibits binding of the aminoglycosides (Falkow, 1975; Holloway & Asche, 1977).

Early observations suggested that R-factor-mediated amino glycoside resistance was not associated with a ribosomal alteration (Smith, 1969; Falkow, 1975). Firstly, in *E. coli* strains partially diploid for chromosomal genes, streptomycin sensitivity was dominant over streptomycin resistance whereas R factor streptomycin resistance was found to be dominant. (Watanabe & Fukasawa, 1961a,c; Lederberg, 1951; Zelle & Lederberg, 1951). Furthermore, whereas chromosomal-mediated resistance was generally antibiotic specific, R-mediated cross-resistance to several aminoglycosides was common. And finally, the level of R-mediated aminoglycoside resistance, but not that of chromosomal based resistance, varied with inoculum size on plates of antibiotic media and on the host species (Smith, 1969). For example, the same R factor will mediate a minimal inhibitory concentration (M.I.C.) of 20µg/ml in *E. coli* but more than 1000 µg/ml in *Salmonella* or *Shigella*. Since an inoculum effect is often associated with drug-destroying enzymes, it was thought that R-streptomycin factors produced an inactivating enzyme. Okamoto and Suzuki (1965) found that extracts of one *E. coli* carrying an R factor inactivated the drug in the presence of magnesium ions and ATP. This work was later confirmed and extended independently by Umezawa et al (1968) and by Yamada et al, (1968). The product of the reaction has been characterised as streptomycin-adenylate. Further

studies also indicate that certain R factors may mediate the synthesis of another enzyme which can inactivate streptomycin, and the produce appears to be a phosphorylated streptomycin. The resulting phosphate ester or phosphodiester derivatives of streptomycin are completely inactive as antibiotics and therefore unable to bind to the target site of the antibiotic on the ribosome (Yamada et al, 1970).

To date, the following enzymes have been characterised which modify aminoglycoside antibiotics. These enzymes are: -

i) Sm phosphotransferase which inactivates Sm by phosphorylation (Ozanne et al, 1969)

ii) Sm adenylate synthetase, which inactivates Sm (Yamada et al, 1968; Umezawa et al, 1968; Benveniste et al, 1970; Harwood & Smith, 1969) and spectinomycin by adenylylation (Benveniste et al, 1970; Smith et al, 1970)

iii) kanamycin acetyl transferase, which inactivates kanamycin by acetylation (Umezawa et al, 1967a, b)

iv) kanamycin phospotransferase which inactivates kanamycin and neomycin by phosphorylation (Ozanne et al, 1969; Umezawa et al, 1967a, b)

v) gentamicin adenylate synthetase, which inactivates the gentamicins, kanamycins, and tobramycin (Martin et al, 1971; Kabins et al, 1971; Benveniste & Davies, 1971)

vi) gentamicin acetyl transferases I and II, which acetylate the gentamicin complex and tobramycin.

d) Chloramphenicol Resistance

The mechanism of chloramphenicol resistance specified by R factors has been elucidated by Suzuki and Okamoto (1967) and by Shaw (1967), who found that a crude extract of an R^+ strain, in the presence of acetyl coenzyme A, was capable of converting chloramphemicol to an inactive O-acetyl derivative. This is produced by the action of a single enzyme, chloramphenicol acetyl transferase. In *E. coli*, this enzyme is synthesized constitutively and is subject to catabolite repression.

(Harwood & Smith, 1970). The products of the acetyiation are a mixture of 3-acetoxychloramphenicol and 1, 3-diacetoxychloramphenicol, which are both inactive as antibiotics. In general, modification of chloramphenicol is not observed in extracts of strains in which chloramphenical resistance is due to a chromosomal mutation (Shaw, 1967). According to Shaw and Brodsky, 1967, there are differences in the enzyme levels observed among different R-factor-chloramphenicol isolates or when the same R factor is transferred from host to host. There are no definitive data on the control of enzyme activity but it has become apparent that exponential growth of R-factor-chloramphenicol-containing bacteria occurs only after there has been complete inactivation of the drug, and this increase in lag before exponential growth is related to the specific activity of the acetylating enzyme.

1.7 CLINICAL ASPECTS OF THE RELATIONSHIP BETWEEN ANTIBIOTIC USAGE AND RESISTANCE

R plasmids became widespread in commensals and pathogens during the 1960s. Even though transfer between strains is not always frequent, it does occur in vivo and, because of the very strong selective pressures, organisms that are or can become resistant prosper at the expense of their drug-sensitive competitors. This situation is seen most dramatically in intensive care units of hospitals and under conditions of intensive animal production (Broda, 1979).

Thus far, an increasing incidence of resistance among bacteria has been counted by the introduction of further antibiotics. It is the selective pressure exerted by the large and sometimes indiscriminate use of antibiotics and chemotherapeutic agents which has led to the present situation and which may possibly lead to its further deterioration (Finland, 1970, 1972). The most common errors in the use of antibiotics are: administration in

cases not requiring this therapy, inappropriate administration of broad spectrum antibiotics, inappropriate administration of combinations of antibiotics and inappropriate prophylaxis. Although semi-synthetic derivatives of established antibiotics are produced with the hope that resistance to these drugs may arise less readily, the real response must be a restraint in the use of antimicrobial agents used consistently, both in the long term and on a world-wide scale (Broda, 1979). This can take several forms: -

1. <u>The avoidance of the prescription of unnecessary antibiotics in both therapy and prophylaxis</u>

   One of the most effective ways to reduce the incidence of resistance is to avoid the prescription of unnecessary antibiotics. For example, it is unnecessary to prescribe antibiotics in treating minor upper respiratory tract infections of viral origin, unless an established bacterial infection coexists (Grassi, 1977). However, if emergency treatment of the patient is necessary, it should be based on using a narrow spectrum agent directed against the most likely causative organism assessed from the clinical data, whilst awaiting culture and sensitivity tests (Holloway, 1977). With regard to the prevention of post-operative wound infection, there is actually no need for antibiotic cover. However, if other cases, decrease in the incidence of surgical infection can be achieved by the administration of three doses, one immediately before, one during, and the last, a few hours after operation, of a broad spectrum antibiotic or a combination of antibiotics active against Gram negative bacteria. Because of the variety of the flora possibly involved, this is one example in which the use of broad spectrum antibiotics may be indicated in prophylaxis. Adverse effects are not produced because of the brief period of treatment involved (Garrod, 1975).

2. General policies for restricted or reserved use of antibiotics

Another way to reduce the incidence of resistance is to restrict on the use of antibiotics. Firstly, through better education of physicians and practitioners, this could be achieved (Grassi, 1977). The problems acquire a primary importance in hospitals where the widespread use of antibiotics in a closed environment allows rapid dissemination of highly resistant strains. These may give rise to infections that are difficult to eradicate (Finland, 1970; McGowan & Finland, 1974; Lorian & Topf, 1972). As regards the use of individual drugs, Garrod (1976) suggested three possibilities: one is restriction to specific uses, especially for a valuable drug such as rifampicin to which resistance is readily acquired. The second possibility is rotation, that is, using a drug until resistance to it appears and then replacing it with another, in the hope that sensitivity to the first will be restored. The third is diversification, which consists of prescribing as wide a variety of drugs as possible, no single one being used enough to provoke frequent resistance. Perhaps the fourth possibility is the regular use of combined treatment, on the principle that resistance to either drug in a combination is unlikely to be acquired (Barber et al, 1960). Unfortunately, it is too costly for general use and presents frequent difficulties in prescribing appropriately in individual cases.

3. Restrictions of antibiotics in animal feeds

This subject has long been debated and legislations have been introduced in order to restrict the use of antibiotics in feeds. These are unlikely to affect the resistance of *E. coli* and *Salmonellae* that are pathogenic for animals as it is probable that the evolution of resistance among pathogenic strains is dependent on the

therapeutic use of antibiotics rather than their addition to feeds (Pohl, 1977). For example, after the introduction of ampicillin in veterinary medicine, strains were soon isolated with the R type Su, Sm, Tc, Cm, Km and Ap, and after the introduction of trimethoprim, strains with the R type Su, Sm, Tc, Cm, Km, Ap and Tmp were found.

The results obtained in large veterinary trials (Finland, 1975; Walton, 1972) showed that the potential and actual danger of a spread of R factor carrying Gram-ve enteric bacteria from animals to humans exist, but is limited to persons in close contact with animals and does not seem to have had a proven influence in epidemics of infectious diseases in man (Finland, 1975; Walton, 1972; Linton, 1972). These considerations do not imply that a problem does not exist, it is one that deserves close surveillance. A reduction in the use of antibiotics in animal feeds can decrease, though not abolish the incidence of transferable bacterial resistance.

4. Adequate dosage

Correct dosage must be established on the basis of antibacterial activity in vitro, on the relationship between the inhibiting concentration in vitro and the drug levels attainable in vivo, on the pharmacokinetic behaviour of the antibiotic and on its toxicity. Too low a dose can be harmful because it may not result in cure of the infection and at the same time will permit an overgrowth of a mostly resistant flora (Grassi, 1977). Low levels can occur by incorrect route of administration, poor absorption of orally administered agents, missed or incorrect doses, inadequate initial dosage, inadequate duration of therapy, a combination of agents which are antagonistic and lack of knowledge of pharmacological properties of tissue distribution and stability (Holloway, 1977). On the other hand, too high a dosage can also be harmful, as it may exert a massive selection

pressure allowing the growth of highly resistant organisms. After the cure of the initial disease, these may spread in the environment and transfer the resistance to other organisms. However, if an antibacterial agent is ineffective and deemed nontherapeutic, it is best to withdraw it from the patient (Holloway, 1977).

5. Choice of an antibiotic

The choice of the most effective antibiotic is not an easy task. This explains in part the popularity of broad spectrum antibiotics. However, their use produce an imbalance in the normal flora, particularly in the bowel, and can operate to select resistant strains among a variety of species of organisms. Therefore the use of these drugs should be restricted except where a definite indication exists (Grassi, 1977).

The use of combinations of antibiotics is also not justifiable because it produces a broad spectrum effect and normal flora will be removed from body surfaces, allowing colonisation of resistant organisms derived from the species to which treatment is directed, or from others selected out of acquired (Holloway, 1977). However, a combination of antibacterial agents which are not closely related may prevent or at least delay the emergence of resistant mutants and even prevent plasmid determined resistance. This applies particularly to the treatment of chronic infections. If organisms develop resistance to certain antibiotics readily, e.g. of *Staphylococcus* to fusidic acid and rifampicin, then, double therapy could be contemplated. It should be noted that when a combination of antibacterial agents is used to prevent emergence of resistant strains, the individual agents should be used in full dosage.

6. Molecular manipulation of antibiotics, as a means of combating transferable resistance.

A lot of work has been done and remarkable results have been achieved both in the knowledge of mode of action of antibiotics and in the mechanism of bacterial resistance (Refer Section 1.6). However, already there are signs that bacteria can succeed in this field more successfully than man. For example, DKB (3'-4'-dideoxikanamycin) lacking the group that is phosphorylated in kanamycin is effective against R factor bearing strains of *E. coli* and *Ps. aeruginosa*. But, in 1972, Yagisawa et al found a strain of *E. coli* bearing a different R factor and capable of inactivating DKB.

Another approach that can be attempted in order to control transferable bacterial resistance is the 'chemotherapy' or 'cure' of plasmids. This could be accomplished through: -

a) selective inhibition of R factor replication
b) interference with R factor genetic expression
c) prevention of R factor transformation
d) suppression of R factor transfer

Other factors that could affect the spread of transferable resistance are the spontaneous loss of R factors (Anderson, 1968); the possibility that in some cases R factors make bacteria more susceptible to antibacterial agents (Watanabe, Ogata, Sugawara & Oda, 1972; Mitsuhashi et al, 1970; Romero et al, 1971), and the possibility that R factors carrying bacteria are endowed with reduced virulence (Watanabe, 1975).

Anderson (1971) stated that, "it is derisory to think of controlling transferable drug resistance by "curing" bacteria of their R factors with chemical agents such as acridine; and to believe that we can always keep at least one step ahead of the bacteria with new antibiotics. If we wish to reduce the incidence and effects of drug

resistant organisms, the only effective method is drastically to reduce the use of antibacterial drugs".

There will be no simple or immediate solutions to the problems of antibiotic resistance in disease-producing bacteria. Only the further understanding of these problems by all participants - antibiotic manufacturers, clinicians, hospital administrators, epidemiologists and microbiologists - will have any impact to prevent the 'further and detrimental involvement of homosapiens in the evolutionary history of bacterial plasmids and antibiotic resistant bacteria' - (Holloway, 1977).

MATERIALS AND METHODS

SEWAGE SAMPLES

From the 8th of February to the 8th of October, 1980, a total of nine samples of raw sewage was collected from the pre-aeration tanks at the Palmerston North Sewage Treatment Plant. All samples were collected in sterile, stoppered universal bottles and processed in the laboratory within three hours of their collection.

ISOLATION OF RESISTANT BACTERIA (DIRECT PLATING)

Upon arrival of the sample in the laboratory, the sample was serially diluted in sterile distilled water to give dilutions of 10^{-1} to 10^{-3}. Triplicate 0.1 ml portions from appropriate dilutions were plated onto plain MacConkey agar (Difco) plates, and distributed evenly over the agar surface using a sterile, glass spreader, in order to obtain estimates of the total number of lactose-fermenting bacteria. Similarly, estimates of the number of antibiotic-resistant lactose fermenters in each sewage sample were obtained by plating triplicate 0.1 ml portions of appropriate dilutions onto MacConkey agar plates containing the following antibiotics, separately or in combination: -

Ampicillin (Ap),	20 ug/ml
Tetracycline (Tc),	20 ug/ml
Streptomycin (Sm),	20 ug/ml
Chloramphenicol (Cm),	25 ug/ml

Ampicillin	+ Tetracycline	each separate antibiotic at 20 ug/ml
Tetracycline	+ Streptomycin	each separate antibiotic at 20 ug/ml
Tetracycline	+ Chloramphenicol	except for chloramphenicol at 25 ug/ml
Ampicillin	+ Streptomycin	except for chloramphenicol at 25 ug/ml

All of the MacConkey agar plates were incubated at 37°C for 1½ hours prior to incubation at 44.5°C overnight. This temperature was selected specially for the selection of fecal coliforms and *E. coli*. At the same time, one set of plain MacConkey agar plates was incubated overnight at 37°C as a control. All the red, lactose-fermenting colonies on the plain MacConkey agar plates, incubated at 37°C were counted to obtain a total number of lactose-fermenting bacteria. Similarly, all the lactose-fermenting colonies on the plain and on the antibiotic-containing MacConkey agar plates incubated at 44.5°C, were counted to give a total number of fecal coliforms and antibiotic-resistant fecal coliforms respectively.

IDENTIFICATION OF FECAL *E. coli*

a) Production of indole at 44.5°C

Lactose-fermenting colonies growing on each set of MacConkey agar plates were picked using a sterile straight wire. Each isolate was inoculated into a separate tube of sterile peptone water, and incubated in a water bath set at 44.5°C for 48 hours. The development of an intense pink colour on the addition of Kovac's Reagent indicated the presence of indole in the test cultures.

b) Production of acid and gas in MacConkey broth

After 24 hours of incubation at 44.5°C in sterile peptone water (used for the Indole test), a loopful of the individual cultures was inoculated into a separate tube containing sterile MacConkey broth and a Durham tube. These were then incubated in a water bath set at 44.5°C for 24-48 hours. Production of acid was detected by the change in colour (from red to yellow) of the MacConkey broth. Gas production was determined visually by accumulation in the Durham

tubes.

c) Citrate utilization test

Just as above, after 24 hours of growing in sterile peptone water at 44.5°C, each of the isolates was streaked onto Simmons Citrate Agar plates, and incubated overnight at 37°C. Failure to grow on this agar was an indication of an *E. coli* organism.

ISOLATION OF RESISTANT FECAL *E. coli* (SPOT METHOD)

From the cultures grown in sterile peptone water (used for the Indole Test), a loopful of the individual cultures was spotted onto Brain Heart Infusion agar plates seeded with antibiotics in the following specified concentrations:

Ampicillin (Ap),	20 ug/ml)	
Tetracycline (Tc),	20 ug/ml)	
Streptomycin (Sm),	20 ug/ml)	Singly or in combination
Chloramphenicol (Cm),	25 ug/ml)	

The plates were all incubated at 37°C overnight. In this way, estimates of the number of singly or multiply-resistant fecal *E. coli* were obtained. This method is in contrast to that described earlier in this study where the sewage sample was diluted and aliquots plated onto MacConkey agar containing the various antibiotics.

Similarly the above cultures were also spotted onto plain Brain Heart Infusion (BHI) agar plates for stock cultures. These plates were also incubated at 37°C overnight.

FURTHER CHARACTERIZATION OF THE SM-RESISTANT ISOLATES

a) Minimal Inhibitory Concentration (M.I.C.) Determination of Streptomycin using Nutrient Broth

The Sm-resistant isolates were grown separately in tubes containing sterile nutrient broth. After overnight incubation at $37^{o}C$ the cultures were each diluted with sterile nutrient broth to give a dilution of 10^{-4}.

A standard volume (1ml) of sterile nutrient broth was added to a series of sterile test tubes. A series of doubling dilutions of the antibiotic was prepared in sterile nutrient broth to give concentrations of 20 ug/ml, 10 ug/ml, 5 ug/ml and 2.5 ug/ml. In certain cases, a two-third dilution of the antibiotic was prepared to give concentrations raning from 100 ug/ml - 8.7 ug/ml. A separate tube containing sterile nutrient broth and no antibiotic was set up as a control. Each of the tubes was inoculated with 0.05 ml of the 10^{-4} dilution of the isolate. These tubes were incubated overnight at $37^{o}C$, and then examined for turbidity.

The minimal inhibitory concentration was taken as the lowest concentration among the tubes which showed no growth.

b) Minimal Inhibitory Concentration Determination of Streptomycin Using MacConkey Broth

The same procedure was carried out as in the M.I.C. determination using nutrient broth. In this case, the minimal inhibitory concentration was taken as the lowest concentration which showed no acid production. Acid production was detected by the change in colour (from red to yellow) of the MacConkey broth.

c) Minimial Inhibitory Concentration Determination of Streptomycin using Plate Method

Just as above, overnight cultures of the isolates were used for this experiment. Each of the cultures was then diluted in sterile distilled water to give a dilution of 10^{-6}.

Sterile MacConkey agar plates were prepared containing different concentrations of streptomycin to give a final concentration of 30 ug/ml, 20 ug/ml, 10 ug/ml, 5 ug/ml, 2 ug/ml and 1 ug/ml respectively. A control MacConkey agar plate containing no streptomycin was also prepared. At the same time, a similar set of Brain Heart Infusion agar plates with and without streptomycin was also prepared.

0.1 ml of the 10^{-6} dilution of the isolates was then plated onto each BHI and MacConkey agar plates and then spread evenly on the surface of the agar with the use of a sterile glass spreader. The plates were then incubated at 37^{o}C for 18-24 hours, and then examined for the presence or absence of growth.

The minimal inhibitory concentration was taken as the lowest concentration that showed no growth.

TRANSMISSIBILITY OF STREPTOMYCIN-RESISTANCE

In this experiment, a strain of *E. coli* K12 (J62-1) was used as recipient. The fecal *E. coli* organisms isolated from sewage, which were Sm-resistant and which were all sensitive to nalidixic acid, were used as prospective donors.

The mating experiment was performed by mixing equal 0.05 ml amounts of overnight BHI broth cultures of the prospective donor and recipient respectively, in 2.5 mls of sterile BHI

broth. The mixture was then incubated for 18-20 hours at 37°C. After mixed growth, the mixture was centrifuged, resuspended in 0.5 ml sterile physiological saline, centrifuged and finally resuspended in 0.5 ml sterile physiological saline. With a sterile wire loop, each of the mixture was streaked onto BHI agar plates containing 50 ug/ml nalidixic acid together with one of either streptomycin (20 ug/ml), tetracycline (20 ug/ml) or ampicillin (20 ug/ml) according to the resistance pattern of the donor. The nalidixic acid in the media prevented growth of the prospective donor, while growth of the recipient strain was prevented by the other antibiotic in the BHI agar. Therefore, the media used was selective for those recipient organisms that had received a resistance factor from the donor, conferring resistance to one or more antibiotics on the recipient.

All plates were incubated at 37°C for 24 hours, and then examined for 'recombinant' colonies. These colonies were then picked off with a sterile straight wire and restreaked onto BHI agar plates containing the respective antibiotics, for confirmation of growth.

CHARACTERIZATION OF THE 'RECOMBINANT' COLONIES

The 'recombinant' colonies that appeared on the above plates, were picked up and each inoculated into a separate tube of sterile BHI broth and incubated overnight at 37°C. The minimal inhibitory concentration of Sm for these isolates was then determined to find out if their level of resistance is due to the nature of the Sm-marker on the plasmid or the nature of the host. The M.I.C. was determined using the three different methods listed below, in the same manner as was described for the Sm-resistant isolates: -

a) M.I.C. determination using nutrient broth
b) M.I.C. determination using MacConkey broth
c) M.I.C. determination using Plate method.

NOTE: Considering that J62-1 is a lac^- mutant, therefore in the M.I.C. determination of streptomycin for the 'recombinants' above, glucose to a level of 0.05% was added to the MacConkey broth.

RESULTS

I VIABLE COUNTS

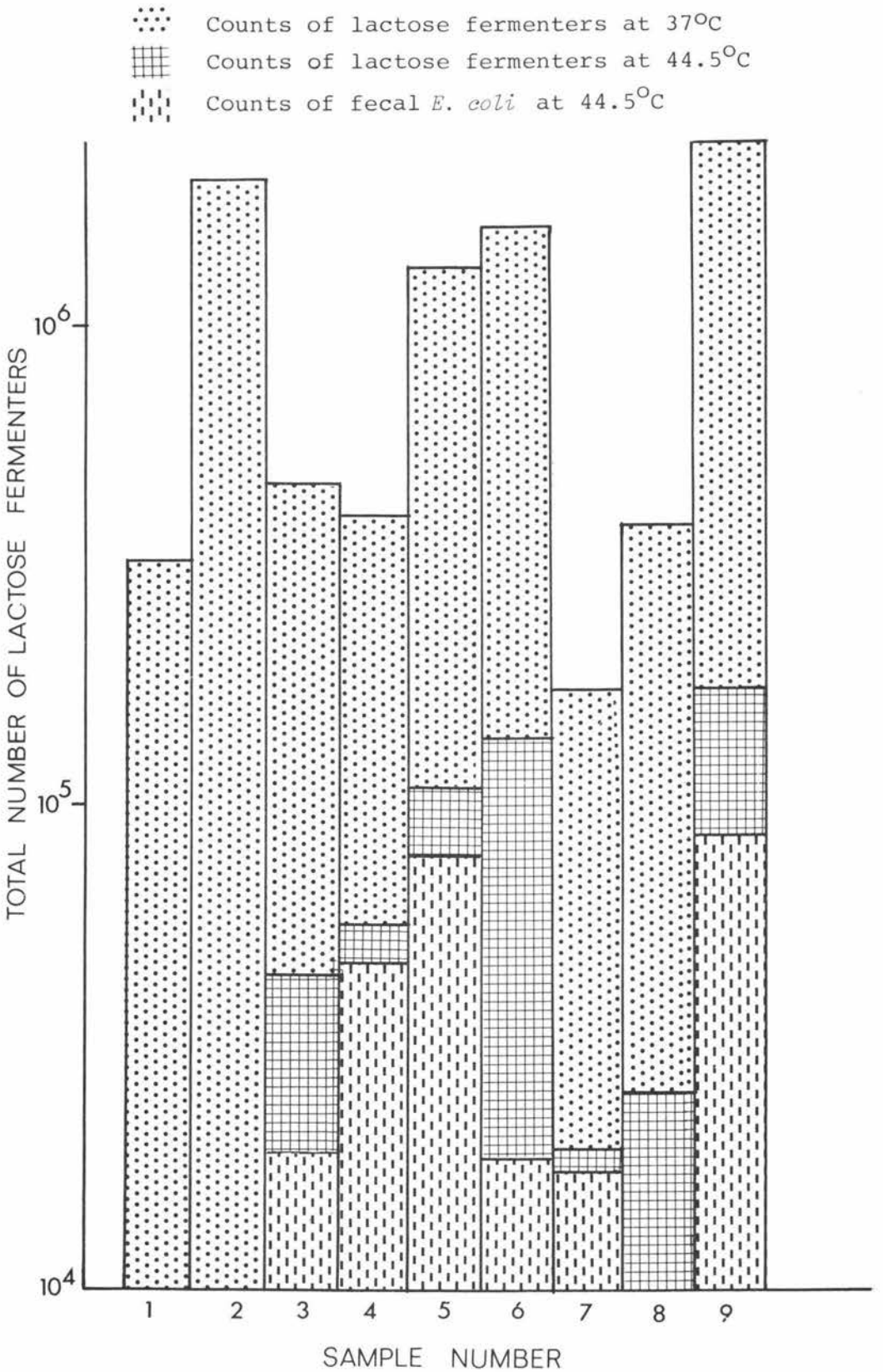
The total numbers of lactose-fermenting colonies which appeared on MacConkey agar plates, after incubation at 37°C and 44.5°C, are shown in Table 2. Similarly, the total number of fecal *E. coli* which appeared on MacConkey plates, and identified by the methods described earlier, are also shown in Table 2 (Refer also to Figure 1).

TABLE 2:

Sample No.	No. of lactose-fermenting colonies/ml		No. of fecal *E. coli*/ml (at 44.5°C)
	37°C	44.5°C	
1	3.2×10^5)	not plated	not plated
2	2.0×10^6)		
3	4.8×10^5	4.6×10^4	1.8×10^4
4	4.1×10^5	5.8×10^4	4.9×10^4
5	1.3×10^6	1.1×10^5	8.1×10^4
6	1.6×10^6	1.4×10^5	1.9×10^4
7	1.8×10^5	2.0×10^4	1.8×10^4
8	3.9×10^5	2.6×10^4	1.0×10^4
9	2.4×10^6	1.8×10^5	9.0×10^4

The incidence of fecal coliforms varied from 6% to 14% of the total lactose-fermenting bacteria, while the incidence of fecal *E. coli* ranged from 1% to 12% of the total lactose-fermenting bacteria. The numbers of fecal *E. coli* in the sewage can be affected by various factors, one of which might be the amount of rainfall. It was thought that perhaps, with the high amount of rainfall, this would dilute the sewage and thus decrease the number of fecal *E. coli* in

FIGURE 1: Counts of lactose fermenters obtained from plates incubated at different temperatures.



the sample. The rainfall data obtained for this survey included the day on which the sample was taken, as well as a few days before that, to investigate not only the effect of rain that falls on a particular day, but, in particular, the cumulative effect of the rain on the numbers of fecal *E. coli*. The results of the findings are shown below (Refer also to Figure 2).

TABLE 3:

Sample No	Date (rainfall data taken)	+Amount of rainfall (mm)	No. of fecal *E. coli*/ml
1	5/Feb	-	
	6/Feb	-	Not plated
	7/Feb	trace	
	*8/Feb	0.1	
2	2/March	13.3	
	3/March	3.9	Not plated
	4/March	0.2	
	*5/March	-	
3	1/June	5.6	
	2/June	-	
	3/June	3.6	
	*4/June	12.5	1.8×10^4
4	14/June	-	
	15/June	1.1	
	16/June	0.6	
	*17/June	trace	4.9×10^4
5	4/July	7.2	
	5/July	-	
	6/July	1.4	
	*7/July	4.1	8.1×10^4
6	20/July	-	
	21/July	-	
	22/July	8.0	
	*23/July	7.3	1.9×10^4
7	1/Aug	37.0	
	2/Aug	14.8	
	3/Aug	-	
	*4/Aug	-	1.8×10^4

* Date: sewage sample was taken
\+ Rainfall data: obtained from DSIR, Palmerston North

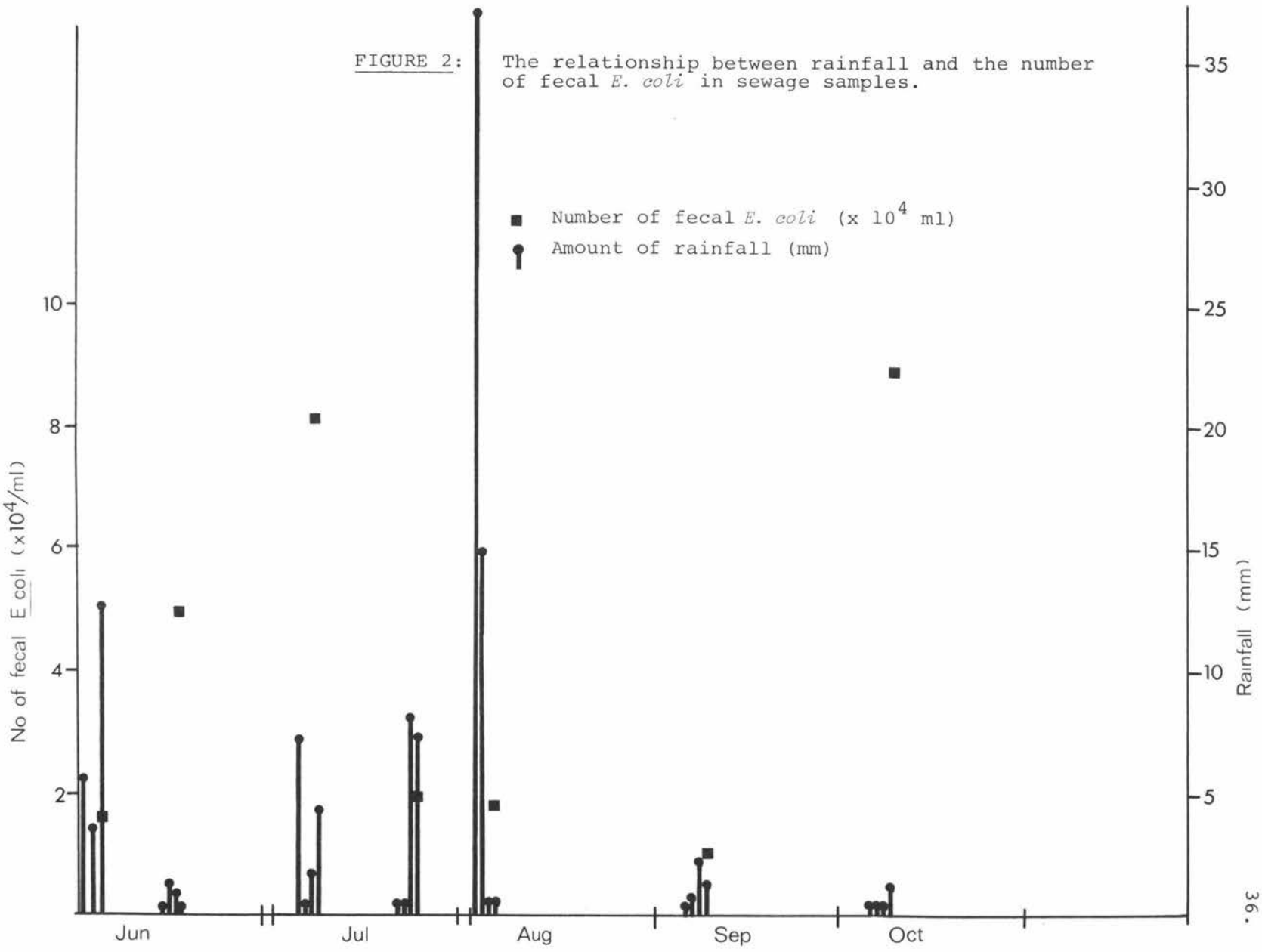


FIGURE 2: The relationship between rainfall and the number of fecal *E. coli* in sewage samples.

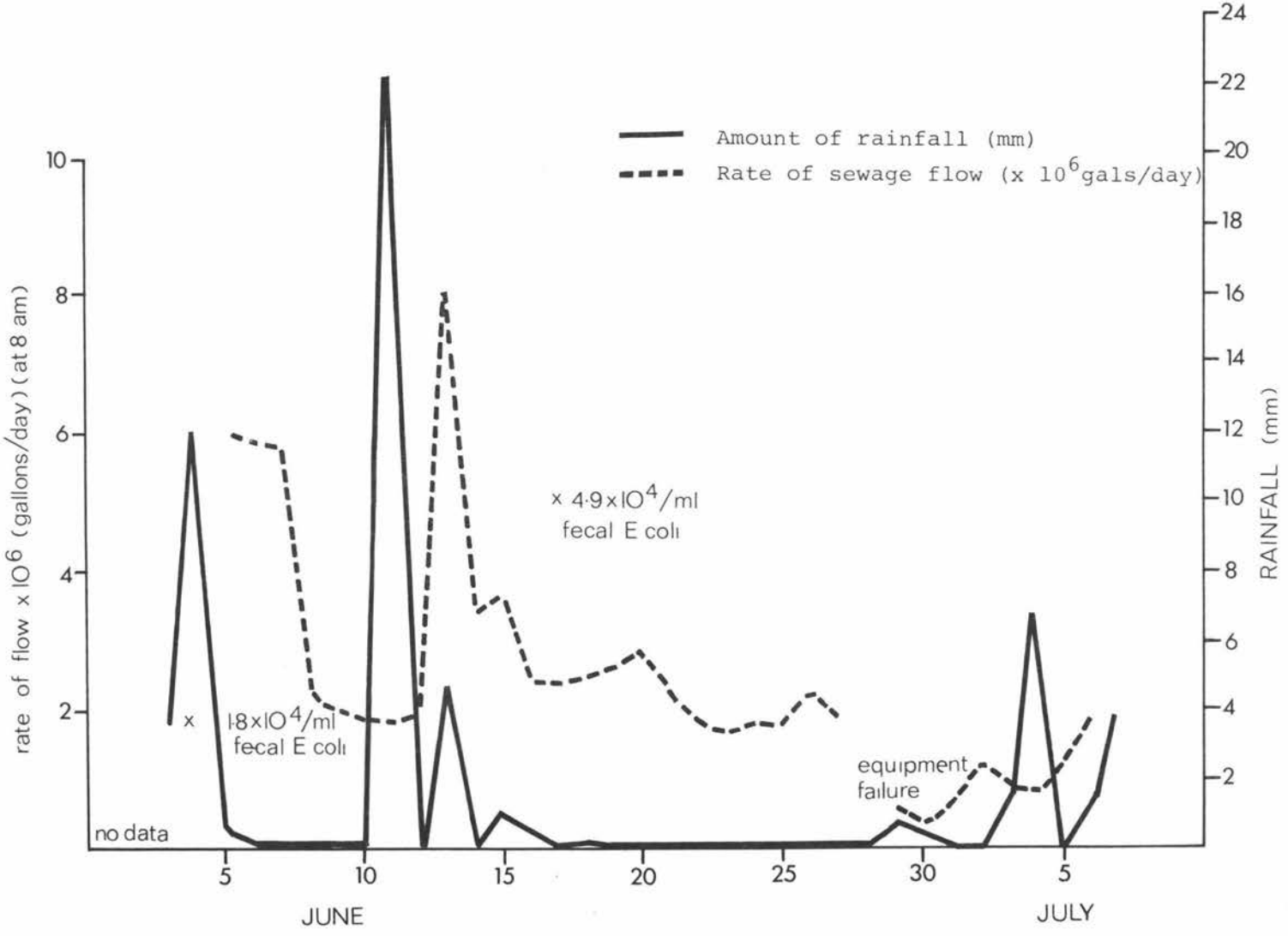
TABLE 3: CONTINUED

Sample No	Date (rainfall data taken)	+Amount of rainfall (mm)	No. of fecal *E. coli*/ml
8	5/Sept	-	
	6/Sept	0.6	
	7/Sept	2.4	
	*8/Sept	1.2	1.0×10^4
9	5/Oct	-	
	6/Oct	-	
	7/Oct	-	
	*8/Oct	0.9	9.0×10^4

* Date: Sewage sample was taken
\+ Rainfall data: obtained from DSIR, Palmerston North

It was hoped earlier on that there be a relationship between the amount of rain and the number of fecal *E. coli* in the sewage. Although these values may be affected by many other factors, but from what is shown in Figure 2, there is some correlation between the amount of rainfall and the number of fecal *E. coli*. There is a general pattern which showed that on days where there was little or no rain, the number of fecal *E. coli* was high. On the other hand, when there was heavy rain, even days before a sample was taken, the number of fecal *E. coli* seemed to be very much reduced. This cumulative effect of the rain on the fecal *E. coli* is best shown in Sample 7. Although no rain was detected on the day before the sample was taken, but the very heavy rain which fell consecutively for 2 days before the sample was taken, had a great effect on the number of fecal *E. coli* in the sewage. This was confirmed when a study of the rate of flow of the sewage was made (Figure 3). Although the data obtained was not sufficient (due to equipment failure, etc) to include all information, but the overall picture seemed to agree with the above observation. It showed that there is a time lag (about 1-2 days) before the rain has a significant effect on the rate of flow of the sewage, which in turn affects the number of fecal *E. coli* in the sewage.

FIGURE 3: The effect of rainfall on the rate of sewage flow.
(Counts of fecal *E. coli* obtained from two samples have been included).



II RECOVERY OF RESISTANT FECAL *E. coli*

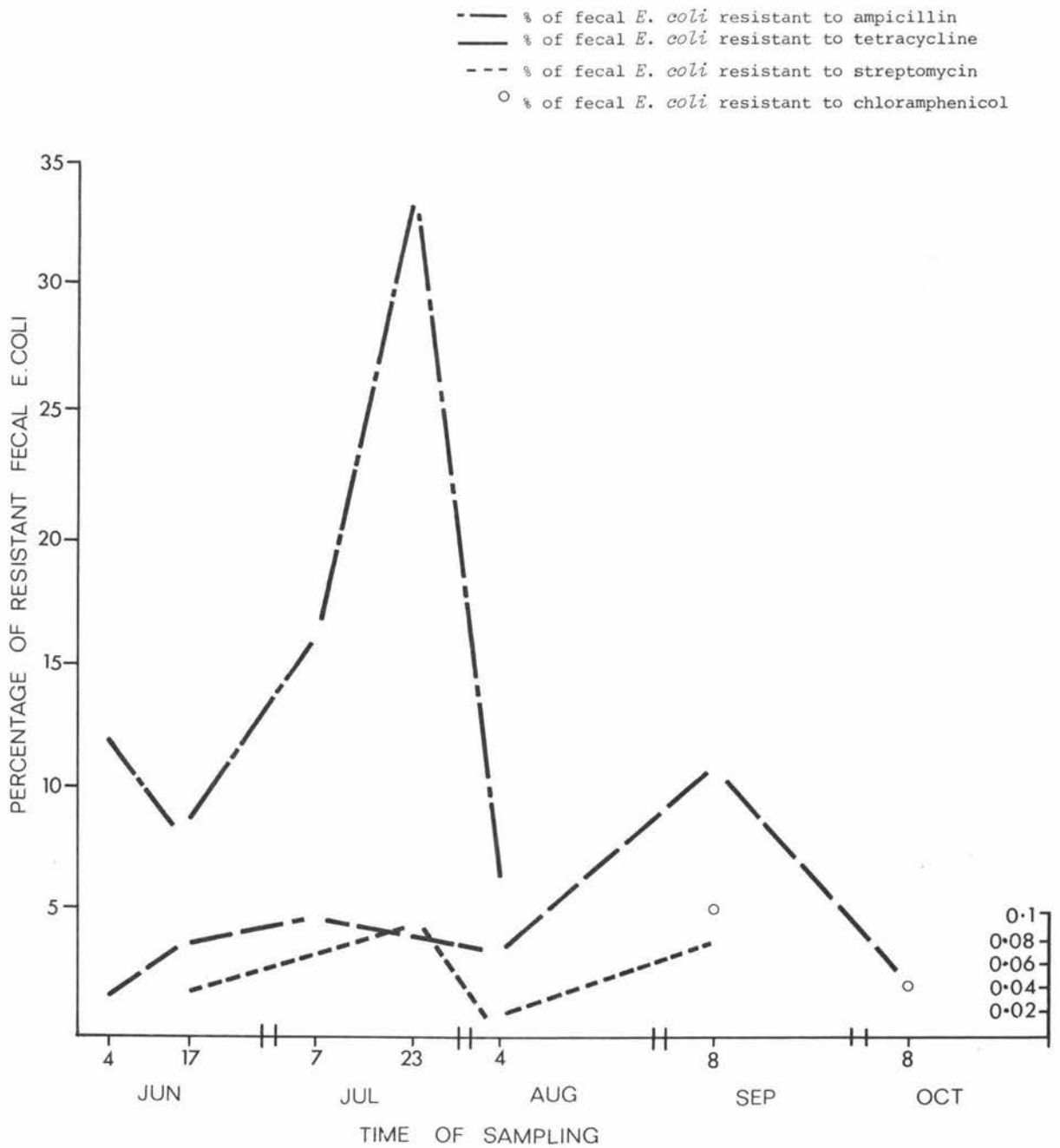
The number and percentage of antibiotic-resistant fecal *E. coli* which were obtained by plating of diluted sewage onto MacConkey agar seeded with ampicillin (Ap), Tetracycline (Tc), streptomycin (Sm) and chloramphenicol (Cm), either individually or in combination, are shown below (Refer also to Figure 4).

TABLE 4: INCIDENCE OF ANTIBIOTIC-RESISTANT FECAL *E. coli* RECOVERED ON MacCONKEY AGAR CONTAINING ANTIBIOTICS INCUBATED AT 44.5°C.

Sample No.	Total No of fecal *E.coli*/ml	Total no. of fecal *E. coli*/ml resistant to						
		Ap	Tc	Sm	Cm	Ap+Sm	Tc+Sm	Tc+Cm
1 2	not plated							
3	1.8×10^4	2.4×10^3 (13.3%)	2.8×10^2 (1.5%)					
4	4.9×10^4	4.0×10^3 (8.2%)	1.8×10^3 (3.6%)	8.5×10^2 (1.7%)				
5	8.1×10^4	1.3×10^4 (16%)	3.8×10^3 (4.7%)					
6	1.9×10^4	6.5×10^3 (34.2%)	7.8×10^2 (4.1%)	8.5×10^2 (4.5%)		7.5×10^2 (3.9%)	1.8×10^2 (0.9%)	
7	1.8×10^4	1.1×10^3 (6.1%)	5.9×10^3 (3.3%)	1.5×10^2 (0.83%)		4.5×10^1 (0.3%)	7.3×10^1 (0.4%)	
8	1.0×10^4		1.1×10^3 (11%)	3.8×10^2 (3.8%)	1.2×10^1 (0.12%)		1.1×10^2 (1.1%)	2×10^1 (0.2%)
9	9×10^4		1.9×10^3 (2.1%)		3.9×10^1 (0.04%)			8.5×10^1 (0.09%)

The antibiotic resistance of the fecal *E. coli* isolates could be determined by the spot method, where colonies that come off plain MacConkey agar plates were picked and then spotted onto BHI + antibiotics plate. This method is in contrast to that described above, where the sewage sample was diluted and aliquots plated onto MacConkey agar seeded with the various antibiotics, either singly or in combination.

FIGURE 4: Percentage of resistant *E. coli* to four antibiotics.



These two methods allow us not only to compare the results obtained but also allows us to validate the methods and techniques involved.

The significance and results of this finding are shown in Table 4a.

IIa TABLE 4a: THE NUMBER OF FECAL *E. coli* RESISTANT TO SINGLE ANTIBIOTIC

Ampicillin-resistant fecal *E. coli*

Sample No	Direct Plating	Spotting
5	1.3×10^4/ml	1.4×10^4/ml
6	6.5×10^3/ml	8.5×10^3/ml
7	1.1×10^3/ml	1.6×10^3/ml

Tetracycline-resistant fecal *E. coli*

Sample No	Direct Plating	Spotting
5	3.8×10^3/ml	7.3×10^3/ml
6	7.8×10^2/ml	9.0×10^2/ml
7	5.9×10^2/ml	6.2×10^2/ml
8	1.1×10^3/ml	1.2×10^3/ml
9	1.9×10^3/ml	2.4×10^3/ml

Streptomycin-resistant fecal *E. coli*

Sample No	Direct Plating	Spotting
6	8.5×10^2/ml	9.0×10^2/ml
7	1.5×10^2/ml	1.8×10^2/ml
8	3.8×10^2/ml	5.2×10^2/ml

Chloramphenicol-resistant fecal *E. coli*

Sample No	Direct Plating	Spotting
8	1.2×10^1/ml	1.4×10^1/ml
9	3.9×10^1/ml	4.9×10^1/ml

IIb Fecal *E. coli* isolates resistant to two antibiotics

This can be determined in the following ways: -

a) by plating sample directly onto MacConkey + 2 antibiotics
b) by spotting method, where colonies that appeared on MacConkey agar plates containing a single antibiotic were picked off and then spotted onto BHI agar plates containing the second antibiotic.

By doing this, the frequency of doubly-resistant fecal *E. coli* isolates obtained by the two methods can be compared. Furthermore, this method also allows us to examine the effect of one antibiotic on the scoring of the resistance to the second antibiotic, and vice versa.

The results obtained from the two methods are shown in Table 4b.

TABLE 4b: THE NUMBER OF FECAL *E. coli* RESISTANT TO TWO ANTIBIOTICS

Ampicillin + Tetracycline resistant fecal *E. coli*

	Sample 5	Sample 6	Sample 7
MacConkey+Tc+Ap - direct plating	1.2×10^3/ml	2.0×10^2/ml	2.5×10^2/ml
MacConkey+Tc - spotted for Ap^R	1.7×10^3/ml	1.4×10^2/ml	3.1×10^2/ml
MacConkey+Ap - spotted for Tc	2.9×10^3/ml	9.3×10^2/ml	5.8×10^2/ml

Ampicillin + Streptomycin resistant fecal *E. coli*

	Sample 6	Sample 7
MacConkey + Ap + Sm - direct plating	7.5×10^2/ml	4.5×10^1/ml
MacConkey + Ap - spotted for Sm^R	2.0×10^3/ml	1.8×10^2/ml
MacConkey + Sm - spotted for Ap	5.6×10^2/ml	1.0×10^2/ml

Tetracycline + Streptomycin resistant fecal *E. coli*

	Sample 6	Sample 7	Sample 8
MacConkey+Tc+Sm - direct plating	1.8×10^2/ml	7.3×10^1/ml	1.1×10^2/ml
MacConkey+Tc - spotted for Sm^R	4.5×10^2/ml	3.9×10^1/ml	2.6×10^2/ml
MacConkey+Sm - spotted for Tc	4.1×10^2/ml	5.4×10^1/ml	1.9×10^2/ml

TABLE 4b: CONTINUED

Tetracycline + chloramphenicol resistant fecal *E. coli*

	Sample 8	Sample 9
MacConkey + Tc + Cm - direct plating	2×10^1/ml	8.5×10^1/ml
MacConkey + Tc - spotted for Cm^R	5.3×10^1/ml	6.7×10^1/ml
MacConkey + Cm - spotted for Tc^R	0.7×10^1/ml	3.5×10^1/ml

IIc Fecal *E. coli* isolates resistant to three antibiotics

These were determined in the following ways:

a) Colonies that appeared on MacConkey plates + single antibiotic (e.g. ampicillin) were picked off and tested for resistance to the other two antibiotics (e.g. Tc and Sm) separately, by the spot method on BHI agar plates. Since these isolates were initially ampicillin-resistant, therefore, all those colonies that showed resistance to both the latter drugs, were then taken to be Ap Tc and Sm-resistant.

b) Alternatively, the colonies that appeared on the MacConkey plates containing two antibiotics (by direct plating method) were picked off and then spotted onto BHI agar plates containing the third antibiotic.

The results obtained from these 2 methods are shown in Table 4c.

TABLE 4c: THE NUMBER OF FECAL *E. coli* RESISTANT TO THREE ANTIBIOTICS

Tetracycline + Streptomycin + Ampicillin resistant fecal *E. coli*

	Sample 6	Sample 7
MacConkey + Ap - spotted for Tc^R and Sm^R	5.6×10^2/ml	1.3×10^2/ml
MacConkey + Sm + Tc - spotted for Ap^R	1.3×10^2/ml	6.8×10^1/ml
MacConkey + Tc - spotted for Ap^R and Sm^R	1.1×10^1/ml	3.9×10^1/ml
MacConkey + Ap + Sm - spotted for Tc^R	9.9×10^2/ml	3.9×10^1/ml
MacConkey + Sm - spotted for Ap^R and Tc^R	2.0×10^2/ml	2.7×10^1/ml

TABLE 4c: CONTINUED

Tetracycline + Streptomycin + Chloramphenicol resistant fecal *E. coli*

		Sample 8
MacConkey + Sm	- spotted for Tc^R and Cm^R	3.2×10^2/ml
MacConkey + Tc + Cm	- spotted for Sm^R	1.6×10^1/ml
MacConkey + Cm	- spotted for Tc^R and Sm^R	0.7×10^1/ml
MacConkey + Tc + Sm	- spotted for Cm^R	3.4×10^1/ml
MacConkey + Tc	- spotted for Sm^R and Cm^R	5.3×10^1/ml

Ampicillin + Tetracycline + Chloramphenical resistant fecal *E. coli*

		Sample 9
MacConkey + Cm	- spotted for Ap^R and Tc^R	2.7×10^1/ml
MacConkey + Tc + Cm	- spotted for Ap^R	7.4×10^2/ml

III STREPTOMYCIN-RESISTANT FECAL *E. coli*

In the course of screening for streptomycin-resistance among isolates of fecal *E. coli*, an interesting observation was made. When cultures of fecal *E. coli* were spotted on to Brain Heart Infusion agar seeded with 20 ug/ml Streptomycin, two different kinds of growth were observed: One was a confluent type of growth, as is normally associated with resistance and a second type, where the *E. coli* grew as well-defined, isolated colonies. In the results that follow, the latter kind of growth is designated as (R). The two kinds of growth were further analysed; initially, the minimal inhibitory concentrations (M.I.C.) were determined in an attempt to distinguish between these two patterns of resistance.

TABLE 5: DETERMINATIONS OF THE MINIMAL INHIBITORY CONCENTRATION OF STREPTOMYCIN FOR FECAL *E. coli* STRAINS USING NUTRIENT BROTH

(the following isolates were taken off MacConkey + Tc and MacConkey + Ap agar plates respectively).

Sample 10	Resistant	(R)
Tc^R $Sm^{(R)}$ - 10*		20 - 30 ug/ml
Tc^R $Sm^{(R)}$ - 8		13 - 20 ug/ml
Ap^R $Sm^{(R)}$ - 5		8 - 13 ug/ml
Ap^R $Sm^{(R)}$ - 8		13 - 20 ug/ml
Tc^R Sm^{R+} - 1	<8.7 ug/ml	
Tc^R Sm^R - 2	<8.7 ug/ml	
Ap^R Sm^R - 18	<8.7 ug/ml	
Ap^R Sm^R - 20	<8.7 ug/ml	

Abbreviations: *(R): isolated colonies
+ R : confluent colonies

TABLE 5: CONTINUED

Sample 11	Resistant	(R)
Tc^R Sm^R - 8	5-10 ug/ml	
Tc^R Sm^R - 17	5-10 ug/ml	
Tc^R Sm^R - 28	5-10 ug/ml	
Tc^R Sm^R - 24	5-10 ug/ml	
Tc^R Sm^R - 6	5-10 ug/ml	
Tc^R $Sm^{(R)}$ - 14		10 - 20 ug/ml
Tc^R $Sm^{(R)}$ - 11		10 - 20 ug/ml
Tc^R $Sm^{(R)}$ - 9		10 - 20 ug/ml
Tc^R $Sm^{(R)}$ - 1		> 20 ug/ml
Tc^R $Sm^{(R)}$ - 3		10 - 20 ug/ml

Such characteristics were mainly observed when the isolates were picked off MacConkey + Tc plates and spotted onto BHI + Sm plates. It was then decided to do the same thing for isolates that came off MacConkey + Sm plates but which were either Tc-sensitive or Tc-resistant. The results were as follows: -

TABLE 6: DETERMINATIONS OF THE MINIMAL INHIBITORY CONCENTRATION OF STREPTOMYCIN FOR FECAL *E. coli* STRAINS USING NUTRIENT BROTH

(the following isolates were taken off MacConkey + Sm plates)

Sample 11	Resistant	(R)
Tc^S Sm^R - 2	10 - 20 ug/ml	
Tc^S Sm^R - 12	10 - 20 ug/ml	
Tc^S Sm^R - 10	10 - 20 ug/ml	
Tc^S Sm^R - 7	10 - 20 ug/ml	
Tc^S Sm^R - 3	10 - 20 ug/ml	

TABLE 6: CONTINUED

Sample 11	Resistant	(R)
Tc^R Sm^R - 5	> 20 ug/ml	
Tc^R Sm^R - 6	10 - 20 ug/ml	
Tc^R Sm^R - 13	> 20 ug/ml	
Tc^R Sm^R - 16	> 20 ug/ml	
Tc^R Sm^R - 17	10 - 20 ug/ml	

The results of this initial finding were intriguing. It was expected that the fully-resistant colonies would have a higher M.I.C. value than the (R)-type colonies. But, as shown in the above table, when these dates were picked off MacConkey + Tc plates, the (R)-type colonies seemed to have a higher M.I.C. value (20-30 ug/ml) as compared to the fully-resistant colonies (<8.7 ug/ml). It was then decided to pick colonies that came off MacConkey + Sm plates, but which were either Tc-resistant or Tc-sensitive. On these plates, no (R)-type colonies were found, which explains why the M.I.C. was not done on these isolates. The results obtained were again puzzling. Although the M.I.C. values of these fully-resistant isolates appeared as initially expected, but, comparing these with the results earlier on, it showed that their M.I.C. values were similar to those of the (R)-type colonies (10-20 ug/ml). It was this finding that prompted the investigation into the transmissibility of the streptomycin resistance.

The results of the transfer experiment is shown on the next page (Table 7).

TABLE 7: TRANSFER EXPERIMENT BETWEEN THE STREPTOMYCIN-RESISTANT ISOLATES AND *E. coli* J62-1 ($pro^-his^-trp^-lac^-Nal^R$)

Donor	Recipient	Transmissiblity
$Tc^R\ Sm^R$ - 1	J62 - 1	+
$Tc^R\ Sm^R$ - 2	J62 - 1	+
$Tc^R\ Sm^{(R)}$ - 8	J62 - 1	+
$Tc^R\ Sm^{(R)}$ - 10	J62 - 1	+
$Tc^S\ Sm^R$ - 2	J62 - 1	+
$Tc^S\ Sm^R$ - 12	J62 - 1	+
$Ap^R\ Sm^{(R)}$ - 5	J62 - 1	+
$Ap^R\ Sm^{(R)}$ - 8	J62 - 1	+

All the above 'recombinants' that appeared after the transfer were picked up and the M.I.C. of Sm done on them to determine if the resistance exhibited by them was due to the nature of the plasmid or the nature of the host. The M.I.C. was done using Nutrient broth and then MacConkey broth to compare the two results. Considering that all the donors were picked off originally from MacConkey agar, it was hoped that by using MacConkey broth, the level of resistance of the organisms would be maintained and that it would not vary as much as it might have been in a rich medium as Nutrient broth. To double check on this, an M.I.C. of Sm using MacConkey broth was also done on the donors and the results compared with the M.I.C. values obtained using Nutrient broth.

The results of these two experiments are shown on the next page (Table 8).

TABLE 8: DETERMINATIONS OF THE MINIMAL INHIBITORY CONCENTRATION OF STREPTOMYCIN FOR 'RECOMBINANTS' USING NUTRIENT BROTH AND MacCONKEY BROTH

Donor	Recipient	Transmissibility	'Recombinants' MIC (Nutrient Broth)	'Recombinants' MIC (MacConkey Broth)
Tc^R Sm^R - 1	J62-1	+	5 - 10 ug/ml	(not done)
Tc^S Sm^R - 2	J62-1	+	5 - 10 ug/ml	10 - 20 ug/ml
Tc^S Sm^R - 12	J62-1	+	5 - 10 ug/ml	10 - 20 ug/ml
Tc^R $Sm^{(R)}$ - 8	J62-1	+	< 2.5 ug/ml	2.5 - 5 ug/ml
Tc^R $Sm^{(R)}$ - 10	J62-1	+	< 2.5 ug/ml	2.5 - 5 ug/ml
Tc^R Sm^R - 2	J62-1	+	2.5 - 5 ug/ml	5 - 10 ug/ml
Ap^R $Sm^{(R)}$ - 5	J62-1	+)	not done	not done
Ap^R $Sm^{(R)}$ - 8	J62-1	+)		

TABLE 9: DETERMINATIONS OF THE MINIMAL INHIBITORY CONCENTRATION OF STREPTOMYCIN FOR FECAL *E. coli* (DONOR) STRAINS USING NUTRIENT BROTH AND MacCONKEY BROTH

Isolate No	M.I.C. (Nutrient broth)	M.I.C. (MacConkey Broth)
Tc^S Sm^R - 2	10 - 20 ug/ml	> 20 ug/ml
Tc^S Sm^R - 12	10 - 20 ug/ml	10 - 20 ug/ml
Tc^R $Sm^{(R)}$ - 8	13 - 20 ug/ml	10 - 20 ug/ml
Tc^R $Sm^{(R)}$ - 10	20 - 30 ug/ml	10 - 20 ug/ml
Tc^R Sm^R - 2	< 8.7 ug/ml	2.5- 5 ug/ml

A final attempt made to study on these Sm-resistant isolates was a minimal inhibitory concentration determination of Sm for the donors as well as the J62-1 that had already received the Sm-resistant marker. The M.I.C. was done using the plate method, that is, BHI agar plates and MacConkey agar plates. The results obtained were as follows: (Table 10).

TABLE 10: DETERMINATIONS OF THE MINIMAL INHIBITORY CONCENTRATION OF STREPTOMYCIN FOR FECAL *E. coli* STRAINS USING PLATE METHOD

Isolate No: Tc^R $Sm^{(R)}$ - 8

M.I.C. using Nutrient Broth : 13 - 20 ug/ml
M.I.C. using MacConkey Broth: 10 - 20 ug/ml

	Viable Count	
Conc. of Sm (ug/ml)	BHI agar	MacConkey agar
Control (no antibiotic)	76	>43
5 ug/ml	68	48
10ug/ml	46	54
20ug/ml	21	80
30ug/ml	17	50

Isolate No: J62-1/Tc^R $Sm^{(R)}$ - 8

M.I.C. using Nutrient Broth: <2.5 ug/ml
M.I.C. using MacConkey Broth: 2.5 - 5 ug/ml

Conc. of Sm (ug/ml)	BHI agar	MacConkey agar
Control (no antibiotic)	22	34
1 ug/ml	26	18
5 ug/ml	16	17
10 ug/ml	1	27
20 ug/ml	-	14
30 ug/ml	-	16

Isolate No: Tc^R Sm^R - 2

M.I.C. using Nutrient Broth: <8.7 ug/ml
M.I.C. using MacConkey Broth: 2.5 - 5 ug/ml

Conc. of Sm (ug/ml)	BHI agar	MacConkey agar
Control (no antibiotic)	40	34
5 ug/ml	47	39
10 ug/ml	29	50
20 ug/ml	23	28
30 ug/ml	5	20

TABLE 10: CONTINUED

Isolate No: J62-1/Tc^R Sm^R - 2

M.I.C. using Nutrient Broth: 2.5 - 5 ug/m.
M.I.C. using MacConkey Broth: 5 - 10 ug/ml

	Viable Count	
Conc. of Sm (ug/ml)	BHI agar	MacConkey agar
Control (no antibiotics)	97	100
2 ug/ml	88	73
5 ug/ml	109	85
10 ug/ml	68	81
20 ug/ml	35	77
30 ug/ml	17	88

Isolate No: Tc^S Sm^R - 2

M.I.C. using Nutrient Broth: 10 - 20 ug/ml
M.I.C. using MacConkey Broth: > 20 ug/ml

Conc. of Sm (ug/ml)	BHI agar	MacConkey agar
Control (no antibiotics)	81	64
5 ug/ml	93	61
10 ug/ml	67	56
20 ug/ml	60	77
30 ug/ml	55	69

Isolate No: J62-1/Tc^S Sm^R - 2

M.I.C. using Nutrient Broth: 5 - 10 ug/ml
M.I.C. using MacConkey Broth: 10 - 20 ug/ml

Conc. of Sm (ug/ml)	BHI agar	MacConkey agar
Control (no antibiotic)	86	85
5 ug/ml	53	65
10 ug/ml	86	80
20 ug/ml	9	55
30 ug/ml	5	47

TABLE 10: CONTINUED

Isolate No: Tc^S Sm^R - 12

M.I.C. using Nutrient Broth: 10 - 20 ug/ml
M.I.C. using MacConkey Broth: 10 - 20 ug/ml

	Viable Count	
Conc. of Sm (ug/ml)	BHI agar	MacConkey agar
Control (no antibiotic)	65	52
5 ug/ml	79	38
10 ug/ml	50	69
20 ug/ml	52	76
30 ug/ml	17	48

Isolate No: J62-1/Tc^S Sm^R - 12

M.I.C. using Nutrient Broth: 5 - 10 ug/ml
M.I.C. using MacConkey Broth: 10 - 20 ug/ml

Conc. of Sm (ug/ml)	BHI agar	MacConkey agar
Control (no antibiotic)	78	67
5 ug/ml	69	38
10 ug/ml	69	92
20 ug/ml	41	75
30 ug/ml	17	55

Isolate No: Tc^R $Sm^{(R)}$ - 10

M.I.C. using Nutrient Broth: 20 - 30 ug/ml
M.I.C. using MacConkey Broth: 10 - 20 ug/ml

Conc. of Sm (ug/ml)	BHI agar	MacConkey agar
Control (no antibiotic)	93	(spoiled plate)
5 ug/ml	62	85
10 ug/ml	46	59
20 ug/ml	15	63
30 ug/ml	1	30

TABLE 10: CONTINUED

Isolate No: J62-1/Tc^{R} $Sm^{(R)}$ - 10

M.I.C. using Nutrient Broth: < 2.5 ug/ml

M.I.C. using MacConkey Broth: 2.5 - 5 ug/ml

	Viable count	
Conc. of Sm (ug/ml)	BHI agar	MacConkey agar
Control (no antibiotic)	62	79
1 ug/ml	78	54
5 ug/ml	9	71
10 ug/ml	17	30
20 ug/ml	4	2
30 ug/ml	7	-

DISCUSSION

In the present study, the fecal coliform population ranged from 6.0-14.0% of the total coliform population. In a similar investigation by Sturtevant et al (1970), in four of the five sewage treatment plants sampled, it was found that the fecal coliform population ranged from 30-60% of the total coliform population. These values are very much higher than the values obtained in the present study. The number of fecal coliforms/coliforms in sewage could vary a lot depending on the source of sewage. Sewage that comes from hospitals might be expected to have a high percentage of coliforms and fecal coliforms. Fontaine and Hoadley (1976) showed that the number of fecal coliforms per 100 ml, from hospital wastes was 1.23×10^7 which was higher than the number obtained from municipal wastes (7.9×10^6). Certain industrial wastes may be toxic to fecal coliforms thus lowering their percentage in the sewage (Bonde, 1974).

The number of bacteria in sewage is directly related to the strength of the sewage, and is expected to vary due to the heterogeneity of the sewage (Gameson, 1978; Bonde, 1974). The sewage may show hourly, daily and seasonal variations. Generally speaking, hourly variations are mainly the result of the pattern of defecation in a community; daily variations may reflect variations in domestic and industrial water usage, and both daily and seasonal variations the result of variations in rainfall (McCoy, 1971). The diurnal fluctuations in the flow of sewage on any normal day at the Palmerston North Sewage Treatment Plant does not appear to vary greatly. The flow chart obtained from the Treatment Plant (personal communication) for the June period, 1980 showed, on average, a consistent flow rate. Within any 24 hour period, there tends to be a general increase in the flow, between 1000-1300 hours, an increase from an average of 2×10^6 gals to 6×10^6 gals/day. This increase is associated with the period when most of the

domestic and industrial activity are considered to be taking place. Although the flow decreases after 1300 hours, it still remains just above average (about 3 x 10^6gals/day) with a subsequent decline after 2000 hours. The investigation made by Gameson (1978) showed an increase of coliform counts in the period, late morning to early afternoon (1000-1300 hours) and an increase in the fecal coliform counts in the period, 0700-0900 hours. Since the present study was not aimed at studying the diurnal variations of the coliforms/ fecal coliforms, therefore the numbers of the latter at particular times throughout the day were not determined. However, the numbers of coliforms obtained by Gameson were within the range of 5 x 10^5 - 2 x 10^6/ml, a result similar to the figures obtained in the present study. According to McCoy (1971), the pattern of defecation in a community is more or less constant. In general, in a large community, 50-60% of the daily load is present in the crude sewage between 0600 and 1200 hours, and 15-20% in the period noon to 1800 hours. In a small community, most of the load may be concentrated in the crude sewage before noon.

Entry of rain water to combined sewerage systems can produce 50-fold rises in the rate of flow. The composition of storm sewage is also high variable (Gameson, 1978). Investigations by Gameson showed that the concentrating suspended solids was often higher in storm sewage than in dry-weather sewage, particularly during the early stages of increased flow. It also showed that during the first few hours (while the flow rose to about four times the dry-weather value) the coliform count also increased, but that throughout the last 10 hours of a 24 hour period (while the flow was still well above average) the coliform counts were about only half of the overall medians. These results suggested the scouring out of fecal material from the sewerage system, followed by dilution of the sewage by rain. Although the present study of the Palmerston North sewage does not involve a specific study of the effect of rain on the coliforms/fecal coliforms within a day, yet from the results, it was observed that

these were very much affected by rain, although there was a lag in time before the effect could be detected.

The changes in coliforms/fecal coliforms associated with delays in analysis have also been studied on a number of occasions. As shown by Gameson (1978) the delays in analysis of the sample seemed to affect both the coliform counts as well as the fecal coliform counts. Our present study also showed similar effects. The samples were initially examined as soon as practicable, and reexamined after storage for 24 hours in the cold room. Although the total count of lactose fermenters was slightly lower than the initial total count, the recovery value for fecal *E. coli* tended to be high. It is possible that, during storage of the sample that, while the other bacteria may have been killed due to toxic substance present in the sewage, the fecal *E. coli* survived.

The reduction in the number of lactose fermenters was observed only when the stored sample was plated directly. However, if the stored sample was 'pre-warmed' in a 37^{o}C water bath and then plated at a definite time, e.g. say 30 min. time interval, the initial total count of lactose fermenters could be recovered. From the experiment that was carried out, it was found that it took 30-60 mins. before the initial total count of lactose fermenters could be recovered. Our results also showed that this value would increase up to 120 minutes, after which no further follow up of the numbers was done. However, according to Gameson, if a sample is stored for a long time (e.g. a pump house), the total count would tend to increase for the first few hours with a subsequent decline.

As Palmerston North is an urban centre within a rural environment, the sources of *E. coli* in the sewage would be expected to vary. It is not surprising that multiply drug resistant *E. coli* were isolated from the sewage of Palmerston North, particularly as antibiotics are widely used, both medically and agriculturally (Meekin et al, 1979). However, for a sewage system should contain such antibiotic

resistant organisms as were found in this study can be considered a problem.

R^+ coliform and fecal coliform bacteria found in raw and treated sewage enter rivers and seawater through effluent disposal and run-off (Sturtevant & Feary, 1969); Smith, 1970, 1971; Feary et al, 1972; Cooke, 1976a). If these coliform bacteria carrying R factors enter drinking water, their ingestion may result in establishment or in the transfer of antibiotic resistance to the normal intestinal flora, which may then act as a reservoir of resistance. Grabow et al (1974) calculated that $2R^+$ coliforms may be ingested each day by a person drinking 1000 ml of water containing 10 coliforms of sewage origin per 100 ml. Antibiotic therapy will kill or inhibit sensitive components of the bacterial flora, increasing the selective pressure for establishment of an antibiotic resistant bacterial population.

The significance of the presence of fecal *E. coli* in aquatic environments is that the water in question has been contaminated with fecal material from warm-blooded animals or humans. This is because this genus is found in the feces of men and aminals in higher densities and with greater frequency than any other coliform. However, according to Dufour (1977), the predominance of *E. coli* in feces is not reflected in the distribution of coliforms found in sewage. In a survey of a sewage treatment plant (Dufour, 1977) which receives mainly domestic waste water, showed that in raw waste water, *E. coli* makes up only about one-third to one-fourth of the coliforms detected, while the other two-thirds to three-fourths were identified presumptively as belonging to the genera *Klebsiella*, *Enterobacter* and *Citrobacter*. In a survey of the sewage treatment plant here in Palmerston North carried out in 1976 (personal communication) a similar observation was made. Although the samples taken for that survey were small, it did show that at that time, that one-third of the coliforms detected were *E. coli* while the other two-thirds were *Klebsiella* and in some cases *Citrobacter*. However, in the

present study, the fecal *E. coli* makes up about 10% of the coliforms. In another survey of the coliforms in the Palmerston North sewage in 1975/76 (personal communication) the numbers of coliforms seemed to fluctuate, this being influenced very much by *Klebsiella*. In the determination of ampicillin resistance of the coliforms, a dramatic rise in resistance to ampicllin was observed over the winter months. But in later investigations, it was established that some of the resistance observed was not due to R factors, and that 50% of the isolates were not fecal *E. coli*. It was this observation that prompted the investigation of fecal *E. coli* and in particular the antibiotic resistance within these isolates. As shown from the results obtained in the present study, there was also a dramatic rise of ampicillin resistance among the fecal *E. coli* isolates during the winter months. This probably coincides with the increased use of this broad-spectrum antibiotic over the winter months.

The fecal coliform test selects mainly for the genera *Escherichia* and *Klebsiella* with occasional positive reactions being given by other genera. This lack of specificity was one of the determining factors which prompted the call for a re-examination of the definition for fecal coliforms, or more properly, the definition of a coliform indicator consistently and specifically associated with fecal contamination (Dufour, 1977). One of the earliest efforts to differentiate the coliforms to those coliforms related to feces was carried out by Eijkman in 1904 (cited by Dufour, 1977), who used gas production from glucose at an elevated incubation temperature to detect coliforms associated with fecal pollution. Modifications of his medium by others has led to the methods used today to detect the fecal coliforms. The improved specificity of the fecal coliform index relative to that of the coliform index led to its acceptance and subsequent widespread use. In the identification of fecal *E. coli*, the elevated temperature of 44.5^{o}C is included because the ability to grow and ferment lactose at an elevated temperature has been shown over the years to be

highly characteristic of *E. coli* (American Public Health Association, 1971).

In the identification of fecal *E. coli* in the present study, great care was taken in the techniques carried out, and, in particular, in the maintenance of the incubation temperature. In a surveillance study like this one, it is important to have some standardisation in the methods used for assessing the numbers and types of coliform bacteria. By doing so, the results of laboratory findings can be compared one with another, and be validated. Any variations in the techniques or in the incubation temperature can result in higher or lower counts, or disallow the comparison of one series with another (New Zealand Microbiological Society, 1976).

In the use of the 44.5°C as the selective temperature for fecal coliforms and *E. coli*, it was found that in order to assess the numbers of fecal coliforms on the MacConkey plates, it would be preferable if the plates were pre-incubated at 37°C prior to overnight incubation at 44.5°C. The length of time adopted for pre-incubation at 37°C was 1½ hours. In the survey carried out, it was found that if the inoculated plates were placed in the 44.5°C incubator immediately, the number of fecal coliforms and *E. coli* were very much reduced. It was this observation that prompted the proposal to pre-incubate the inoculated plates at 37°C for 1½ hours. However, another set of plates was routinely inoculated and incubated at 37°C; these served as a control and thus allowed comparisons to be made of the number of fecal coliforms to the total number of coliforms. In this particular survey, the incubation temperature seemed to be particularly important and therefore, the temperature of the incubator and the water bath were constantly monitored.

The numbers of lactose-fermenting bacteria resistant to any single antibiotic has been determined by plating the sewage sample onto MacConkey plates containing the particular

antibiotic. From plates of this type, several lactose-fermenting colonies were picked and their drug resistance determined. When this was done, it was frequently found that the isolates examined were resistant to two or more antibiotics. This has been shown by many workers who have worked with resistance transfer factors (Sturtevant, 1969, 1970; Linton et al, 1974; Grabow & Prozesky, 1973; Fontaine & Hoadley, 1976; Woods et al, 1972). It was this observation that prompted Sturtevant and Feary (1969) to select for antibiotic-resistant bacteria by the use of selective media containing more than one antibiotic.

In the present study, an attempt was made to validate the above technique using a "spotting" method, whereby the cultures were spotted onto media containing antibiotics, either singly or in combination. By comparing the results obtained from the two methods (direct plating vs the "spot" method), it was hoped that some kind of conclusion could be made about the validity of the two methods and the techniques involved, and perhaps a proposal as to the preferred method might be made and used in the future as a rapid preliminary means of isolating and screening for antibiotic resistant bacteria.

It was a matter of concern that the inoculum size, inherently a feature of the "spotting" method might increase the score of isolates recorded as resistant as compared to the direct plating method where single bacteria must survive the challenge, initiate growth and develop a colony. However, the present study had as a major goal, the validity of readily applied screening methods, appropriate to routine surveillance, and therefore some restraints had to be applied to the methods used. In spite of the above concern, there were no significant differences in the results obtained by these two methods.

The values obtained for single antibiotic resistance by both methods were in close correlation. In obtaining the number of fecal *E. coli* resistant to two antibiotics, the values obtained fluctuated. In some cases, the values obtained from direct plating and spot method, showed close agreement, in some cases they do not. However, the differences observed were not great. In most of the cases observed, the numbers of resistant bacteria obtained by direct plating were slightly lower than those obtained by the spot method. This could perhaps be due to the effect of dilution or the combined effect of the two antibiotics in the medium. In the low formulated by Jawetz and Gunnison in 1952 (Garrod, Lambert & O'Grady, 1973) concerning antibiotic combinations, bacteristatic antibiotics may antagonize the action of bactericidal drugs, and that the latter may be synergistic when used together. This was later reviewed in 1957 by Dowling who showed that mixtures in vitro of bactericidal drugs frequently showed synergism, sometimes indifference, never antagonism. Mixtures of bacteristatic drugs are neithersynergistic nor antagonistic to each other but simple additive effects were often observed. However, when a bactericidal drug is used with a bacteristatic drug, the effect may be synergism, indifference or antagonism; depending on the relative amounts of the antibiotics used in the combination and the susceptibility or resistance of the microorganism to the individual antibiotic. However, within this general scheme, many possible variations exist. For example, when penicillin (bactericidal) is combined with tetracycline (bacteristatic), it was shown that penicillin antagonized the action of tetracycline (Lepper & Dowling, 1951). However this antagonism was not fully corroborated by studies of penicllin (bactericidal) and chloramphenicol (bacteristatic). Depending on the organism and the amounts of the antibiotics used in the combination, antagonism of penicillin by chloramphenicol may or may not occur (Wallace, 1967; Bodine et al, 1977; Carrizosa et al, 1975). Similarly, aminoglycosides plus tetracycline or chloramphenicol have been shown to be antagonistic (Jawetz et al, 1951; White & White, 1964; D'Alessandri et al, 1976). But to date, no

clinical evidence is available regarding the use of this combination for Gram-ve infections to support or refute the possibility of antagonism (Rahal, 1978). Despite a large volume of in vitro and other experimental data, clinical evidence for synergy or antagonism between antimicrobial agents is sparse.

Our results suggest that, within experimental error, it is valid to plate the sample directly on to antibiotic-containing MacConkey agar plates for the isolation of antibiotic-resistant organisms.

With regard to the streptomycin (Sm)-resistant isolates, because of the fact that the 'intriguing' behaviour of some isolates was observed only when these were picked from MacConkey + tetracycline (Tc) plates, it is suggested that their behaviour was due to the nature of the plasmid carrying the Tc-resistant marker. A wide variation in the characteristics of expression of resistance to the tetracyclines by naturally occurring R plasmids in *E. coli* has been found (Foster & Walsh, 1974). The different phenotype classes of R plasmid could result from differences in the expression of a common Tc determinant on different plasmids. The Tc resistance genes are often contained in an element called transposon and this element can be translocated from one replicon to another by an insertion process (Chopra & Howe, 1978). These transposable elements may serve as biological "switches" capable of turning on and off the expression of nearby genes as a consequence of their insertion into or excision from plasmid genome (Cohen, 1976). Translocation (transposition) of DNA segments carrying antibiotic resistance genes have been reported in a number of cases. Mitsuhashi and coworkers (quoted by Cohen, 1976) described the spontaneous integration of R-plasmid genes for chloramphenicol resistance of various sites of the *E. coli* chromosome, and the subsequent movement of the chloramphenicol resistance trait on to another R plasmid or on to bacteriophage PI, which could then transfer the resistance inter-

bacterially. Other observations suggested that Tc-resistance genes were also capable of moving from plasmid to plasmid or from plasmid to chromosome (Harada et al, 1967; Mitsuhashi, 1969). In 1968, Anderson et al reported observations suggesting that ampicillin (Ap)-resistance determinant from one plasmid could become associated with another plasmid. Other observations were also made by Kopecko and Cohen (1975); and Barth et al (1976). At this time, structurally defined DNA segments coding for resistance to ampicillin, streptomycin-spectinomycin, sulphonamides, chloramphenicol, tetracycline and trimethoprim have been shown to be capable of translocation from genome to genome, and it seems that the sequential translocation of such segments may be the principle mechanism by which plasmids can accumulate multiple antibiotic resistance determinants (Cohen, 1976).

In *Staph. aureus*, Asheshov (1975) observed two phenotypic patterns of high-level resistance to the tetracyclines. One phenotype was determined by plasmid-located genes, and the other is determined by chromosomal genes. And the different phenotypes observed was explained by assuming that the resistance genes of the plasmid were contained in a transposon, the expression of which is altered by integration into the chromosome. There is evidence that genes for resistance to some autibiotics are part of transposous in *Staph. aureus* (Schweisinger, 1977) and in Gram negative bacteria, transposition of Tn10 from a plasmid to a chromosomal locus has occurred under natural conditions (Elwell et al, 1977)

More detailed work needs to be done to characterise the Sm-resistant isolates obtained in this study; for the moment, only speculation can be made on the observed behaviour of these isolates. The fact that two phenotypic pattersn of Sm-resistance was observed, could suggest that one phenotype was determined by plasmid-located genes and the other was by chromosomally-located genes, as was observed with *Staph. aureus*. Another speculation is that perhaps two different plasmids were involved - one with a Sm marker which gives rise

to high-level of resistance to Sm, and the other contains a Sm marker which gives a low level of resistance to Sm. From the colonial morphology, it was expected that the confluent colonies were associated with high level resistance while the scattered colonies (R) were associated with low level resistance. However, on the basis of the M.I.C. values of these isolates, this does not appear to be so.

Sm resistance can be mediated by R factors that contain genes for adenylylating or phosphorylating enzymes (Benveniste et al, 1970; Harwood & Smith, 1969; Okamoto & Suzuki, 1965; Ozanne et al, 1969; Yamada et al, 1968). The two distinct enzymes Sm adenylate synthetase and Sm phosphotransferase are synthesized constitutively. According to Nordstrom et al (1972), there is a linear correlation between resistance and enzyme production. The results obtained in the present study indicate that if the resistance of the Sm isolates were actually due to the action of the inactivating enzyme then, the effect of this could be seen right from the time when the isolates were being spotted onto BHI + Sm agar plates. Since all the isolates were spotted onto one common plate containing 20ug/ml Sm, it would be expected that the enzymes produced would cause all the isolates on the plate to be resistant and hence grew confluently. Furthermore, if the resistance observed is actually due to drug-inactivating enzymes, then the isolates that gave confluent growth on these plates, would also be expected to give high M.I.C. values. But the fact that these isolates gave very low M.I.C. values as opposed to the (R) colonies that gave very high M.I.C. values, this could not be explained by the action of the drug-inactivating enzymes.

The level of R-mediated aminoglycoside resistance, but not that of chromosomal-based resistance, was shown to vary with inoculum size (Harwood & Smith, 1969; Okamoto & Suzuki, 1965). A few of the confluent and the (R) isolates were picked and the effects of inoculum size tested on them. No

difference was observed. Perhaps too few isolates were tested and therefore the results obtained may not have been conclusive enough. According to Richmond (1965), the inoculum effect has been associated with extra-cellular drug-destroying enzymes, but the results obtained in the present study seemed to rule out that possibility.

The levels of resistance conferred by R factors vary from drug to drug, from factor to factor and also the same factor in different hosts gives different levels of resistance (Watanabe, 1966; Smith, 1969). The resistance of the Sm isolates obtained in the present study was shown to be plasmid-mediated. The isolates that were chosen for the transfer experiment were resistant to Sm at a concentration of 10-30 ug/ml. However, when this resistance was transferred to J62-1, the resistance of the recipients that had received this resistance, was found to be very much lowered, ranging from <2.5-10 ug/ml Sm, except for a few cases where the M.I.C. values ranged from 10-20 ug/ml Sm. This difference in resistance to Sm is thought to be due to the nature of the host receiving the plasmid.

The M.I.C. of these Sm-resistant isolates was also determined by using MacConkey broth to check if the difference in the MacConkey and nutrient broth would have an effect on the M.I.C. However, the results showed that there was no significant difference in the M.I.C. values of the isolates. No difference was also observed in the M.I.C. values of the recombinants. A similar attempt was also made by the plate method using BHI agar and MacConkey agar. The results showed that agar and broth M.I.C.'s correlate poorly for the Sm isolates. In almost all cases, it showed that the M.I.C. obtained by the plate methods were very much higher than those obtained by the broth dilution method. One of the factors that could contribute to the high M.I.C. value obtained by the plate method is the heterogeneity of the agar (Rylander et al, 1979). Furthermore, the number of bacteria exposed to antibiotic activity is larger in the

recommended broth dilution method, hence lowering the M.I.C. value as compared to those obtained by the plate method (Ericsson et al, 1971; Washington & Barry, 1974).

However, considering the two media used in the determination of M.I.C., the results showed that BHI agar proved to be the better medium of the two; in most cases, regardless of whether the M.I.C. was done on the donors or the recombinants, there was a marked reduction in the number of resistant bacteria on BHI agar containing 20-30ug/ml Sm. However, this was not observed with the MacConkey agar, which seemed to maintain the number of resistant bacteria even when the concentration of Sm in the plates was as high as 30 ug/ml.

GENERAL CONCLUSION

Since its discovery in Japan in 1959 (Watanabe, 1963), infectious drug resistance, mediated by episomal elements known as R factors, has been shown to be an important factor in the spread of multiple antibiotic resistance among all members of the *Enterobacteriaceae* as well as to unrelated Gram-ve bacteria such as *Ps. aeruginosa*, *Vibrio cholerae* and *Pasteurella pestis*. The major selective force favouring the emergence of drug-resistant bacteria is antibiotic usage and thus it is not surprising that the wide distribution and high incidence of R factors among Gram-ve bacteria have been noted mainly among clinical isolates associated with human and animal disease (Anderson, 1968). However, R factors have also been identified among the Gram negative intestinal flora of presumably healthy individuals (Datta, 1969; Moorhouse, 1969) and therefore their detection and characterisation among bacteria found in sewage could serve as an indication of the level of infectious drug resistance existing in the general population (Sturtevant & Feary, 1969; Grabow & Prozesky, 1973; Linton et al, 1974; Smith, 1970; Sturtevant et al, 1971).

The percentage of resistant bacteria varies greatly depending on a multiplicity of factors, from as little as 0.1% to as much as 100% (WHO Technical Report, 1978) but in the present study, fecal *E. coli* was particularly chosen because they are inherently sensitive and specific indicators of fecal contamination whether human or animal. They are indicators of fecal contamination, and the frequency with which they prove resistant to antibiotics will indicate how frequently they are resistant in the human flora. These bacteria are often abundant in the aquatic environment; they survive relatively well and theoretically do not multiply. The concentration of antibiotic resistant fecal *E. coli* in water will be an indicator of the utilisation of antibiotics in hospitals or as additives in animal feeds. Furthermore,

E. coli are virtually ubiquitously distributed in the intestines of man and the warm-blooded animals, where they comprise about 1% of the total bacterial flora (Mitsuoka & Hayakawa, 1972). Therefore sewage is likely to contain *E. coli* in relatively large numbers from these sources.

One of the advantages of bacteria as indicators is that they may be identified by simple techniques, so that a large number of samples can be handled at one time with greater sensitivity than that of physico-chemical methods (Bonde, 1974). Apart from fecal *E. coli* as the indicator organism in sewage, other organisms that are becoming particularly important in the study of plasmids and transferable drug resistance are *Ps. aeruginosa* and *Cl. perfringens*. *Ps. aeruginosa*, while it is often considered to be a ubiquitous bacterial inhabitant of surface waters and soil, appears to enter the environment mainly with human fecal wastes or with the fecal wastes of animals associated with man. The isolation of this organism from surface waters suggests the influence of man, and its numbers reflect the degree of pollution. (Hoadley, 1977). However, they survive only for short periods, and there frequently appears to be little relation between populations of *Ps. aeruginosa* and those of other pathogens or fecal indicators.

Ps. aeruginosa is an opportunistic pathogen of man and animals which may be spread by water. Particularly important is that *Ps. aeruginosa* is resistant to many antibiotics. Gentamicin has been the most effective antibiotic for pseudomonas infections and initially, the majority of strains were sensitive to it. However, recent reports showed that the incidence of gentamicin-resistant strains of *Ps. aeruginosa* is increasing (Meyer et al, 1976), that this resistance is frequently plasmid-borne (Knothe et al, 1973; Bryan et al, 1974; Jacoby, 1974) and that it is often associated with resistance to other aminoglycoside antibiotics. Although the gentamicin-resistant isolates of *Ps. aeruginosa* isolated in

Auckland, New Zealand (Bremner, 1979) did not transfer their resistance to *E. coli*, but it can be transferred within their own species. All these findings support the view that this mechanism is responsible for the emergence of resistant strains that are reducing the value of gentamicin in the management of infections caused by *Ps. aeruginosa*.

The enumeration of *Cl. perfringens* spores as a water quality indicator appears to have specific and limited applications, primarily as an adjunct to the commonly used coliform indicator. As Cabelli (1977) pointed out, it appears to be a microbial indicator of choice when the requirement is for measuring remote and intermittent sources of pollution reaching an area (such as, tracing sewage sludge dumped into bodies of water or the infiltration of ground water supplies with fecal wastes) and in instances where other microbial indicators are rapidly destroyed, such as chlorinated water supplies. In addition, it has promise as a conservative tracer for use in field studies on the movement and decay of pathogenic micro-organisms in sewage and sludge.

Clostridium perfringens can cause gas gangrence or enterotoxemic disease in man and animals. Very few plasmid-determined functions have been reported in this organism. However, in 1974/75, Sebald (1975) reported two strains of *Cl. perfringens* that were resistant to clindamycin, chloramphenicol, erythromycin and tetracycline. For one of these strains, two plasmids have been demonstrated - one is a transferable plasmid encoding resistance to tetracycline and chloramphenicol (Sebald & Brefort, 1975), and the other a nontransferable plasmid encoding resistance to erythromycin and clindamycin. In 1978, Rood et al isolated multiple-drug resistant *Cl. perfringens*, and although attempts made to transfer this resistance was unsuccessful, this does not necessarily rule out the possibility that the resistance was plasmid determined. It is possible that the R plasmid present was of a nontransferable type (Rood et al, 1978).

The spread of R^+ bacteria may be limited by better control of drug use as proved in isolated environments such as hospitals (Gardner & Smith, 1969; Bulger et al, 1970; Lowbury et al, 1972; Richmond, 1972). It seems, however, unlikely that drug consumption could be controlled to such an extent as to affect the general incidence of R^+ bacteria (Jukes, 1972; Richmond, 1982). The dissemination of these organisms may also be limited by reducing their transmission (Gardner & Smith, 1969) by sewage polluted water. Smith (1971), Feary et al (1972) and Grabow et al (1975) showed that insufficiently purified sewage is the main source of drug resistant bacteria in rivers, dams and bathing beaches. These findings and the properties of drug resistant bacteria support the view that sewage-polluted water may play an important role in the spread of R^+ and R^- bacteria. Sewage should therefore be treated by more advanced methods, prior to discharge into water destined for drinking, irrigation or recreation, and thus protect the population against this hazard (Smith, 1971, 1973; Feary et al, 1972; Grabow et al, 1973, 1974; Gardner & Smith, 1969; Dixon, 1972; Richmond, 1972). Routine surveillance of sewage at periodic intervals for the detection and characterization of prevailing R factors may serve as a means of detecting significant changes in the resistance patterns of prevailing R factors and of detecting changes in the incidence of specific R factors to be found in the general population (Sturtevant & Feary, 1969).

APPENDIX I

All media were sterilised by autoclaving at 15 psi for 15 minutes.

BRAIN HEART INFUSION BROTH (BHI) BROTH Difco

Calf Brain Infusion	200 g
Beef Heart Infusion	250 g
Proteose peptone, Difco	10 g
Bacto - Dextrose	2 g
Sodium chloride	5 g
Disodium phosphate	2.5 g

20 g of this made up to 1 litre with distilled water. For BHI agar, add 1.5% Davis agar.

NUTRIENT BROTH Difco

Bacto-beef extract	3 g
Bacto-peptone	5 g

8 g of this made up to 1 litre with distilled water.

MacCONKEY BROTH Gibco

Peptone 190	20 g
Lactose	10 g
Bacteriological bile	5 g
Bromcresol purple	0.01 g

35 g of this made up to 1 litre with distilled water.

MacCONKEY AGAR Difco

Bacto-peptone	17 g
Proteose peptone, Difco	3 g
Bacto-Lactose	10 g
Bacto-Bile salts No. 3	1.5 g

MacConkey Agar Continued

Sodium chloride	5 g
Bacto-Agar	13.5 g
Bacto-Neutral Red	0.003 g
Bacto-Crystal Violet	0.001 g

50 g of this made up to 1 litre with distilled water.

PEPTONE WATER

Difco peptone	10 g
Sodium chloride	5 g
Distilled water	1 litre
pH adjusted to 6.8	

KOVAC'S REAGENT

Para-dimethyl-amino benzaldehyde	5 g
Amyl or butyl alcohol	75 ml
Concentrated hydrochloric acid	25 ml

SIMMONS CITRATE AGAR BBL

Ammonium dihydrogen phosphate	1 g
Dipotassium phosphate	1 g
Sodium chloride	5 g
Sodium citrate	2 g
Magnesium sulphate	0.2 g
Agar	15 g
Brom- Thymol Blue	0.08g

24.2 g of this made up to 1 litre with distilled water.
Final pH 6.9 $\pm$ 0.2.

APPENDIX II

PREPARATION OF ANTIBIOTIC STOCK SOLUTIONS

Ampicillin (Sigma Chemical Company)

Supplied as dry white powder in a vial containing 25 gm (Lot No. 39C - 0341). A stock solution of 5 mg/ml was prepared by dissolving 72.5 mg of ampicillin in 14.5 ml solvent. (7 ml deionised water + 0.9 ml $^{N}/10$ NaOH before adding 6.6 mls of minimal salts medium to make up the final volume of 14.5 mls).

Chloramphenicol (Sigma Chemical Company)

Supplied as white, dry powder in a vial containing 25 gm of chloramphenicol. (Lot No: 26C - 0018).
0.1 g of the dry powder was dissolved in 20 mls of ethanol giving a stock solution of 5 mg/ml. Further dilutions were made with sterile distilled water to give the appropriate working solutions.

Nalidixic acid (Sigma Chemical Company)

Supplied as white, dry powder in a vial containing 5 g of malidixic acid (Lot No: 18C - 0457 - 1).
A stock solution of 10 mg/ml was prepared by dissolving 0.5 g of the dry powdered in 50 mls of 0.1N NaOH. Further dilutions were made with sterile distilled water to give the appropriate working solutions.

Streptomycin Sulphate (Glaxo)

Supplied in vials, as a white sterile powder. Contents equivalent to 1 g streptomycin base. (Batch No. 7HP 182D).

Streptomycin sulphate Continued

Volume of sterile distilled water added to the 1 gm vial.

4.25 ml to give 200 mg
3.25 ml to give 250 mg
1.25 ml to give 500 mg

Further dilutions were made with sterile distilled water to gove the appropriate working solutions.

Tetracycline (Sigma Chemical Company)

Supplied as yellow, dry powder in a vial containing 25 g of tetracycline. (Lot No: 49C - 0448).
A stock solution of 6 mg/ml was prepared by dissolving 0.12 g of the dry powder in 20 mls of methanol. Further dilutions were made with sterile distilled water to give the appropriate working solutions.

All antibiotic solutions were filter sterilized through a 0.22 um millipore filter and stored at -20^{o}C.

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