ENTEROBACTERIAL PLASMIDS:
A STUDY OF THEIR DISTRIBUTION, MOBILITY AND EPIDEMIOLOGY AMONG CLINICAL ISOLATES.

by

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SUMMARY

Bacterial plasmids are accessory genetic elements, many of which have the ability to promote their transfer between different bacterial strains, species and genera by conjugation. They also confer diverse additional properties upon their host bacterium, many of which result in phenotypic changes. Among the first to be recognised was resistance to antimicrobial agents which has become a major area of research because of its impact in clinical infection.

The aim of the study was to characterise enterobacterial plasmids in vitro using methods that provided information concerning their behaviour in vivo with particular regard to epidemiology, antibiotic resistance and the development of a clearer view of plasmid ecology. Initial steps included selection of a patient population which provided sites, accessable to repeated sampling without the use of invasive techniques. Patients with vascular disease were chosen, different types of wound defined and patterns of wound colonisation with coliforms were determined; differences in the isolation incidence of coliforms and other potential pathogens from the different types of wounds studied were demonstrated. The severity and duration of different wound

types also varied; however, the administration of topical and/or systemic antimicrobial therapy did not appear to greatly influence the results found.

In addition to the study of resistance (R) plasmids, the distribution and conjugative ability of plasmids not associated with resistance was investigated. A method based on the mobilisation of non-conjugative R-plasmids ($Tra^- R^+$) was established to determine the conjugative ability of plasmids not associated with resistance ($Tra^+ R^-$). A restriction enzyme fingerprinting strategy was developed and fingerprints of plasmids from clinical isolates and transconjugants were compared within various sub-groups of organisms. These included conjugative and non-conjugative plasmids, plasmids with common resistance determinants and plasmids from isolates within the same and between different bacterial genera.

The major difference between the distribution of plasmids in Escherichia coli and other enteric genera (OEG) was the large proportion of the latter group (64%) which was plasmid-free compared to the former group (13%). Moreover, a much higher proportion of plasmid-containing E.coli isolates harboured conjugative R-plasmids ($Tra^+ R^+$) (45%) compared to plasmid-containing OEG isolates (10%). Using the methods described, only 6 per cent of resistant plasmid-containing

E.coli were shown to harbour Tra^+ R^- plasmids compared to 58 per cent of the sensitive sub-population. In contrast, 45 per cent of resistant plasmid-containing OEG harboured Tra^+ R^- plasmids compared to 41 per cent of the sensitive sub-population.

Ampicillin resistance was the most common determinant and was transferred from 81 per cent of isolates that harboured Tra^+ R^+ plasmids. It was detected both as a single determinant (31%) and in combination with at least one additional resistance determinant (50%). Resistance to tetracycline, streptomycin and sulphamethoxazole were each transferred from more than 30 per cent of isolates harbouring Tra^+ R^+ plasmids, either individually or in combination with other agents. Resistance to kanamycin, chloramphenicol and trimethoprim were each transferred from less than 15 per cent of these isolates. Transferable resistance determinants were found in a much larger percentage of resistant E.coli (73%) compared to resistant OEG (14%), whereas non-transferable resistance was more common in OEG (92%) than in E.coli (55%).

Plasmid fingerprinting analysis was used to identify pairs of coliforms isolated from wound and rectal swabs from a number of patients. This confirmed that many of the coliforms isolated from wounds were from the patients' gastro-intestinal

tract. The results also indicated that some coliform genera, especially Proteus spp., were better able to colonise/infect wounds compared to E.coli. Failure to demonstrate parallel isolation of a given coliform from wound and rectal swabs suggested an environmental source of organisms colonising wounds rather than faecal contamination. In few instances were identical organisms (defined by plasmid profiles, resistance determinants and fingerprints) associated with different patients which indicated that cross-infection was uncommon.

Plasmid transfer in vivo was considered and the indices "transfer potential" and "transfer rate" were defined. Among the organisms studied the transfer potential was 280 and transfer rate 1:70. This included 151 possible events involving 50 Tra^+ R^+ plasmids and 129 possible events involving 46 Tra^+ R^- plasmids. The actual transfer rate found was 1:50 involving Tra^+ R^+ plasmids and 1:129 involving Tra^+ R^- plasmids. None of these events were associated with a patient receiving antimicrobial therapy to which the plasmids specified resistance. Thus, the detection of 4 in vivo transfer events from three patients indicates that in vivo transfer is a

relatively common occurrence and that the cumulative effect of these events may play an important role in the dissemination of plasmids.

Eleven isolates of Proteus spp. possessed conjugative plasmids; one was a conjugative R-plasmid and 10, although conjugative, did not determine antimicrobial resistance. However, all were unstable on transfer to E.coli K12. These results, together with the results from plasmid fingerprinting suggested that plasmids from Proteus spp. were closely related with respect to both their narrow host range and molecular structure.

E.coli K12 transconjugants from 10 of the 21 Klebsiella spp., Enterobacter spp. and Citrobacter spp. were unstable. This suggested that plasmids from these genera consist of two types, those with a broad host range, stable in E.coli and others with a narrow host range which were stable only in closely related genera.

Plasmid fingerprints from E.coli were difficult to interpret due to the diversity of plasmids, the large numbers present and the fingerprinting strategy used. However, it appears that the plasmid pool in E.coli was large. The majority of the conjugative plasmids from clinical E.coli isolates were stable in E.coli K12 recipients.

Thus, distribution and fingerprinting results demonstrated that the plasmids of E.coli constitute a large and diverse pool of extrachromosomal DNA. The plasmids of Klebsiella spp., Enterobacter spp. and Citrobacter spp. were less diverse than E.coli but more diverse than Proteus spp. plasmids which formed a small, closely related group.

ABBREVIATIONS

Ap	ampicillin
BHI	brain heart infusion
CFA	colonisation factor antigen
CI	clinical isolate
CLED	cysteine lactose electrolyte deficient
Cm	chloramphenicol
Col	colicin
DCA	deoxycholate citrate agar
DNA	deoxyribonucleic acid
EDTA	disodium ethylenediaminetetra-acetic acid
Inc	incompatibility
kb	kilobase
Km	kanamycin
lac^+	lactose-fermenting
lac^-	non-lactose-fermenting
LT	labile toxin
Md	megadalton
Nal^R	naladixic acid-resistant
OEG	enteric genera other than E.coli
pKM	partial kanamycin
pSm	partial streptomycin

PVD	peripheral vascular disease
R	resistance
Rif^R	rifampicin resistant
SDS	sodium dodecyl sulphate
Sm	streptomycin
ST	stable toxin
Su	sulphamethoxazole
Tc	tetracycline
TCT	transconjugant
TE	tris, EDTA buffer
TES	tris, EDTA, salt buffer
TGE	tris, glucose, EDTA buffer
Tn	transposon
Tp	trimethoprim
Tra^+	conjugative
$Tra^+ R^+$	conjugative resistance plasmid
$Tra^+ R^-$	conjugative plasmid not associated with resistance
Tra^-	non-conjugative plasmid
$Tra^- R^+$	non-conjugative resistance plasmid
$Tra^- R^-$	non-conjugative plasmid not associated with resistance

INTRODUCTION

Bacterial Plasmids:

Bacterial plasmids are intracellular, autonomously replicating, covalently closed circular deoxyribonucleic acid (DNA) molecules. They have been described as sub-cellular organisms although whether as endosymbionts or endoparasites remains conjectural and probably reflects not only the host-plasmid relationship but also the prevailing environmental conditions. Plasmids are not essential for the survival of the host cell but can confer properties which may be advantageous in some environments and they increase the gene pool. Most plasmids can be classified by incompatibility (the inability of closely related plasmids to stably co-exist in the same host cell) and phenotypic properties conferred by them such as antibiotic or heavy metal resistance (R-plasmids) and colicin production (Col plasmids). Some plasmids exhibit a restricted host range and replicate stably within a single species whereas others are found among diverse genera and are not limited even to taxonomic families. The transferability of easily detected markers such as antibiotic resistance and colicin, haemolysin or toxin production, has been used to infer the presence of plasmids but recent advances in physical

methods has not only confirmed their presence but has also shown that many naturally occurring organisms harbour plasmids to which no phenotype can be readily attributed at present. These are usually termed cryptic plasmids.

A property of many plasmids is their ability to promote self-transfer to related organisms by conjugation (Tra^+) but non self-transmissible plasmids (Tra^-) have also been found. Tra^- plasmids can be further subdivided on the basis of whether they are mobilised by none, few or many Tra^+ plasmids when co-resident in the same cell. Conjugative R-plasmids (Tra^+ R^+) have been the most intensively studied group of plasmids because of their easy detection and significance in the hospital environment.

Bacterial plasmids vary in size from about 1.5 megadaltons (Md) to over 400 Md. All plasmids are capable of independent replication and contain an origin of replication. The regulation of copy number is determined by the plasmid and is related to incompatibility group. Multiple copies (10-40) of small plasmids are normally found in the host cell where regulation of copy number is under relaxed control, whereas only one to 4 copies of large plasmids are maintained under stringent control. Small plasmids are largely dependent on gene products encoded by the host chromosome for replication

although they may carry a small number of genes in addition to those which are involved in the control of their own maintenance and replication. Large plasmids are less dependent on host functions as they encode a larger proportion (or all) of the genes required for their stable maintenance and replication.

In addition to genes for maintenance and replication a proportion of large plasmids also specify genes that promote conjugal transfer and include the genes involved in the production of sex pili which are essential for conjugation. The structure of sex pili is related to the plasmid incompatibility group.

The discovery of R-plasmids:

R-plasmids were first discovered in 1959 in Japan when Akiba and independently Ochiai (cited by Watanabe, 1963) demonstrated that multi-resistant strains of Shigella flexneri transferred their resistance determinants to Escherichia coli in mixed cultures.

Sh.flexneri isolates resistant to streptomycin, chloramphenicol, tetracycline and sulphonamides, antmicrobial drugs widely used in the treatment of dysentry, had been isolated with increasing frequency after the introduction of

these agents. Akiba and Ochiai demonstrated that resistance determinants could not be transferred from cell-free filtrates of Sh.flexneri to E.coli. Successful transfer was however apparent when both organisms were present in mixed cultures. These results suggested that cell-to-cell contact (conjugation) was essential for the transfer of resistance determinants.

Thus, the discovery of R-plasmids, because of their medical importance, has led to many investigations into the characteristics of infectious drug resistance in bacteria. Genetic and molecular studies of these, and many other plasmids which have subsequently been isolated, has also led to a large area of both fundamental and applied research.

R-plasmids isolated from different bacterial genera:

Although conjugative R-plasmids were first discovered in shigellae in Japan their presence has since been demonstrated world-wide in almost all bacteria in which they have been sought. Many of these are pathogenic for man and animals therefore their capacity to harbour R-plasmids can directly affect their ability to survive and cause infection.

Many members of the family Enterobacteriaceae are human pathogens and R-plasmids have been found within all the main medically-important species. Following their initial

recognition, R-plasmids have been demonstrated in Shigella spp. isolated in many countries. In Britain they have been isolated both from infections with indigenous Sh.sonnei (Davies, Farrant and Tomlinson, 1968a) but more commonly from other Shigella spp. usually associated with foreign travel (Frost and Rowe, 1983; Gross et al, 1981). Outbreaks of infection with resistant Shigella spp. in foreign countries (Frost et al, 1981) continue to cause major therapeutic problems.

Salmonella spp. have also been shown to harbour R-plasmids, the incidence of multiple drug resistance in Sal.typhimurium isolated in Britain having increased from about one per cent in 1971 to about 9 per cent in recent years (Rowe and Threlfall, 1984): this topic has been recently reviewed (Stuart-Harris and Harris, 1982). Other salmonella serotypes have also been shown to harbour R-plasmids (Threlfall et al, 1983). Of particular importance was the demonstration of transferable antibiotic resistance in Sal.typhi, especially resistance to chloramphenicol, which since 1972, has become a major cause for concern in many countries (Anderson and Smith, 1972; Paniker and Vimala, 1972). In the UK the isolation of resistant strains from people returning from countries where the organism is endemic, is not uncommon (Anderson, 1975a).

In addition to being a commensal of the gastro-intestinal tract of man and many animals, some strains of E.coli are human pathogens. Strains that cause diarrhoea in man can be divided into three groups according to the disease symptoms:-

(1) Enteropathogenic E.coli cause gastroenteritis, mainly in infants, and enterotoxin production is involved in the pathogenicity of these strains.

(2) Enterotoxigenic E.coli produce enterotoxins similar to those produced by Vibrio cholerae.

(3) Enteroinvasive E.coli, which cause symptoms similar to shigella dysentery and which have the ability to invade the intestinal epithelium.

On a worldwide basis, the former two groups are the most important and contain many resistant strains. For example, more than half of the enteropathogenic E.coli isolated in Britain in 1980 and 1981 possessed at least one resistance determinant (Gross et al, 1982) and 63 per cent of these resistant strains harboured conjugative R-plasmids. The

proportion of resistant strains among enterotoxigenic E.coli varies greatly. In a study of isolates from 14 different countries 26 per cent were shown to be resistant (Scotland et al, 1979) whereas 72 per cent of isolates from the Far East were resistant (Echeverria et al, 1978). R-plasmid transfer was demonstrated from up to 80 per cent of resistant isolates in the latter study.

Of increasing importance in the hospital environment are nosocomial infections caused by multi-resistant coliforms [1] (Casewell, 1982). The most commonly encountered genera include Escherichia (Hughes, Bauer and Roberts, 1981; Sadowski et al, 1979), Klebsiella (Casewell, Talsania and Knight, 1981; Markowitz et al, 1980), Serratia (Thomas et al, 1977; Bullock et al, 1982), Enterobacter (Markowitz, Smith and Williams, 1983; Tantulavich et al, 1981), Citrobacter (Tompkins, Plorde and Falkow, 1980) and Proteus (Coetzee, Datta and Hedges, 1972) and transferable R-plasmids have been shown to be responsible for resistance within all of these genera.

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[1] Coliforms used as a trivial collective term to describe any member of the Enterobacteriaceae.

During recent years outbreaks of infection caused by multi-resistant Serratia spp. and Klebsiella spp. are among the best documented. For example, an epidemic of colonisation and infection involving more than 400 patients over a two year period which was initially caused by multi-resistant Ser. marcescens was closely followed by the involvement of Kleb. aerogenes (Thomas et al, 1977). Isolates from both genera were shown to harbour a plasmid of approximately 90 Md which transferred resistance to cephalothin, ampicillin, chloramphenicol, tetracycline, kanamycin, streptomycin and sulphonamides.

An unrelated outbreak in which gentamicin-resistant Kleb.aerogenes was shown to colonise and cause infection, was described by Casewell et al, (1977). A plasmid-carrying the gentamicin resistance determinant as well as resistance to a number of other antimicrobial agents was demonstrated in a number of Kleb.aerogenes strains isolated from urine samples from 17 patients. Subsequent plasmid analysis showed that the Kleb.aerogenes isolates each harboured a cryptic 90 Md plasmid and a multi-resistance plasmid of 65 Md (Datta et al, 1979).

In an outbreak of Kleb.pneumoniae infection reported by Sadowski et al (1979), 10 additional coliforms, including different Kleb.pneumoniae serotypes together with E.coli, Ent.

cloacae and Prot.morganii, isolated from 7 patients were shown to harbour similar R-plasmids. These coliforms each transferred a number of common resistance determinants on a single 58 Md plasmid. Restriction endonuclease analysis demonstrated no detectable differences between the plasmids from the different isolates.

From these and similar examples of the spread of multi-resistant coliforms which harboured similar plasmids, and the dissemination of R-plasmids among a variety of coliform genera (Casewell, 1981; Knight and Casewell, 1981; Tompkins et al, 1980; O'Brien et al, 1980), it is clear that the dissemination of plasmid-mediated resistance determinants poses significant problems in the treatment and control of nosocomial coliform infections.

In addition to coliforms many other organisms including other Gram-negative bacteria and Gram-positive genera, both aerobic and anaerobic, have been shown to harbour R-plasmids although they are not all Tra^{+}. Medically important bacteria which harbour R-plasmids include members of the genera Pseudomonas (Ingram, Richmond and Sykes, 1973; Bridges et al, 1979), Acinetobacter (Goldstein et al, 1983; Devaud, Kayser and Bachi, 1982), Vibrio (Dupont et al, 1985, Threlfall, Rowe and Huq, 1980), Haemophilus (Laufs et al, 1981;

Stuy, 1979), Neisseria (Goh et al, 1985; Dillon, Pauze and Yeung, 1983), Bacteroides (Tally et al, 1982; Welch and Macrina, 1981), Staphylococcus (Lacey, 1975; McDonnell, Sweeney and Cohen, 1983), Streptococcus (Buu-Hoi and Horodniceau, 1980; Clewell, 1981), Clostridium (Abraham, Wales and Rood, 1985; Brefort, et al, 1977) and Corynebacterium (Schiller, Groman and Coyle, 1980).

The mechanisms of resistance to antimicrobial agents encoded by R-plasmids can be divided into a number of distinct groups. Thus, according to Davies and Smith (1978), the following groups can be defined.

(1) Alteration of drug target site, e.g. erythromycin.

(2) Interference with drug transport, e.g. tetracycline and aminoglycosides.

(3) Enzymatic detoxification of antibiotics, e.g. chloramphenicol and beta-lactam antibiotics.

(4) By-pass mechanisms, e.g. sulphonamides and trimethoprim.

(5) Unknown resistance mechanisms, e.g. fusidic acid and novobiocin.

Thus, the number and variety of plasmid-mediated antibiotic resistance mechanisms now recognised is large and constantly increasing. Furthermore, as new antimicrobial agents have been developed and used in medicine over the past twenty to thirty years, plasmids conferring resistance to many of the new agents have often appeared within a short time. For example, cefoxitin resistance was shown to be plasmid-mediated soon after it became widely available (Knight and Casewell, 1981). These findings strengthen the argument against the indiscriminate use of antimicrobial agents and for the constant monitoring of numbers of resistant organisms in any population. By the above means it is hoped that resistance will be maintained at an acceptable level and that antimicrobial agents presently effective against a number of bacterial pathogens are preserved.

Individual plasmids may specify only a single resistance determinant but resistance to multiple drugs is also common. Genes that encode resistance properties are often clustered on plasmids such as chloramphenicol, streptomycin, sulphonamide, ampicillin and kanamycin resistance on plasmid

R1 (Sharp, Cohen and Davidson, 1973). There are however, exceptions to this where resistance genes are not closely linked as on RP4 (Thomas, 1981). The organisation of resistance genes within plasmids has become clearer since the recognition of transposable elements.

Transposons:

A transposon (Tn) is a genetic element which consists of one or more genes that confer a demonstrable phenotype such as drug resistance, flanked by a DNA sequence which enables the whole element to undergo illegitimate recombination or "transposition". The flanking regions contain either inverted or direct repeat terminal sequences and neither the rec A gene product nor extensive DNA homology is required for transposition (Datta and Nugent, 1983; Jacoby, 1985).

The first transposon, Tn1, encoding ampicillin resistance was discovered by Hedges and Jacob in 1974. Since then many other transposons carrying various resistance genes and other characteristics, for example toxin production, have been identified. A number of transposons are composite and encode multiple resistance determinants; Tn4, ampicillin, streptomycin and sulphonamide resistance (Kopecko and Cohen, 1975). Transposons that specify drug-resistance range in size

from approximately 2.7 kilobase (kb) pairs (Tn9) (Gottesman and Rosner, 1975) to 20.5 kb pairs (Tn4) (Kopecko and Cohen, 1975). The DNA sequences at each end of a transposon are similar but usually inverted with respect to each other. The size of this inverted repeat varies in length from 38 base pairs (Tn1) (Hedges and Jacob, 1974) to about 1,500 base pairs (Tn5) (Berg et al, 1975). Transposons can insert at many different sites within a DNA molecule although preferential insertion at particular sites or regions is common. This preferential insertion may have resulted in part from the selection of sites which are not essential to plasmid survival. Transposition occurs between the bacterial chromosome, plasmids and phage DNA. Most transposition events result in the duplication of a short DNA sequence at the site of insertion and they can also induce deletions or inversions of DNA sequences adjacent to the insertion site. A copy of the transposon is maintained at the original site during transposition which occurs at frequencies of 10^{-3} to 10^{-6}/cell/generation.

Transposons have been divided into three groups according to their structure and the mechanisms of transposition (Jacoby, 1985):-

(1) The first group, composite transposons, consists of one or more resistance genes flanked by insertion sequences usually in reverse orientation, e.g. Tn5. Transposition is effected by two genes which are encoded by these insertion sequences: the first is a transposase and the second is a transposase regulator.

(2) The second type of transposon encodes a transposase and a transposase regulator which is also involved in resolving cointergrate intermediates. Essential sites include two terminal inverted repeats and an internal sequence which is required for cointegrate resolution, e.g. Tn3.

(3) The third group of transposons is typified by Tn916, a tetracycline resistance transposon from Strep.faecalis. It transfers by a conjugation-like process in the absence of plasmid DNA (Franke and Clewell, 1981). This group is termed conjugative transposons.

Factors other than antibiotic resistance properties encoded by plasmids:

During recent years a large number of phenotypic

characters, in addition to resistance to antibiotics, have been attributed to plasmid genes. These factors include resistance to other agents including heavy metals, antibiotic and colicin synthesis, metabolic characters, phage restriction and virulence factors.

Resistance to metal ions and compounds including mercury, cadmium, lead, tin, iron, manganese, antimony (Summers and Silver, 1978), tellurium (Summers and Jacoby, 1977), silver (McHugh et al, 1975), cobalt and nickel (Smith, 1967b), and arsenate, arsenite and antimony (Silvers et al, 1981), can be plasmid-mediated in staphylococci and/or Gram-negative bacteria. The degradation of toxic organic compounds such as salicylate, camphor, octane and napthalene in Pseudomonas spp. (Stanisich and Richmond, 1975) and pesticides by Alcaligenes spp. (Don and Pemberton, 1981) can also be specified by plasmids. Changes in the sensitivity of bacteria to ultraviolet light and other mutagens (Mortelmans and Stocker, 1976) and to bacteriocins (Sidikaro and Nomura, 1975) may also be plasmid-mediated traits.

Bacteriocins are large molecules consisting mainly of protein which are lethal to bacterial strains closely related to those which produced the bacteriocin but to which the

producer strain is immune. All bacteriocins produced by E.coli (colicins) are encoded by plasmids (Col plasmids) and are not encoded by the bacterial chromosome (Pugsley, 1984). It is thought that bacteriocins produced by other bacterial species, for example staphylococcins and streptococcins, are also plasmid-determined characters.

Antibiotics are small molecules which are secondary metabolites and act against diverse bacterial genera. Plasmid-mediated antibiotic production is mainly found within the genera Streptomyces and Bacillus: antibiotics produced by these genera include oxytetracycline, chloramphenicol, kanamycin, neomycin and streptomycin (Hopwood, 1978).

A third type of antimicrobial product, microcins, are often plasmid-determined traits. They are low molecular weight molecules which are insensitive to proteases, have a broad spectrum of antibacterial activity and are produced by Gram-negative bacteria, especially E.coli, but also by Ps.aeruginosa (Hopwood, 1978; Bacquero et al, 1978).

Plasmid-determined metabolic characters include a number which are not normally detected in the laboratory, such as the ability of Klebsiella spp. to ferment lactose (Reeve and Braithwaite, 1973), because the bacterial species concerned already possesses chromosomal genes encoding the same function.

However, lactose-fermenting Salmonella spp. (Easterling et al, 1969; Synenki et al, 1973: Johnson et al, 1976), Proteus spp. (Falkow et al, 1964) and other normally non-lactose fermenting bacteria have also been identified where the fermentation ability is encoded by plasmid genes. Genes for the fermentation of other sugars, for example, sucrose in E.coli (Smith and Parsell, 1975) and Sal.tennessee (Johnson et al, 1976) and raffinose in E.coli (Shipley, Gyles and Falkow, 1978) may also be plasmid-mediated. Similarly, plasmids which determine citrate utilisation (Smith, Parsell and Green, 1978), H_2S production (Orskov and Orskov, 1973) and urease production (Grant et al, 1981) have also been found.

The presence of certain plasmids in bacterial strains can alter their susceptibility to a number of phages. For example, plasmids of incompatibility group H inhibit the development of bacteriophage lambda, T1 and T7 in E.coli (Taylor and Grant, 1970). Ø1 and other female-specific phages are unable to lyse E.coli strains which harbour the F plasmid. Plasmids unrelated to F can also inhibit phage infection by repression of F pilus production (Gasson and Willets, 1975). The mechanisms of phage inhibition are often unknown. However,

some plasmids encode restriction endonucleases and DNA modification enzymes which may play a role in resistance to phage infection (Falkow, 1975).

Where bacteriophage typing is used as an epidemiological tool to confirm the presence of an endemic organism during an outbreak of infection, the acquisition of plasmids has been shown to alter the phage type in many bacterial species, for example Sal.typhimurium (Threlfall, Ward and Rowe, 1978). This can cause considerable problems in the interpretation of epidemiologic information when attempting to monitor the spread of specific strains.

Many bacteria harbour plasmids which contribute to the virulence of the bacterial strain which harbour them. The best studied of these "virulence" plasmids are those harboured by pathogenic E.coli.

Many strains of E.coli have been identified as the cause of diarrhoeal disease in both man and animals. Enterotoxigenic E.coli are capable of producing either a heat stable toxin (ST), a heat labile toxin (LT) or both toxins. The genes coding for both ST and LT can be present on the same or different plasmids. In addition, the presence of a colonization factor or adherence antigen (CFA), e.g. CFAI and CFAII is also implicated in the enteropathogenicity of these

E.coli strains. Colonization factors are antigenically distinct depending on the different animal species in which the organisms are found but they share a number of chemical and biological properties. Colonization factors are also plasmid-coded. Plasmids for the production of toxins and colonization factor are normally required for virulence in E.coli strains (Smith and Linggood, 1971). Since the discovery of the association of enteropathogenicity and plasmid-determined characters many different toxins and colonization factors have been identified (Elwell and Shipley, 1980) which have similar properties to those described above.

E.coli isolated from extraintestinal infections of humans have a higher probability of possessing features such as haemolytic activity, colicin (Col V) production and the ability to haemagglutinate human red blood cells in the presence of D-mannose than E.coli isolates from healthy individuals (Minshew et al, 1978). Each of these phenotypic characters have been shown to be plasmid-mediated characteristics in E.coli and other bacterial species. Although an increase in virulence often cannot be directly attributed to the presence of these characters, recent studies suggest that they may be closely related. For example, the presence of plasmid Col V appears to increase the resistance of the harbouring E.coli

strain to host defence mechanisms (Binns, Davies & Hardy, 1979). Other E.coli plasmids confer similar properties such as resistance to serum bactericidal activity (Moll, Manning and Timmis, 1980), complement resistance (Ogata and Levine, 1980) and resistance to phagocytosis (Aguero et al, 1984) on the harbouring strain.

Plasmids from other bacterial species have also been implicated in bacterial virulence. The change from smooth to rough colony type and loss of virulence in Sh.sonnei has recently been attributed to the loss of a plasmid associated with virulence (Kopecko, Washington and Formal, 1980). The ability to penetrate tissue culture cells by a number of Shigella spp. has also been associated with the presence of a large molecular weight plasmid (Watanabe and Nakamura, 1985).

Ninety per cent or more of antibiotic-sensitive Sal.typhimurium, Sal.enteriditus and Sal.dublin carry serotype specific plasmids (Helmuth et al, 1985). In virulence studies a much smaller oral dose of the above plasmid containing salmonella serotypes was required to obtain a 50 per cent lethal dose for mice than plasmid-free isolates. These results suggest that the plasmids in these salmonella serotypes are involved in virulence.

The pathogenesis of Yersinia spp. is dependent on the presence of one of a related group of plasmids (Portnoy, Moseley and Falkow, 1981; Wolf-Watz et al, 1985). Virulence associated with the presence of these plasmids is thought to involve the adhesion of the bacterial cell to mammalian tissue via plasmid encoded pili although further investigations are required to confirm this.

The virulence of B.anthracis is dependent on plasmid-mediated toxin production (Mikesell et al, 1983) and the production of a capsule which is also plasmid encoded (Green et al, 1985).

Exfoliative toxin produced by some strains of Staph.aureus is plasmid encoded (Wiley and Rogolsky, 1977). In a number of strains the genes for toxin production are chromosomal, whereas some produce plasmid-coded toxin and still others produce both plasmid and chromosomally encoded toxin.

The presence of resistance determinants and virulence factors on the same plasmid could, under appropriate conditions, result in the dissemination and maintenance of both resistance determinants or virulence factors as a result of the selection for dissemination of the other character. For example, the presence of resistance determinants and heat-stable toxin on the same transferable plasmid was

demonstrated from 5 of 11 strains of enterotoxigenic E.coli (Echeverria et al, 1985) and is thought to have resulted from widespread use of antimicrobial agents leading to the co-selection of genes for resistance and toxin production.

Molecular analysis of plasmid relatedness:

During the last few years a number of methods used in molecular studies of plasmids have been simplified and widely used in investigating the structure and epidemiology of plasmids. The methods developed fall into two categories - direct and indirect techniques.

Direct techniques for plasmid analysis are based on DNA hybridization. These techniques involve the separation of DNA into single strands, mixing of strands from two different sources, one of which had previously been labelled, and allowing the DNA to reanneal. Single stranded DNA can then be digested and the amount of double-stranded DNA gives an estimate of the degree of homology between different DNA molecules (Barth and Grinter, 1975). Electron microscopy of hybridized DNA, hetroduplex analysis, shows loops of single-stranded DNA (Davis, Simon and Davidson, 1971) the amount of which again reflects the relatedness of two DNA molecules. Small sequences of DNA can be studied by the

Southern blot-hybridization technique (Southern, 1975) where radiolabelled DNA is hybridized with DNA fragments on nitrocellulose.

Indirect techniques include agarose gel electrophoresis of plasmids and DNA fragments generated by restriction endonuclease digestion of plasmid DNA. Agarose gel electrophoresis of cell lysates shows the number of plasmids harboured by an individual isolate and also provides an estimate of their molecular weight (Platt and Sommerville, 1981). Digestion of plasmid DNA with restriction endonucleases generates a number of DNA fragments which have been cleaved at specific sites. As different restriction endonucleases recognise different sites the size and number of fragments, a plasmid "fingerprint", is characteristic for different plasmids (Thompson, Hughes and Broda, 1974). Using these methods, especially with the use of two or more restriction endonucleases (Richmond and Petricheilou, 1978) we can demonstrate that two plasmids of the same size are indistinguishable with regard to fingerprints, alternatively if their base sequence is different then this will be apparent from the fingerprints. Similarly, fingerprint analysis of two plasmids of different molecular weight will show if they share common fragments or if they are entirely different. Small

changes in base sequence can produce significant changes in fingerprints and vice versa. However, in general there is good correlation between fingerprints and molecular relatedness of plasmids.

Gel electrophoresis of plasmid DNA is the simplest of these techniques and the most commonly used. However, the similarity of two plasmids of the same molecular weight can only be implied; confirmation of the degree of relatedness requires further analysis. Restriction endonuclease analysis is often employed for this purpose. Nevertheless changes in small segments of plasmid DNA, for example when a transposon is present in plasmid DNA, may not always be apparent from fingerprint analysis. Hybridization techniques and the use of DNA probes to detect the presence of specific sequences may therefore be required to resolve small changes in DNA structure.

Presence of plasmids in "Pre-antibiotic" populations:

The treatment of some infections has been made difficult by the emergence of bacterial resistance to antimicrobial agents. The ever-increasing usage of these drugs maintains the selective pressure that favours the persistence of resistant strains and also the continuing

evolution of resistance mechanisms. Clinically the most important mechanisms of resistance are plasmid-mediated although primary (intrinsic) and mutationally-acquired resistance account for a small proportion of total infections that prove refractory to treatment (Jacoby, 1985). R-plasmids are ubiquitous in nature; not only are they found in organisms from societies where antimicrobial agents are routinely used but also in bacteria isolated in the "pre-antibiotic" era (Smith, 1967a). Smith showed that of 30 coliform strains tested for resistance to 8 antimicrobial agents, three were resistant to one drug and one strain was resistant to two drugs. Streptomycin and tetracylcine resistance was transferable from the latter isolate.

Conjugative R-plasmids have also been isolated from a small proportion of coliforms from human faecal specimens from a community in Borneo where no antimicrobial agents had been used (Davis and Anandan, 1970) and from E.coli isolated from human faeces from inhabitants of the Solomon Islands (Gardiner et al, 1969). Although 10 per cent of coliforms from faeces from bushmen and animals in South Africa were resistant to one or more antimicrobial agent, none of the resistance

determinants were transferable (Mare, 1968). The absence of physical data to confirm the presence of plasmids prevents any further assessment of the representativeness of these isolates.

Hughes and Datta (1983) found that, although no antimicrobial resistance determinants were transferrable, 19 per cent of coliforms collected in the pre-antibiotic era (The Murray collection) possessed conjugative plasmids. These results may not however, be representative of coliforms generally present during the time period the collection was made (1917 to 1954) as 48 per cent were Salmonella spp. and 32 per cent Shigella spp., with no other single genus being represented by 10 per cent or more of the isolates studied.

Thus although the investigations suggest that transferrable R-plasmids were present in bacterial populations before the introduction of antimicrobial agents, the results may not bear quantitative comparison with the situation today. Nevertheless the results of subsequent studies suggest that R-plasmids prevalent today have evolved over many years. In some instances selective pressure has led to the dissemination of individual plasmids throughout a large and diverse bacterial population while in others it is the resistance determinants themselves, as transposable elements, which have become disseminated and contributed to plasmid evolution.

Transfer of R-plasmids between bacteria in the gastro-intestinal tract of man and animals:

Although conjugative R-plasmids were recognised almost 30 years ago, our knowledge of their behaviour *in vivo* remains limited. Many R-plasmids are readily transferred inter-specifically and intragenerically *in vitro* but transfer frequencies in natural environments are much more difficult to assess and are influenced by many factors including the availability of nutrients and the presence of inhibitors. Evidence indicative of R-plasmid transfer *in vivo* has been derived from experiments both with human volunteers and laboratory animals.

The gastro-intestinal tract of both man and animal is the natural habitat of many coliforms and has been the focus of a number of studies in an attempt to discover whether or not R-plasmid transfer occurs in this habitat.

In investigations where human volunteers ingested large numbers of viable bacteria, no transfer (Anderson, 1978; Smith, 1978) or a very low rate (Anderson, 1975b; Smith, 1969; Williams, 1977) was detected either to ingested *E.coli* K12 or to the indigenous flora. In another study, faecal samples from human volunteers who had ingested 10^{10}-10^{11} viable *E.coli* originally isolated from their own faecal flora were studied to

determine in vivo transfer (Anderson, Gillespie and Richmond, 1973). In these experiments chromosomal resistance to either naladixic acid or rifampicin was induced in the E.coli strain from the volunteers and an R-plasmid was transferred into one strain in vitro. After ingestion, by 4 volunteers, of both potential donor and recipient strains, faecal specimens were examined for evidence of in vivo transfer. None was found despite the isolation of donor and recipient organisms in numbers consistent with conjugative transfer had the experiments been carried out in vitro. Further experiments with three of these 4 volunteers revealed that after a 5 day course of treatment with an antibiotic (ampicillin or tetracycline) to which the ingested E.coli donor possessed plasmid-mediated resistance, R-plasmid transfer in vivo was detected at high frequency. In two volunteers in vivo transfer had occurred, not to the chromosomally-resistant potential recipient ingested but to indigenous E.coli strains. Transfer to the chromosomally-resistant potential recipient was however detected in the third volunteer.

It is now generally accepted that in vivo transfer of R-plasmids occurs readily in the gastro-intestinal tract of gnotobiotic animals in the absence of antibiotic selection pressure. Experimental evidence to support this comes from

studies of germ-free mice (Jones and Curtiss, 1970; Reed, Sieckmann and Georgi, 1969; Salzman and Klemm, 1968) and chickens (Sansonetti et al, 1980) which were fed with potential donor and recipient strains. R-plasmids transfer in newborn or very young animals where the indigenous flora is absent or poorly developed also occurred at a significant rate in a number of different animal species (Smith, 1970; Walton, 1966). In contrast, plasmid transfer in animals with a fully developed indigenous gastro-intestinal flora or artificially developed flora was much less frequent (Kasuya, 1964; Smith, 1970) but antibiotic therapy to which the plasmid specified resistance, greatly increased the numbers of transconjugants isolated (Duval-Iflah et al, 1980; Guinee, 1965; Guinee, 1970; Smith, 1970). This occurs by two mechanisms:- direct selection and reduction in overall flora resulting in less competition and in inhibition, for example by free fatty acids and Bacteroides spp. (Anderson, 1975). Alteration of the normal gut flora by starvation also increased the numbers of transconjugants isolated from the rumen of sheep (Smith, 1977).

These studies also showed that the ability of potential donor and recipient strains to multiply in the gut influenced the frequency of R-plasmid transfer. In most instances transfer was either not detected or occurred at a

very low rate if either the donor or recipient strain could not multiply in the gastro-intestinal tract of the animal being studied. The frequency of transfer was greater where both the donor and recipient strains had successfully colonised the gastro-intestinal tract. It would also appear that the frequency of R-plasmid transfer in vivo varies in different animal species. R-plasmid transfer has been demonstrated in vivo in mice, rats, chickens, calves and sheep but two authors (Jarolmen and Kemp, 1969; Smith 1970) concluded that R-plasmid transfer in the gastro-intestinal tract of pigs was a rare occurrence.

Thus, most workers have concluded that individual R-plasmid transfer events in vivo are a relatively rare occurrence under normal circumstances, both in humans and animals. However, the cumulative effect of these events, which although rare in the individual are nevertheless constantly occurring on a global basis, when considered together with the widespread use of antimicrobial agents explains the high incidence of conjugative R-plasmids in coliforms. The worldwide use of antibiotics both in the treatment of disease and their use in animal husbandry as

growth enhancers may have contributed signficantly to the spread of R-plasmids in coliform populations (Stuart-Harris and Harris, 1982).

R-plasmid transfer in vivo in sites other than the gastro-intestinal tract:

The transmissibility of R-plasmids in sites other than the gut may also be of importance in the spread of resistant strains. As coliforms form an integral component of the bacterial flora of the gut, it is generally assumed that this is the main site for in vivo plasmid transfer between coliforms. However, we have no firm evidence that the gut is the main transfer reservoir and alternative sites which do not contain inhibitors, for example inhibition by large numbers of Bacteroides spp., may play an important role in providing a suitable environment for in vivo transfer. Experimental evidence suggests that R-plasmids can transfer in urine and on skin.

A high frequency of R-plasmid transfer in vitro was found between a multi-resistant clinical isolate of Ser.marcescens, isolated from an outbreak of urinary-tract infections, and an E.coli K12 recipient (Schaberg, Highsmith and Wachsmuth, 1977). In these experiments both the donor and

recipient organisms were grown in sterile urine obtained from a catheter collection bag. After mixing cultures in fresh urine R-plasmid transfer was detected after incubation for 4 hours at room temperature.

In a separate study (Nagano and Takahashi, 1985) a number of R-plasmids were found in several different coliforms isolated from urine samples which had been stored for 24 hours. Although these authors did not perform in vitro experiments with known donor and recipient strains, they suggested that R-plasmid transfer had occurred in some urine specimens. However, as no bacterial or plasmid analysis was performed until after the urine samples had been stored for 24 hours, the presence of a common R-plasmid in two or more coliforms from the same specimen was probably the result of R-plasmid transfer in vitro in the stored urine, rather than in vivo transfer.

Thus although the ability of R-plasmids to transfer in urine is recognised the question of whether in vivo transfer occurs in urine in the bladder requires further investigation.

The isolation of strains of Ps.aeruginosa, Prot.mirabilis and Kleb.aerogenes resistant to ampicillin, carbenicillin, cephaloridine, tetracycline and kanamycin from patients in the same burns unit prompted an investigation into

R-plasmid transfer in patients' burns (Roe, Jones and Lowbury, 1971). A burned mouse model was developed and used to demonstrate R-plasmid tranfer in burns.

In each of 10 experiments using 10 pairs of donor and recipient strains designed to parallel the clinical situation, R-plasmid transfer occurred at a high frequency. The clinical isolates of Ps.aeruginosa, Prot.mirabilis and Kleb.aerogenes all acted as donors for R-plasmid transfer to E.coli and Ps.aeruginosa. Transconjugants also acted as donors to Ps.aeruginosa and E.coli recipients respectively. It was therefore likely that the isolation in this burns unit of different bacteria which harboured the same transferrable R-plasmid was a result of in vivo transfer.

Transfer of resistance determinants between strains of Staph.aureus on the skin of healthy volunteers has been reported on a number of occasions. Plasmid analysis was not performed on any of the strains of Staph.aureus studied by Lacey (1971) and Naidoo and Noble (1978) although they suggested that R-plasmids might have been responsible for the transfer of resistance. Transfer of R-plasmids between strains of Staph.aureus both in vitro and in vivo on the skin of healthy volunteers was confirmed by plasmid analysis of

strains carrying resistance determinants to penicillin, erythromycin, neomycin and tetracycline (Lacey and Richmond, 1974).

Evidence of R-plasmid transfer in clinical studies:

Many reports support the hypothesis that R-plasmid transfer *in vivo* is a constant phenomenon. The evidence indicates that in addition to resistant strain cross-infection between patients, R-plasmid transfer between different coliforms has also occurred in a number of individual patients. However, many of these reports are based entirely on epidemiological evidence: for example, given a single patient from whom two organisms were isolated that harboured the same plasmid(s), it is not possible to determine the direction or site of R-plasmid transfer.

In one hospital in the USA, there was an epidemic of multi-resistant *Ser.marcescens* involving 33 patients in 1976 (Tompkins *et al*, 1980). Twenty-three further *Ser.marcescens* infections were reported during 1977 and 15 during 1978. Concurrently with the isolation of *Ser.marcescens* was the isolation of multi-resistant *Kleb.pneumoniae* followed by *Kleb.oxytoca*, *Cit.freundii*, *Ent.aerogenes*, *Prot.morganii*, *Prot.rettgeri* and *Providencia stuartii*. All multi-resistant

coliforms isolated in this study had a constant pattern of resistance determinants which included resistance to sulphonamides and several aminoglycoside and beta-lactam antibiotics, but variable resistance to tetracycline and chloramphenicol. A 45 Md plasmid present in all isolates specified the constant pattern of resistance. Results from conjugation experiments showed that each coliform could transfer resistance to the same 8 antibiotics (streptomycin, kanamycin, gentamicin, tobramycin, ampicillin, carbenicillin, cephalothin and sulphonamide) and restriction endonuclease analysis showed that the 45 Md transferrable plasmid from each of the isolates was identical. Ninety per cent of the multi-resistant strains were isolated from infections of the urinary tract, initially from patients in the same Urology ward. Later, multi-resistant strains were isolated from a number of different in-patient units and from other sites of infection. However, Tompkins et al, (1980) did not state whether two or more multi-resistant strains were isolated from the same specimen. Thus, although cross-infection initially with Ser.marcescens together with R-plasmid transfer in vivo probably took place, no conclusions can be drawn about the

site of transfer, whether more than one strain acted as a donor, or whether the transfer event took place in an environmental reservoir rather than *in vivo*.

In another hospital in the USA gentamicin-resistant *Kleb.pneumoniae* of a single biotype was isolated from 43 patients during February to May 1976 (O'Brien *et al*, 1980). After May, the number of isolations of this biotype was markedly reduced to be replaced by frequent isolation of gentamicin-resistant *Ser.marcescens* and less often, of *Kleb.pneumoniae* of different biotypes and *E.coli*, *Cit.freundii* and *Prot.morganii*. The combined results from 1976 and 1977 showed that gentamicin-resistant coliforms were isolated from 372 patients. Two thousand and twenty isolates of 49 different species or biotypes were collected, 42 per cent from urine and 23 per cent from sputum, in these patients. Over 90 per cent of the gentamicin-resistant coliforms were also resistant to chloramphenicol, sulphonamide, ampicillin and carbenicillin with tetracycline, streptomycin and kanamycin resistance also being common. Resistance determinants were transferred from a number of different species and biotypes and restriction endonuclease digestion was carried out on the plasmid DNA from the *E.coli* K12 transconjugants. Six of the transconjugants had identical fingerprints but two had one

additional large fragment. One of these two transconjugants also transferred tetracycline resistance but did not possess three of the smaller fingerprint fragments. The authors did not give details of the number and size of all plasmids harboured by either the gentamicin-resistant clinical isolates or transconjugants although they stated that 19 transconjugants harboured a 56.6 Md plasmid of incompatability group M. Differences in restriction endonuclease digestion results might therefore have reflected the presence of an additional plasmid and/or the loss of a segment of plasmid DNA when it was transferred to a different host in vivo. Of 188 gentamicin-resistant E.coli, 149 were isolated from patients who also harboured gentamicin-resistant isolates of other coliform species. However, the demonstration of in vivo transfer requires isolation from the same specimen of two coliforms - one which harboured a conjugative plasmid and one which did not possess the plasmid. A subsequent specimen should demonstrate the plasmid in both coliforms in order to suggest in vivo transfer. Although O'Brien et al, (1980) did not show this, it appears that in vivo transfer has occurred although interpretation of results is limited. More detailed descriptions confirm these observations in a few individual patients.

In one patient with enteric fever, Sal.typhi isolated from blood and faeces, was initially sensitive to chlormaphenicol, sulphonamides, trimethoprim and streptomycin (Datta, Richards and Datta, 1981). The patient had at first responded to oral treatment with chloramphenicol but 7 days after the end of treatment, she deteriorated and Sal.typhi was again isolated from blood cultures. At this time Sal.typhi isolates were still sensitive to the original antibiotics. Oral chloramphenicol was resumed together with intravenous cotrimoxazole. After a further 72 hours, blood cultures were negative but Sal.typhi resistant to chloramphenicol, sulphonamides, trimethoprim and streptomycin was isolated from urine and faeces. Plasmid analysis of the sensitive strain showed that it was plasmid-free whereas the resistant strain harboured two plasmids. One plasmid which belonged to incompatibility group I, carried high level resistance to trimethoprim and low level streptomycin which was confirmed as Tn7 by restriction enzyme analysis. The second plasmid carried chloramphenicol, sulphonamide and low level streptomycin resistance determinants and belonged to incompatability group H. Thus it appears that treatment of this patient with chloramphenicol and cotrimoxazole had

resulted in the *in vivo* transfer of two plasmids to the *Sal.typhi*, one of which, the Inc H group plasmid, was detected in a *Kleb.aerogenes* also present in the patient's faeces.

In a separate study involving a single patient, a *Sal.typhimurium* was shown to sequentially acquire two different R-plasmids (Platt, Sommerville and Gribben, 1984). *Sal.typhimurium* isolated from blood and faeces of this patient was initially resistant to trimethoprim and sulphamethoxazole and harboured an 18 Md non-conjugative plasmid. After 4 days of ampicillin therapy *Sal.typhimurium* isolated from peritoneal dialysis fluid and faeces, was additionally resistant to ampicillin and harboured a 55 Md plasmid in addition to the 18 Md plasmid. Therapy was changed to chloramphenicol and two weeks later about 4 per cent of faecal *Sal.typhimurium* were also resistant to chloramphenicol and harboured a third plasmid of 62 Md. Plasmid analysis of other coliform species from the patient suggested that the transferrable R-plasmids were initially harboured by different indigenous faecal coliforms. Experimental evidence suggested that the ampicillin resistance-plasmid was transferred directly to the *Sal.typhimurium* from *E.coli* and the chloramphenicol-resistant plasmid although detected in a *Kleb.pneumoniae* and a further distinct *E.coli* was transferred from the *E.coli* strain. In

this patient treatment with ampicillin followed by chloramphenicol therapy resulted in the sequential acquisition of plasmids encoding resistance to these drugs from coliforms in the gastro-intestinal tract of the patient concerned.

A number of further examples of in vivo transfer of R-plasmids was found in individual patients, for example, a Klebsiella K10 and a Cit.freundii were isolated from one patient, the same resistance determinants were transferred from both strains each of which carried two R-plasmids of incompatability groups FII and G (Datta et al, 1980). A Klebsiella K21 and an E.coli were isolated from a second patient: both isolates transferred the same resistance determinants and harboured an Inc FII plasmid. Datta et al, (1980) also demonstrated that the acquisition of resistance by transposition was an important feature of the development of resistant coliforms in addition to the transfer of R-plasmids between bacterial species. Transposition of ampicillin-resistance was demonstrated on a number of occasions, for example from 6 Inc FII plasmids which also determined resistance to gentamicin, tetracycline, sulphonamide, chloramphenicol and mercuric chloride. Both ampicillin-resistant and ampicillin-sensitive variants of this transferrable R-plasmid were detected. Acquisition of

ampicillin-resistance coincided with an increase in the molecular weight of the plasmid from 57 Md to 59 Md. A second example of the involvement of a transposon was found; in this instance the plasmid transferred resistance to trimethoprim and streptomycin which is indicative of Tn7, in that restriction endonuclease digestion with HindIII yielded two fragments characteristic of Tn7.

Thus our current knowledge of the behaviour of R-plasmids in vivo is based largely on inference from retrospective studies but that the large body of circumstantial evidence firmly supports in vivo transfer. This evidence is based on R-plasmids present in specific genera (Davies, Farrant and Tomlinson, 1968b; Courtney, Miller and Streips, 1980; Markowitz et al, 1980), the transfer of specific R-plasmids between different coliform species (Thomas et al, 1977; Tantulavich et al, 1981; Rubens et al, 1981), the transfer of common resistance determinants (Dubois, Pechere and Letarte, 1981; Knight and Casewell, 1981; Takahashi and Nagano, 1985; Elwell, Inamine and Minshew, 1978) and the influence of transposons on hospital infection (Datta et al, 1979). In contrast there are very few studies involving individual patients where sufficient information is available to distinguish between donor and recipient strains in any outbreak

of infection (Farrar et al, 1972). A study in which the apparent index case was recognised retrospectively to have caused an outbreak of multi-resistant Kleb.pneumoniae infections has been recently discussed. However, the "index" organism was not actually studied because it was isolated before the epidemic was recognised and therefore was not stored (Gerding et al, 1979).

Thus, any attempt to investigate the distribution, mobility and epidemiology of plasmids from coliforms isolated from faecal material will be dependent on a large number of factors. These include that coliforms isolated from faecal material represent only a small proportion of those present in a limited area of the gastro-intestinal tract and a smaller proportion of a much larger and undefinable flora. In addition, the complexity of other factors which may influence coliform growth rate, distribution, physiological state and plasmid content may be expected to influence the results obtained. Analysis of results would therefore be complex and difficult to interpret. In contrast, open wounds are often colonised/infected with coliforms accessible for sampling without the complexity associated with studies of isolates from the gut.

Peripheral vascular disease and associated infections:

Disorders of the vascular and lymphatic systems involve every organ in the body. Peripheral vascular disease (PVD) affect the limbs and generally results in reduced perfusion of blood to the affected tissue which becomes clinically apparent by the development of ischaemia (Nolan et al, 1974) Most arterial disease is due to atheromatous degeneration of the arterial wall followed by the superimposition of acute thrombosis on the artheromatous plaque or the lodging of an embolus in a major artery to the limb.

Patients with PVD may present with symptoms additional to the development of ischaemia. These include rest pain, muscle atrophy, and paralysis may occur after a sudden complete arterial blockage. A number of superficial arteries may exhibit abnormal pulsations which is presumptive evidence of a block in the main artery, distal to the superficial artery. Colour changes of the skin and increased temperature sensitivity may also occur. Vascular insufficiency results in deterioration of the nutrient supply to the skin and consequent inability to repair minor day-to-day trauma which leads to the development of ulcers which are slow to heal and may deteriorate progressively to gangrene.

The development of ulceration of the skin and gangrene in severe cases of ischaemia is a result of tissue death which can result in two clinical forms of necrosis. Dry gangrene and mumification, especially of the toes, is common where no underlying infection is present. The development of ulcers and/or wet gangrene results in swelling and sloughs forming with a line of granulating tissue separating the infected area. Wet ulceration and gangrene are often found in patients with diabetes mellitus. Ulcers in patients with PVD are characteristically found on the lower part of the leg and around the ankle.

In a study of 40 patients with untreated ulcers secondary to PVD the predominant bacteria isolated were Staph.aureus and coliforms (Friedman and Gladstone, 1969). Specimens were obtained by mixing 1 ml of sterile water over a 20 mm diameter area of the ulcer and culturing the resulting rinse water. Staph.aureus was isolated from 17 and coliforms from 9 of these 40 ulcers. More than one organism was isolated from 20 ulcers. Good correlation was found between the presence of inflammation in the ulcer and the isolation of Staph.aureus and coliforms. Although the presence of invasive infection could not be determined as collection of biopsy specimens was contra-indicated in these patients since adequate

circulation was not present, it appears that the presence of Staph.aureus and coliforms in these ulcers was contributing to the deterioration and inflammation of the ulcers.

Treatment of PVD is diverse and is dependent upon its severity. The treatment of associated infection with dressings soaked with hypertonic solutions is recommended as is both topical and systemic antimicrobial therapy in some instances, to prevent cellulitis. Surgical debridement is often undertaken although amputation to remove an infected area is not advised unless absolutely necessary.

Surgical treatment to increase the flow of blood to an affected limb is often peformed. Arterial reconstruction is carried out only in patients with localised arterial disease. Bypass operations involving insertion of either an autologous saphenous vein or a prosthesis such as terylene (dacron) are often performed. Specific patients may be selected for sympathectomy where division of the sympathetic nerve supply results in increased blood supply to the limb especially to the skin. Sympathectomy may not benefit the muscles directly but can relieve coldness and assist healing of skin lesions.

Infection of surgical wounds is a well recognised problem in a number of patients who have undergone these procedures. The incidence of infection in clean wounds, those

performed through uninfected tissue where no hollow organ is opened and where there is no break in aseptic technique, is generally low, at about two per cent (Galland, 1982). However, in vascular surgery the rate of infection is much higher when prosthetic material is implanted, in operations involving the groin and in lower limb amputations.

In a study of surgical wound infection from 1971 to 1978, a gradual decrease was seen, from 7.1 per cent in 1972 to 3.6 per cent in 1978 with a mean of 5.4 per cent (Leigh, 1981). This study included results for all types of surgical wounds in two UK hospitals. Thirty-nine per cent of wounds were infected with Staph.aureus and 55 per cent with coliforms. Anaerobes were isolated from 14 per cent of all infected wounds. Sixty-two per cent of infections were caused by a single organism of which Staph.aureus was the most common. In contrast, the majority of the 38 per cent of wounds infected with more than one organism were associated with infections with bacterial species other than Staph.aureus which included infections mainly with coliforms but also with anaerobes.

Decubitus ulcers are caused by venous stasis and pressure resulting in the development of ulcers typically on the buttocks, sacral area and heels. A number of patients with PVD develop decubitus ulcers as a result of the disease

but they are also common in both geriatric and orthopaedic patients. The bacterial genera and species isolated from decubitus ulcers is similar to those isolated from other skin ulcers and surgical wounds. Staph.aureus is the most commonly isolated organism with coliforms and anaerobes also isolated frequently (Dagher, Alongi and Smith, 1978; Daltrey, Rhodes and Chattwood, 1981; Lookingbill, Miller and Knowles, 1978). The isolation of a mixed bacterial flora appears to be more common than the isolation of a single pathogenic species.

Thus, the patterns of colonisation and infection in post-surgical lesions and peripheral ulcers indicate that these sites provide a suitable system in which to study coliforms, plasmids and the in vivo transfer of plasmids. Such a system has advantages over the gastro-intestinal tract in that it is simpler to study and may parallel the burned mouse model used by Roe et al, (1971) to study the in vivo transfer of R plasmids. The following reasons also contributed to the decision to study these patients:-

(1) Patients selected were normally available for follow-up over a reasonable time period.

(2) The site(s) to be sampled was expected to contain coliforms (ideally more than one) as a predominant colonist without large numbers of other organisms and ideally in a site where they represent potential pathogens.

(3) In general, sites were expected to be more evenly colonised and show less species diversity than the gastro-intestinal tract.

(4) Specimen collection did not require the use of invasive techniques, disturb the routine of the clinical unit, nor disturb the patient in such a way that compliance affected future sampling.

AIMS OF THE PRESENT STUDY

(1) To assess the bacterial colonisation of vascular ulcers and surgical wounds in patients with PVD and decubitus ulcers in geriatric patients and to correlate the findings with the clinical situation.

(2) To determine the presence and distribution of plasmids present in the coliforms isolated. To assess the mobility of R-plasmids and plasmids not associated with resistance. Compare in a limited number of patients, the plasmids present in coliforms isolated from rectal swabs.

(3) Apply plasmid profile and fingerprinting techniques to the resolution of both epidemiological problems including identification of instances of cross-infection and distinguishing between endogenous and exogenous sources of wound infection. Instances of plasmid transfer in vivo involving conjugative R-plasmids or conjugative plasmids not associated with resistance, will also be identified.

MATERIALS AND METHODS

Patients:

Patients studied were either in-patients of the PVD unit, Glasgow Royal Infirmary, or of geriatric units in hospitals in the Eastern District of Glasgow during the period February 1981 to June 1983 as detailed in Table 1.

Relevant patient details were recorded on a questionnaire (Appendix 1) on each occasion a wound was sampled. Information recorded included a clinical summary and details of antimicrobial therapy together with a detailed description of the type of wound, site and clinical appearance. Wounds were classified on the basis of appearance:

Grade I - Clean, no evidence of infection.

Grade II - Inflammation present with or without oedema; no purulent exudate.

Grade III - Inflammation, oedema and purulent exudate present.

Dry wounds were excluded on the basis of the results from a pilot study which established that no coliform isolation was associated with this type of wound.

TABLE 1: **Patient populations studied and types of specimens collected.**

Number of Patients	Hospital Unit	Period of Collection	SPECIMENS COLLECTED Wound Swab	Rectal Swab
25	PVD	Feb.81-Mar.81	Yes	No
53	PVD	Nov.81-Apr.82	Yes	No
26	PVD	Oct.82-May 83	Yes	Yes
13	Geriatric	Mar.83-Jun.83	Yes	Yes

PVD = peripheral vascular disease

* Mean age 69 years (range 37 - 91)

Specimens:

Wound swabs were obtained at weekly intervals from patients in the PVD unit. Rectal swabs were also collected in parallel each week from those patients studied during the period October 1982 to March 1983. Wound swabs from patients in geriatric units were also obtained at weekly intervals. Parallel rectal swabs were collected when the wound was first sampled and thereafter, at two to three-week intervals.

Wounds were divided on clinical grounds into -

(a) **Vascular ulcers;** wounds arising as an indirect result of PVD where no surgical procedure was involved.

(b) **Amputation sites;** wounds in PVD patients resulting from surgical amputation.

(c) **Surgical vascular repair sites;** surgical wounds which appeared clinically to be infected and/or slow to heal after surgery to repair or by-pass blood vessels.

(d) **Decubitus ulcers;** ulcers in geriatric patients arising as a result of vascular insufficiency and pressure.

All wound swabs from patients in the PVD unit were obtained using either a dry swab, or in the case of particularly dry wounds, a saline moistened swab. The swab tip was broken into a bijou containing 0.5 ml brain heart infusion (BHI) broth (Oxoid, CM225). All rectal swabs and wound swabs obtained from geriatric patients were taken using a dry swab and transported in Amies transport media (Exogen Vi-Pak). All specimens were processed within two hours of collection.

Isolation of Organisms:

Wound swabs in BHI broth were vortexed thoroughly and 0.1 ml broth inoculated onto each of:-

(1) cysteine-lactose-electrolyte-deficient (CLED) agar (Mast, DM110)

(2) Columbia agar base (Oxoid, CM331) containing 5 per cent defibrinated horse blood

(3) neomycin blood agar (Brucella agar base (Difco, 096001), which contained 70 µg/ml neomycin (Sigma), 5 per cent defibrinated horse blood and 10 ml/l vitamin K haemin solution (Gibco)).

Wound swabs in transport media were inoculated directly onto solid media as above. Rectal swabs were inoculated directly onto plates of CLED and MacConkey (Oxoid, CM7B) agar. In addition, to facilitate isolation of resistant coliforms present in small numbers in the original specimen a direct sensitivity test was carried out (Platt, Chesham and Kristinsson, 1986). Isosensitest agar (Oxoid, CM471) and the following antibiotic discs (Oxoid):- carbenicillin 100 µg, cephazolin 30 µg, tetracycline 10 µg, kanamycin 30 µg, streptomycin 10 µg, chloramphenicol 10 µg, sulphamethoxazole 25 µg and trimethoprim 1.25 µg were used.

All CLED, MacConkey, Columbia blood and Isosensitest agar plates were incubated overnight at 37°C in air enriched with 5 per cent CO_2. Neomycin blood agar plates were incubated for 48 hr at 37°C anaerobically (Oxoid gas generating kit BR38).

After incubation all potential pathogens from wound swabs were purified and identified using standard methods (Lennette et al, 1980). Results were recorded and cultures of anaerobes and Gram positive aerobes were discarded.

Estimation of mean healing time of wounds:

To compare different types of wounds (vascular ulcers, amputation sites, surgical vascular repair sites and decubitus ulcers) a mean value was derived from each group. As wounds were only sampled when their appearance suggested that they were colonised/infected, the mean number of weeks sampled was an indication of the time it took for the wound to heal, i.e. the mean healing time.

The number of weeks a wound was sampled from geriatric patients was not, in general, an accurate indication of healing time as many of the patients either died or were transferred to other units without resolution of the wound. This has therefore led to inaccuracy in calculating the mean healing time from geriatric patients, resulting in an underestimate in the value obtained.

Calculation of healing time of wounds in the presence and absence of potential pathogens:

To correlate wound healing with the isolation of one

or more potential pathogen, the data collected was reviewed as described below on the assumption of equivalence between wound healing and sampling, i.e. wounds were, with few exceptions, sampled until healing was complete. Thus the number of weeks that samples were collected provided an estimate of the healing time. This was only applied to patients in the PVD unit. Geriatric patients were excluded on the grounds that the assumption above was not valid as described earlier.

(1) The number of weeks taken for wounds, from which no potential pathogens were isolated, to heal was calculated.

(2) The number of weeks that a wound was sampled, including the week of initial isolation of a potential pathogen, was taken as the number of weeks it took for the wound to heal.

(3) This was repeated for all wounds from which potential pathogens were isolated.

(4) The chi squared test was used to compare healing time of wounds from which no potential pathogens were isolated with wounds from which potential pathogens were isolated.

(5) This analysis was performed for wounds from which coliforms, Staph.aureus, Pseudomonas spp. and any potential pathogen, were isolated.

Further investigation of enterobacteria, pseudomonas and acinetobacter isolates:

All isolates of enterobacteria, pseudomonads and acinetobacter from wounds and rectal swabs even if only present as single colonies and including those from wound swabs considered to be of significant or doubtful clinical significance, were purified as follows. In the case of wound swabs enterobacteria, pseudomonads and acinetobacter were purified from Columbia blood and CLED plates, from rectal swab cultures on CLED and MacConkey plates were used. Up to 6 colonies of each distinct colony type were pooled in 2.5 ml Isosensitest broth (Oxoid, CM473). Any colonies growing within the inhibition zones on the direct sensitivity plate were similarly suspended in Isosensitest broth. All

suspensions were then plated for purity on CLED and disc-diffusion sensitivity tests performed (Platt and Sommerville, 1981) on Isosensitest agar and using discs containing the following antimicrobial agents; rifampicin 50 µg (Mast), naladixic acid 30 µg, ampicillin 10 µg, carbenicillin 100 µg, cephazolin 30 µg, cephamandole 30 µg, colistin sulphate 25 µg, tetracycline 10 µg, kanamycin 30 µg, streptomycin 10 µg, gentamicin 10 µg, tobramycin 10 µg, amikacin 10 µg, sulphamethoxazole 25 µg, trimethoprim 1.25 µg and chloramphenicol 10 µg (all Oxoid). If the resulting culture was not homogeneous with respect to either colony type or sensitivity pattern, purity and sensitivity testing were repeated for each variant until homogeneous cultures were obtained.

Many members of the family Enterobacteriaceae possess chromosomally-determined ampicillin resistance. Coliforms other than E.coli were therefore classified as being resistant to ampicillin, for the purpose of designation as resistant isolates, only if they produced a beta-lactamase enzyme. This was tested using betatest strips (Medical Wire Co. Ltd.) where only strong positive reactions detected within 5 min were regarded as confirmatory.

Each isolate was then identified by the API 20E system and the detection of plasmid DNA was performed using a development of the method described in detail below (Platt and Sommerville, 1981). Each isolate was tested on three seperate occasions to determine the number and size of plasmids present or to confirm a negative result. Plasmid detection was carried out on clinical isolates prior to storage.

All 674 strains of enterobacteria, pseudomonads and acinetobacter were stored in columbia agar base stabs containing 15 per cent BHI broth at 0-4°C for further investigation. Each isolate was coded to identify the patient number, patient specimen number and organism identity as in Table 2. Patients and specimens were numbered sequentially. The two letter organism identity code was that used in this Department as an organism computer identity code.

Examples of organism identity code

12AA1: 12 = patient number, AA = E.coli, 1 = 1st specimen from patient number 12.

TABLE 2: **Organism identity code, based on identification by the API 20E system, used for the identification of coliforms, pseudomonas and acinetobacter isolated from clinical specimens.**

Organism Identification by API 20E	Organism Code
Escherichia coli (lac^+)	AA
Escherichia coli (lac^-)	AB
Proteus mirabilis	BA
Proteus morganii	BB
Proteus vulgaris	BC
Klebsiella pneumoniae	CD
Klebsiella oxytoca	CH
Providencia stuartii	DA
Pseudomonas aeruginosa	EA
Pseudomonas fluorescens	EB
Pseudomonas maltophilia	ED
Pseudomonas species	EF
Enterobacter cloacae	JC
Enterobacter agglomerans	JE
Hafnia alvei	JH
Serratia marcescens	KA
Serratia laquifaciens	KB
Yersinia ruckeri	PA
Acinetobacter calcoaceticus var anitratus	QA
Citrobacter freundii	RA

lac^+ = lactose-fermenting: lac^- = non-lactose-fermenting.

12aBA1a: 12a = 2nd anatomical wound site from patient number 12, BA = Prot.mirabilis, 1a = 1st specimen, 2nd colony/sensitivity variant of Prot.mirabilis from specimen.

12RCD3: 12R = rectal swab from patient number 12, CD = Kleb.pneumoniae, 3 = 3rd specimen.

Multiple isolates from the same patient:

Enterobacteria, pseudomonads and acinetobacter isolated either from the same wound on more than one occasion, or from different wounds in the same patient, were excluded from further investigation if their API identity, antibiogram and plasmid profile were the same. Rectal isolates were treated similarly.

Recipient strains:

Determination of which strains were the best recipients was investigated as follows:- spontaneous rifampicin-resistant mutants were isolated, from strains under test for recipient ability, by spreading 0.25 ml of an overnight BHI broth culture onto an Isosensitest agar plate containing 50 µg/ml rifampicin (Sigma) and 5 µg/ml ascorbic

acid (Martindale, 1977). After overnight incubation at 37°C, single colonies were tested to confirm their resistance to rifampicin (and other antimicrobial agents to detect mutational resistance to other agents) using sensitivity testing methods and antibiotic discs as previously described.

Four plasmids (RP4, R1, 7679 and 8080a), were used to test the recipient ability of rifampicin-resistant mutants. Plasmid characteristics are shown in Table 3.

Eleven rifampicin-resistant mutants of clinical E.coli from the routine diagnostic laboratory and E.coli K12 J62-2 were tested for their ability to act as recipients for the 4 plasmids carried by E.coli K12 J53-1. Conjugation experiments with each plasmid-containing donor isolate and each potential recipient strain and the characterisation of transconjugants were performed as described later.

Four E.coli strains were found to act as recipients for all 4 plasmids tested. Three of the recipient strains contained plasmids and/or were resistant to antimicrobial agents in addition to rifampicin. The fourth strain, E.coli K12 J62-2 possessed no plasmids, was resistant only to rifampicin and was therefore chosen as the best recipient (Table 3). In a similar experiment E.coli K12 J53-1, J53-2

TABLE 3: **Bacterial strains and plasmids used in the study, their characteristics and source or reference.**

Bacterial strain and Plasmids	Characteristics	Source
E.coli K12 J53-1	Recipient, lac^+, Nal^R	Bachmann, 1972
E.coli K12 J53-2	Recipient, lac^+, Rif^R	Bachmann, 1972
E.coli K12 J62-1	Recipient, lac^-, Nal^R	Bachmann, 1972
E.coli K12 J62-2	Best *E.coli* recipient, lac^-, Rif^R	Bachmann, 1972
Ent.aerogenes GRI 9880-1	Best *Enterobacter* spp. recipient, Nal^R	Clinical isolate, Platt and Sommerville, 1981
Ent.aerogenes GRI 9880-2	Best *Enterobacter* spp. recipient, Rif^R	Clinical isolate

Kleb.aerogenes NCTC 8172-2	Best Klebsiella spp.recipient, Rif^R	NCTC collection
Cit.freundii GRI 182-2	Best Citrobacter spp.recipient,Rif^R	Clinical isolate
Hafnia alvei GRI 38882-2	Best Hafnia spp.recipient, Rif^R	Clinical isolate
Prot.mirabilis GRI 17381-2	Best Prot.mirabilis recipient, Rif^R	Clinical isolate
Prot.morganii GRI 16181-2	Best Prot.morganii recipient, Rif^R	Clinical isolate
Prot.vulgaris GRI 21181-2	Best Prot.vulgaris recipient, Rif^R	Clinical isolate
Sal.typhimurium LT2	Intermediate in triple cross	ATCC 23564
RP4	Donor plasmid, 36 Md molecular weight standard, mobilising plasmid, resistant to Ap, Cb, Tc, Km.	Jacob et al, 1977
R1	Donor plasmid, 62 Md molecular weight standard, resistant to Ap, Cb, Km, pSm, Su, Cm.	Jacob et al, 1977

7679	Donor plasmid, resistant to Ap, Cb, Tc, Km, pGm, Su.	Clinical isolate
8080a	Donor plasmid, resistant to Tc, Sm, Su, Tp	Clinical isolate
R702	46 Md molecular weight standard	Jacob et al, 1977
RA-1	85 Md molecular weight standard	Jacob et al, 1977
pMR5	Temperature-sensitive derivative of RP4, resistant to Ap, Cb, Tc, Km	Robinson, et al, 1980
pHH1310a	Tra^- R^+ resistant to Ap, Cb	Hughes and Datta 1983
R300B	Tra^- R^+ resistant to pSm, Su	Hughes and Datta 1983

Ap = ampicillin: Cb = carbenicillin: Tc = tetracycline; Km = kanamycin: Sm = streptomycin: pSm = partial streptomycin: pGm = partial gentamicin: Su = sulphamethoxazole: Cm = chloramphenicol: Tp = trimethoprim.

Nal^R = naladixic acid resistant: Rif^R = rifampicin resistant: lac^+ = lactose-fermenting: lac^- = non-lactose-fermenting: Md = megadaltons.

Tra^- R^+ = non-conjugative resistance plasmid.

and J62-1 were shown to be equally good recipients as J62-2. These strains were therefore employed as recipients in conjugation experiments with clinical E.coli isolates.

The 4 test plasmids were transferred into Ent. aerogenes NCTC 10006, Kleb.aerogenes NCTC 8172 and Cit. freundii NCTC 9750. These plasmid-containing strains were then used as donors to assess the recipient ability of Enterobacter spp., Klebsiella spp., Citrobacter spp. and Hafnia spp. The method of plasmid transfer and selection of transconjugants was as described above for screening to find the best E.coli recipients. The strain from each genus used as a recipient in conjugation experiments with clinical isolates was one which was able to accept transfer of all or a majority of the plasmids tested. If two or more recipients gave the same results then the one which possessed the least number of resistance determinants, in addition to rifampicin and which possessed the smallest number of plasmids in addition to those being tested, was chosen as a recipient in further conjugation experiments with clinical isolates (Table 3).

A similar procedure was adopted to determine the best Proteus recipients. All 4 plasmids were transferred into a clinical strain of Prot.mirabilis and were subsequently re-transferred to a number of Proteus spp. The best recipients are listed in Table 3.

Inhibition of growth of E.coli K12:

During some conjugation experiments it was noted that a number of clinical E.coli isolates appeared to produce factor(s) with colicin-like activity which inhibited the growth of E.coli K12. This inhibition was confirmed and the resistance of Ent.aerogenes GRI 9880 and Sal.typhimurium LT2 to these factor(s) was demonstrated using the following method:- one loopful of an overnight BHI broth culture of the bacteria to be tested for sensitivity to factor(s) produced by clinical E.coli isolates was evenly spread over the surface of an Isosensitest agar plate with a sterile dry swab and the plates allowed to dry completely at room temperature. Ten microlitres of an overnight BHI broth culture of each clinical E.coli under test for production of presumptive colicins was dispensed onto the plate and allowed to dry. After overnight incubation at 37°C, sensitivity was detected as areas where the test organism lawn had failed to grow around the drop of

the clinical E.coli isolate. Resistance to presumptive colicins produced by the clinical E.coli was demonstrated by growth right up to the clinical E.coli drop.

Ent.aerogenes GRI 9880-2 was subsequently used as the recipient in all conjugation experiments with clinical E.coli which produced presumptive colicins to which E.coli K12 was sensitive since this Ent.aerogenes strain was resistant.

Conjugation: R-plasmid transfer from clinical isolates:

The method employed to demonstrate R-plasmid transfer and characterise transconjugants was adapted from an earlier method developed in this Department (Kraft, Platt and Timbury, 1983):- 1 ml of a BHI broth culture of donor was mixed separately with 1 ml of recipient culture. 5 ml fresh BHI broth was added and the mixtures incubated for 24 hr at 37°C. Samples were then plated by multipoint inoculator (Denley) onto CLED selection plates containing either 50 µg/ml rifampicin and 5 µg/ml ascorbic acid or 25 µg/ml naladixic acid (Sigma), to select against the donor. In addition, one of the following antimicrobial agents was added to select against the recipient:-

100 µg/ml ampicillin (Sigma)

400 µg/ml	carbenicillin	(Sigma)
10 µg/ml	tetracycline	(Sigma)
25 µg/ml	kanamycin	(Sigma)
15 µg/ml	streptomycin	(Sigma)
5 µg/ml	gentamicin	(Roussel)
100 µg/ml	sulphamethoxazole	(Wellcome)
30 µg/ml	trimethoprim	(Wellcome)
20 µg/ml	chloramphenicol	(Sigma).

If a donor isolate possessed more than one resistance marker the conjugation mixture was inoculated onto selection plates to select separately for the transfer of each resistance marker. Each resulting potential transconjugant was tested separately for purity and a homogeneous resistance pattern. If any of the zones around antibiotic discs appeared to be mixed, sensitivities and further purification of variants was carried out as follows:- resistant colonies from within the zones were purified and sensitivity tests repeated. Sensitive variants were obtained by streaking single colonies from the CLED purity plate onto Isosensitest agar with the appropriate antibiotic disc in the centre of the streak. Sensitive clones identified after incubation were then plated for purity and sensitivity tests performed as previously described.

These purification steps were repeated as necessary until morphologically pure cultures were obtained on CLED agar which were homogeneous with respect to sensitivity pattern. Plasmid DNA in crude lysates from all resulting transconjugant cultures was correlated with those present in the donor organism. If antibiotic sensitivity and plasmid profile was the same from all selection plates only one transconjugant was stored for further investigation. When the sensitivity pattern and/or plasmid profile differed in transconjugants from a single donor isolate, one example of each variant was stored for further investigation.

Strains used as recipients when clinical E.coli isolates were the potential donors were E.coli K12 J53-1, J53-2, J62-1, J62-2; Ent.aerogenes GRI 9880-1 or GRI 9880-2. In addition to E.coli K12 and Ent.aerogenes, each of the best recipient strains listed in Table 3 were also used for R-plasmid transfer from enteric genera other than E.coli.

Transfer of plasmids not associated with antibiotic resistance:

The characteristics of non-conjugative resistance (Tra^- R^+) plasmids pHH1310a and R300B are listed in Table 3. These plasmids were obtained in E.coli K12 hosts, pHH1310a in J62-2 and R300B in W3110 (Hughes and Datta, 1983). Both

plasmids were mobilised into Sal.typhimurium LT2 using the following method. Plasmid pMR5 (Table 3) was transferred into the E.coli K12 isolates that carried pHH1310a and R300B at 30°C. Both Tra^- R^+ plasmids were then mobilised by pMR5 into Sal.typhimurium LT2. Selection plates for the isolation of Sal.typhimurium LT2 transconjugants containing plasmids pMR5 and pHH1310a or pMR5 and R300B were deoxycholate citrate agar (DCA) (Oxoid, CM227) and appropriate antimicrobial agents in the concentrations previously described. In the case of plasmid pHH1310a, carbenicillin selection was employed and single transconjugant clones were tested for the presence of both plasmids by sensitivity testing and plasmid visualisation. Selection plates for the mobilisation of R300B by pMR5 contained sulphamethoxazole or streptomycin. The presence of both plasmids was confirmed by sensitivity testing and plasmid visualisation. Both cultures were then incubated overnight in BHI broth at 40°C to eliminate pMR5. Direct sensitivity tests and purity plates were then inoculated. The presence of plasmid pHH1310a in Sal.typhimurium LT2 and curing of plasmid pMR5 was confirmed by retention of resistance to carbenicillin, loss of resistance to tetracycline and kanamycin and by plasmid visualisation. Similarly the retention of plasmid R300B and

loss of pMR5 was confirmed by streptomycin and sulphamethoxazole resistance, carbenicillin, tetracycline and kanamycin sensitivity and plasmid visualisation.

To determine the conjugative ability of plasmids in sensitive coliforms and resistant coliforms which did not transfer resistance markers, the ability to mobilise pHH1310a and/or R300B was tested using a modification of the method of Anderson (1965) and described in detail below. Clinical isolates were used as donors; intermediate strains were E.coli K12 J62-2 pHH1310a, W3110 R300B, Sal.typhimurium LT2 pHH1310a and LT2 R300B. The final recipients were E.coli K12 J53-1 or Ent.aerogenes GRI 9880-1. Organisms were inoculated separately into BHI broth and incubated overnight at 37°C. 1 ml of each donor organism was mixed separately with 1 ml of either each E.coli K12 intermediate or each Sal.typhimurium intermediate. 3 ml BHI broth was added and the mixtures were incubated at 37°C for 2 hr. 1 ml of recipient was then added to each donor/intermediate mixture and further incubated at 37°C overnight. E.coli K12 intermediates and recipients were used for all mobilisation experiments with the following exceptions. When the donor was a clinical E.coli, producing

presumptive colicins to which E.coli K12 was sensitive, Sal.typhimurium LT2 was employed as an intermediate and Ent.aerogenes GRI 9880-1 as a recipient.

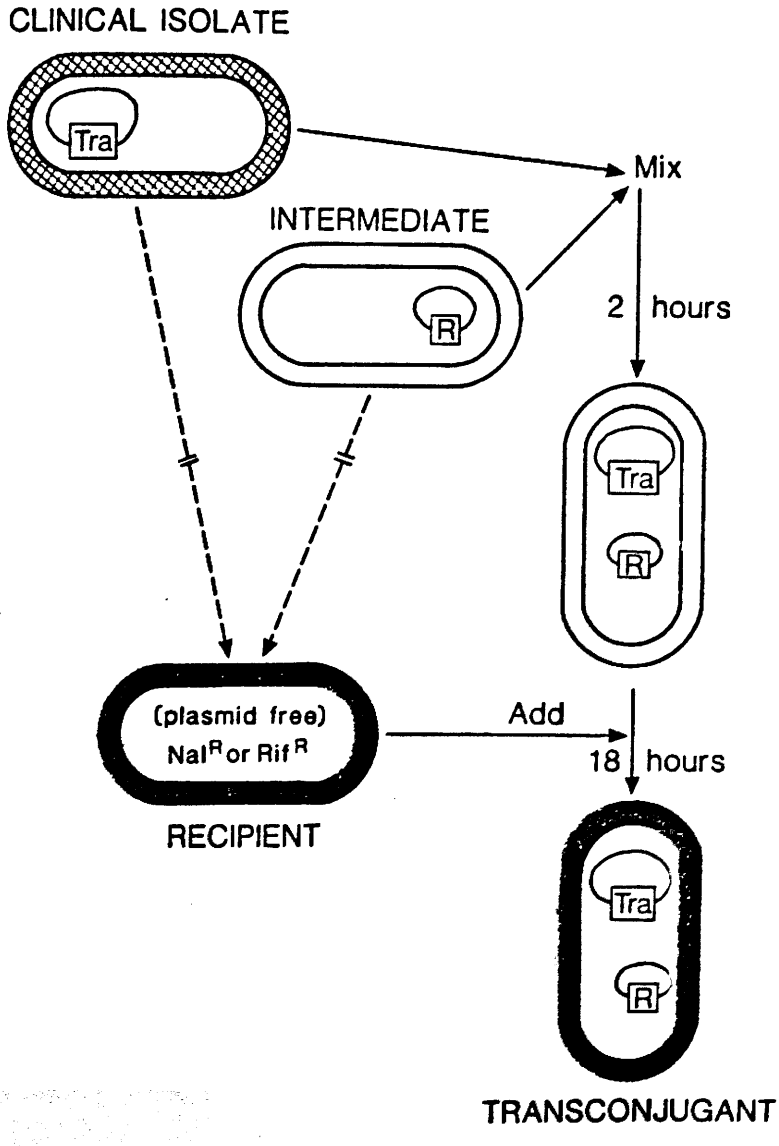
0.25 ml of each triple culture was inoculated onto CLED agar containing 25 μg/ml naladixic acid to select against the donor and intermediate and 400 μg/ml carbenicillin to select for mobilised pHH1310a or 100 μg/ml sulphamethoxazole for selection of mobilised R300B. Colonies on selection plates after incubation at 37°C overnight were tested for sensitivity and the presence of plasmids was confirmed by agarose gel electrophoresis, Figure I.

In a number of instances transconjugants acquired the appropriate mobilised Tra^- R^+ plasmid but no plasmid DNA corresponding to that seen in the clinical isolate was observed. In these instances re-mobilisation of the Tra^- R^+ plasmid was attempted by mixing the Nal^R transconjugant harbouring the Tra^- R^+ plasmid with a Rif^R E.coli K12 recipient. After overnight incubation the mixture was inoculated onto the plates selecting for the presence of the recipient containing the Tra^- R^+ plasmid. Mobilisation failure on the second conjugation was taken to indicate that the first mobilisation event resulted in either the

FIGURE I: Conjugative transfer of a Tra^+ R^- plasmid from a clinical isolate into an intermediate strain harbouring a Tra^- R^+ plasmid followed by mobilisation of a Tra^- R^+ plasmid into a recipient strain by the Tra^+ R^- plasmid.

Tra = conjugative ability
R = resistance determinants
Nal^R = rifampicin resistant
Rif^R = rifampicin resistant

FIGURE I


CLINICAL ISOLATE
Tra
Mix
INTERMEDIATE
R
2 hours
Tra
R
(plasmid free)
NalR or RifR
Add
18 hours
RECIPIENT
Tra
R
TRANSCONJUGANT

mobilisation of the Tra^- R^+ plasmid with failure of the Tra^+ R^- plasmid to transfer or unstable maintenance of the Tra^+ R^- plasmid.

Tra^- plasmid mobilisation by RP4:

A triple cross system was employed to detect the mobilisation of Tra^- plasmids in clinical isolates by RP4 using a similar method as described for the detection of mobilisation of Tra^- R^+ plasmids by clinical Tra^+ R^- plasmids.

Intermediate strains were clinical isolates containing plasmids which were not found to be Tra^+ by the methods as previously described. The donor strain was either E.coli K12 J53 RP4 or Sal.typhimurium LT2 RP4: recipient strains were E.coli K12 J62-1 or Ent.aerogenes GRI 9880-1. After overnight incubation of the donor, RP4 containing strain and recipient mixture, a sample was plated out onto media selecting for RP4 in the recipient strain. Twelve single colonies were examined by agarose gel electrophoresis to detect the presence of any plasmids in addition to RP4. Clones containing plasmid DNA in addition to RP4 were sensitivity tested as previously described.

Furthermore, clinical isolates which possessed resistance determinants in addition to intrinsic resistance were tested for the ability of RP4 to mobilise any Tra^- R^+ plasmids, if present. In these instances any resistance determinants in addition to those specified by RP4 were selected after overnight incubation of the mixture of clinical isolate, RP4 containing strain and potential recipient.

Detection and characterisation of plasmid DNA in crude lysates:

A modification of a method of DNA extraction and electrophoretic separation of plasmids as described by Platt and Sommerville (1981) was employed. The equivalent of 12-20 colonies of an overnight 37°C culture on nutrient agar (Oxoid, CM3) was suspended in 300 µl of tris-borate buffer (89 mM tris, 89 mM boric acid, 1.25 mM disodium ethylenediaminetetra-acetic acid (EDTA), pH 8.2) in 1.5 ml Eppendorf tubes. Two hundred microlitres of sodium dodecyl sulphate (SDS) (10% in tris-borate buffer) was added and the tubes mixed gently by inversion. After heating at 50°C for 5 min the crude lysates were centrifuged at 9,980G for 15 min in an Eppendorf centrifuge (Model 5414) at room temperature. One hundred microlitres of the supernate was loaded onto 0.7 per cent vertical agarose (Sigma) gels (15x10x0.5 cm). Five

microlitres of bromophenol blue tracking dye (25% sucrose, 8 mM sodium acetate, 3.5 mM SDS and 0.7 mM bromophenol blue) was added and the gels sealed with molten agarose. Electrophoresis was at room temperature in tris-borate buffer for 1 hr at 100V constant voltage, followed by 4 hr at 200V. After electrophoresis, gels were stained in 50 mM tris, 5 mM EDTA, 40 mM sodium chloride, (TES) buffer, pH 8.0 containing 0.165 mM ethidium bromide for 15 min. Plasmids were visualised using a long wavelength transilluminator (Ultra-Violet Products Inc.) and were photographed using polaroid film (Type 665) and a Polaroid MP4 land camera through Kodak filters 25, 12 and 2E. Four plasmids of known molecular weight RA-1, R1, R702 and RP4 (see Table 3) were included in each gel for callibration to estimate the molecular weight of unknown plasmids.

Preparation of purified plasmid DNA for restriction endonuclease digestion:

The method used to prepare purified plasmid DNA was adapted from a method developed in this Department (Platt *et al*, 1986). Twelve to 20 colonies from an overnight nutrient agar culture were harvested using a sterile dry swab and the organisms suspended in 0.5 ml 25 mM tris, 50 mM glucose, 10 mM

EDTA, (TGE) buffer, pH 8.0 in 1.5 ml Eppendorf tubes. The suspension was centrifuged in an Eppendorf centrifuge for 30 sec, the supernate discarded and 200 µl TGE containing 5 mg/ml lysozyme (Sigma) (freshly prepared) added. After vigorous mixing, to resuspend the pellet, tubes were incubated on ice for 5 min. 400 µl alkaline/SDS (freshly prepared, 1 ml 2M NaOH + 1 ml 10% SDS + 8 ml H_2O) was added, inverted gently to mix contents and further incubated on ice for 5 min. 300 µl 3 M sodium acetate was added, tubes mixed gently until a white precipitate formed then mixed vigorously. After 5 min incubation on ice, tubes were centrifuged for 2 min and the supernate transferred to a fresh tube. 0.5 ml phenol/chloroform (5 g phenol + 5 ml chloroform + 1 ml TGE buffer) was added, contents mixed, spun for 2 min and the upper aqueous layer transferred to a clean tube. 0.5 ml propan-2-ol was added, tubes mixed and left at room temperature for 5 min. After centrifugation for 2 min to precipitate nucleic acids the supernate was discarded and the pellet resuspended in 100 µl 10 mM Tris, 1 mM EDTA, (TE) buffer (pH 8.0). 50 µl 7.5 M ammonium acetate was added, the contents mixed and 300 µl ice cold ethanol added. The tubes were then left for a minimum of 30 min at -20°C. After centrifugation for 2 min the pellet was resuspended in 80 µl TE buffer, 9 µl 1 mg/ml ribonuclease (heat

inactivated) was added, tubes mixed and incubated at 37°C for 30 min. After addition of 10 µl of 2.5 M NaCl the phenol/chloroform extraction procedure, isopropanol precipitation and ethanol reprecipitation steps were repeated. The final DNA pellet was resuspended in 30 µl TE buffer. Duplicate purified plasmid DNA was prepared from each strain and the suspensions in TE buffer were pooled and 15 µl samples used for restriction endonuclease digestion.

Restriction endonuclease digestion:

The method developed in this Department (Platt et al, 1986) was used for restriction endonuclease digestion and visualisation of plasmid fragments. The following restriction enzymes were obtained - PstI, SmaI, HindIII, EcoRI, AvaII (BRL/Gibco) and Bspl286 (New England Biolabs, Inc.) - and 20 units of each enzyme used to digest plasmid DNA. Each restriction digestion mixture contained 15 µl DNA (or 2 µl phage lambda DNA), 5 µlx10 buffer as recommended by the enzyme manufacturer, 20 units enzyme and water to a total of 50 µl. After incubation for 4 hr at 37°C (SmaI at 30°C) 5 µl bromophenol blue tracker dye was added to each tube and the contents loaded onto 0.8 per cent horizontal agarose gels (15x15x0.3 cm) made in Tris-borate buffer containing 0.3 µg/ml

ethidium bromide. After overnight electrophoresis at 18 mA constant current in the above buffer, gels were photographed as previously described. A restriction digest of bacteriophage lambda DNA with PstI was included at both ends of each agarose gel to calibrate the gel for determination of molecular weight of restriction fragments of plasmid DNA.

Plasmid DNA was extracted and purified from each clinical isolate that contained plasmids and all transconjugants. A sample of the DNA was digested with enzyme PstI. The following criteria were then used to determine which further enzymes were used to produce plasmid fingerprints:-

(1) If the number of DNA fragments detected was 6 or greater but less than 21, the enzyme Sma I was used.

(2) If more than 20 DNA fragments were seen, EcoRI and HindIII were used.

(3) When less than 6 fragments were detected, Bspl286 and AvaII were used.

Following these general rules, fingerprints from all plasmid-containing clinical isolates and transconjugants were obtained using a pair of enzymes. Exceptions to the above criteria arose, for example when a clinical isolate possessed multiple plasmids not all of which transferred into the recipient strain; in such cases more than 20 DNA fragments may have been generated with the enzyme PstI from plasmids in the clinical isolate but between 6 and 20 from the transconjugant. In this instance, providing the number of fragments from the clinical isolate did not exceed 30 with either enzyme, SmaI was used as the second enzyme. If the number of PstI or SmaI fragments from the clinical isolate had exceeded 30 then EcoRI and HindIII was used. If the number of fragments in the transconjugant was less than 6 with either EcoRI or HindIII then PstI and SmaI were used for digestion of transconjugant plasmid DNA and EcoRI and HindIII for the clinical isolate. Thus, different clinical isolates and transconjugants were digested with the enzymes which gave the best fit to the general criteria described above.

Standardisation of estimates of fingerprint fragment molecular weight by computer analysis:

The mean distance travelled by 12 of the largest

fragments generated by PstI digestion of phage lambda DNA on 6 occasions was used to construct a calibration line for the standardisation of all subsequent digest gels (Table 4). The distance travelled by PstI fragments of phage lambda DNA was then measured using a Hewlett Packard 2648A digitiser from photographs of each agarose gel of plasmid digests. The distance travelled by fragments from each subsequent PstI digest of phage lambda DNA was then normalised against this callibration line. The distance travelled by plasmid DNA fragments was then digitised and normalised using the standardised line for each agarose gel photograph.

The standardised distances travelled for each plasmid DNA fragment were then transferred to the main-frame computer (ICL 2988) and the molecular weight of each fragment was calculated by reference to a standard curve of the reciprocal of mean distances travelled by phage lambda DNA fragments against molecular weight.

Each fingerprint was then plotted using a semi-logarithmic scale into tracks representing those obtained by agarose gel electrophoresis of plasmid DNA fragments. The relationship of proportionality between molecular weight and

TABLE 4: **Molecular weight of 12 of the largest fragments of phage lambda DNA generated by PstI digestion and mean distance travelled on 6 occasions tested by agarose gel electrophoresis.**

Phage lambda DNA fragment size (kb pairs)	Mean distance travelled on agarose gel after electrophoresis (cm)
11,501	10
5,077	18.3
4,749	19.5
4,507	20.5
2,838	30.2
2,443	34.5
2,104	37.7
1,986	40
1,700	44
1,159	54.7
1,093	56.7
805	60.7

kb = kilobase: cm = centimetre

the inverse of distance was chosen for optimal linearity whereas graphical output using log molecular weight proportional to distance provided greater similarity to the visual appearance of gels.

Estimation of reproducibility of fragment size:

Plasmid DNA from a single plasmid-containing clinical isolate was prepared, digested with PstI and gel electrophoresis carried out on 7 separate occasions. The molecular weight of each fragment was then calculated as described above.

The 7 fingerprints were then compared to assess the reproducibility of the method used to generate fingerprints from DNA which had been purified, digested and electrophoresed on different occasions. In addition, the standard error of the mean molecular weight of 18 fragments common to all 7 fingerprints was calculated to assess the reproducibility of the method used for determination of molecular weight of individual fragments.

Statistical analysis:

Statistical analyses were performed using methods as described by Siegel (1956). These included calculation of standard deviation, standard error, chi-squared (χ^2) test with n degrees of freedom (df_n) and a χ^2 2x2 contingency test incorporating Yates' correction for continuity.

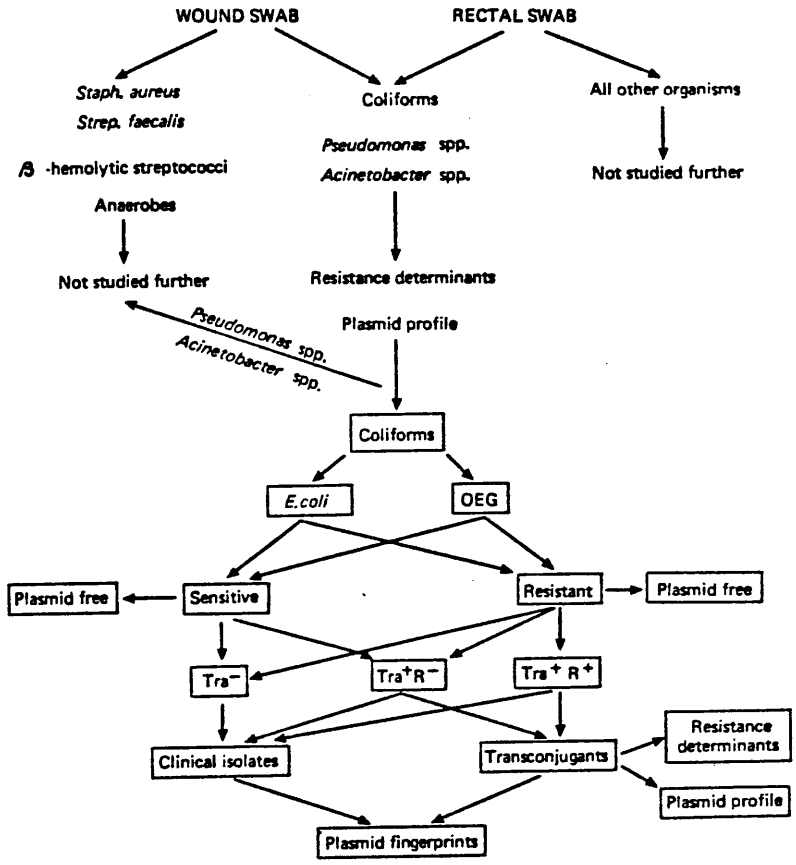
Summary experimental approach:

A summary of the experimental approach used in this study is outlined in Figure II.

FIGURE II: **Outline of the methods used to characterise coliforms isolated from clinical specimens and techniques used to investigate the plasmids present in the coliform isolates and transconjugants resulting from conjugation experiments.**

OEG = enteric genera other than E.coli
$Tra^+ R^+$ = conjugative resistance plasmid
$Tra^+ R^-$ = conjugative plasmid not associated with resistance
Tra^- = non-conjugative plasmid

FIGURE II


WOUND SWAB
RECTAL SWAB
Staph. aureus
Strep. faecalis
β -hemolytic streptococci
Anaerobes
Not studied further
Coliforms
Pseudomonas spp.
Acinetobacter spp.
Resistance determinants
All other organisms
Not studied further
Plasmid profile
Pseudomonas spp.
Acinetobacter spp.
Coliforms
E.coli
OEG
Plasmid free
Sensitive
Resistant
Plasmid free
Tra⁻
Tra⁺R⁻
Tra⁺ R⁺
Clinical isolates
Transconjugants
Resistance determinants
Plasmid profile
Plasmid fingerprints

RESULTS

Patients and wounds studied:

One hundred and seventeen patients were studied between February 1981 and May 1983 where clinical appearance suggested delayed healing and/or infection of the wound. Wounds from patients in the PVD Unit were divided into three groups - vascular ulcers, amputation sites or surgical vascular repair sites. Wounds from geriatric patients were pressure sores with the exception of one patient with minor burns. For the purpose of this study wounds from geriatric patients are referred to as decubitus ulcers.

Of the 117 patients studied, single wounds were present in 89 and 28 possessed multiple wound sites as shown in Table 5.

One hundred and sixty three wounds were studied, of which 48 were vascular ulcers, 39 amputation sites, 50 surgical vascular repair sites and 26 decubitus ulcers. Table 6 shows the number of weeks each wound was investigated. For vascular ulcers the mean healing time was 1.77 weeks (range 1-6), 2.33 weeks (range 1-8) for amputation sites and 1.70 weeks (range 1-5) for surgical vascular repair sites. This indicates that vascular ulcers and surgical vascular repair sites took a

TABLE 5: **Classification of wounds into groups according to type of wound (163 wounds from 117 patients).**

No. of Patients	No. of Vascular Ulcers	No. of Amputation Sites	No. of Surgical Vascular Repair Sites	No. of Decubitus Ulcers
27	1	-	-	-
29	-	1	-	-
25	-	-	1	-
8	-	-	-	1
4	2	-	-	-
2	-	2	-	-
2	-	-	2	-
1	1	1	-	-
5	1	-	1	-
3	-	1	1	-
1	3	-	-	-
1	1	-	2	-
1	-	-	3	-
1	1	-	3	-
1	1	1	2	-
1	1	1	3	-
3	-	-	-	2
1	-	-	-	4
1	-	-	-	8
TOTAL	48	39	50	26

TABLE 6: **Distribution of the number of weeks 163 wounds were sampled.**

No.of Weeks Sampled	Vascular Ulcers	Amputation Sites	Surgical Vascular Repair Sites	Decubitus Ulcers
1	25	16	26	8 *
2	14	11	16	6 *
3	6	3	6	4 *
4	2	5	1	2 *
5	-	2	1	3 *
6	1	1	-	-
7	-	-	-	-
8	-	1	-	1
9	-	-	-	-
10	-	-	-	2
MEAN	1.77	2.33	1.70	3.19

* The wounds in these patients were not observed to resolution because the patient either died or was transferred and therefore represents an underestimate of the healing time.

similar length of time to heal, but that amputation sites healed more slowly. Despite the fact that the mean healing time for decubitus ulcers could not be measured accurately, it appeared that decubitus ulcers healed slowly (mean 3.19 weeks, range 1-10) compared to wounds from PVD patients.

Wounds were classified on clinical grounds and in terms of increasing severity, as Grade I, II or III.

The results for the initial specimen collected from each wound are detailed in Table 7. Vascular ulcers were of the lowest wound grade with a mean of 1.88, amputation sites, and surgical vascular repair sites were similar with mean grades of 2.26 and 2.24 respectively. Decubitus ulcers were on average of a higher wound grade with a mean of 2.77.

In patients in the PVD Unit, the mean grade of wounds that were sampled on only one occasion and which therefore resolved quickly, was lower than that of the wounds which healed more slowly and from which subsequent wound swabs were obtained. The mean wound grade of wounds only sampled on one occasion was higher than the mean grade of the initial assessment from wounds which were sampled on more than one occasion from geriatric patients.

TABLE 7: **Comparison of the initial clinical severity (Grades I-III) of wounds with the wound type site.**

Grade of Wound	Vascular Ulcers			Amputation Sites			Surgical Vascular Repair Sites			Decubitus Ulcers		
	A	**B**	**A+B**	**A**	**B**	**A+B**	**A**	**B**	**A+B**	**A**	**B**	**A+B**
I	8	3	11	2	2	4	1	3	4	-	-	-
II	16	16	32	9	12	21	20	10	30	1	5	6
III	1	4	5	5	9	14	5	11	16	7	13	20
MEAN	1.72	2.04	1.88	2.19	2.30	2.26	2.15	2.33	2.24	2.88	2.72	2.77

A = Wounds sampled on only one occasion

B = Initial wound from which subsequent specimens were obtained

Isolation of potential pathogens

The incidence of potential pathogens in the first specimen from each wound is shown in Table 8. These results indicate whether or not any species of potential pathogen was isolated from the wound but does not indicate how many wounds were colonised/infected by more than one type of organism. Potential pathogens were isolated from the initial specimen from 79 per cent of vascular ulcers, 59 per cent of amputation sites, 44 per cent of surgical vascular repair sites and 96 per cent of decubitus ulcers. This indicates that vascular ulcers are more commonly colonised/infected with potential pathogens than are amputation sites, with surgical vascular repair sites being colonised/infected to a lower degree. Colonisation/infection of decubitus ulcers with potential pathogens was more common than in any of the other groups.

Eight vascular ulcers, 10 amputation sites and 17 surgical vascular repair sites were sampled on only one occasion and gave negative results.

Of the remaining sites where no potential pathogens were isolated from the first specimen, 9 (1 vascular ulcer, 2 amputation sites and 6 surgical vascular repair sites) still

TABLE 8: **Isolation incidence of potential pathogens from the initial specimen from different wound groups.**

	Vascular Ulcers			Amputation Sites			Surgical Vascular Repair Sites			Decubitus Ulcers		
	A	**B**	**A+B**	**A**	**B**	**A+B**	**A**	**B**	**A+B**	**A**	**B**	**A+B**
Potential pathogens isolated	17	21	38	6	17	23	9	13	22	8	17	25
No potential pathogens isolated	8	2	10	10	6	16	17	11	28	0	1	1

A = Wounds sampled on only one occasion

B = Initial wound from which subsequent specimens were obtained

gave negative results on the second occasion sampled. These wounds were not sampled again. One surgical vascular repair site gave negative results on each of three occasions sampled.

Ten wounds gave negative results from the first specimen but a second yielded potential pathogens. These wounds were sampled between two and 5 occasions. One or more species of potential pathogen was isolated on the second and subsequent occasions sampled.

Seventeen vascular ulcers, 6 amputation sites, 9 surgical vascular repair sites and 8 decubitus ulcers were sampled on one occasion and at least one species of potential pathogen was isolated from each specimen.

Twenty one vascular ulcers, 17 amputation sites, 13 surgical vascular repair sites and 17 decubitus ulcers yielded potential pathogens on the first occasion sampled and were sampled on at least one subsequent occasion.

Sixty one of the 163 wounds sampled yielded potential pathogens on two or more occasions: however, only 12 wounds produced the same species of potential pathogen on all occasions sampled. To investigate the possibility of patterns of wound colonisation with particular species of potential pathogens a summary of all organisms isolated from each wound including multiple specimens are shown in Table 9. Isolation

TABLE 9: **Isolation incidence of potential pathogens from 48 vascular ulcers, 39 amputation sites, 50 surgical vascular repair sites and 26 decubitus ulcers**

	Vascular Ulcers		Amputation Sites		Surgical Vascular Repair Sites		Decubitus Ulcers	
	No.	**(%)**	**No.**	**(%)**	**No.**	**(%)**	**No.**	**(%)**
Coliforms	23	(48)	17	(44)	15	(30)	26	(100)
Pseudomonas spp.	14	(29)	7	(18)	3	(6)	17	(65)
Acinetobacter spp.	2	(4)	1	(3)	1	(2)	-	-
Staph.aureus	17	(35)	8	(21)	10	(20)	5	(19)
Strep.faecalis	3	(6)	9	(23)	9	(18)	1	(4)
Beta-haemolytic streptococcus	2	(4)	2	(5)	-	-	6	(23)
Anaerobes	2	(4)	3	(8)	1	(2)	8	(31)

of more than one species of coliform, or isolates which varied with regard to colonial morphology or antibiotic sensitivity within any single potential pathogenic species, were not distinguished.

There was very little difference in the rate of isolation of coliforms from vascular ulcers, amputation sites or surgical vascular repair sites. However, at least one species of coliform was isolated from each decubitus ulcer studied. Pseudomonas spp. were isolated from a larger number of vascular ulcers than amputation sites. A small proportion of surgical vascular repair sites contained Pseudomonas spp. However, a much larger proportion of decubitus ulcers contained Pseudomonas spp. compared to other wound groups. Staph.aureus was isolated more commonly from vascular ulcers than from amputation sites, surgical vascular repair sites or decubitus ulcers. Strep.faecalis was isolated from similar numbers of amputation sites and surgical vascular repair sites with a lower incidence of isolation from vascular ulcers and decubitus ulcers. Acinetobacter spp., beta-haemolytic streptococci and anaerobes were isolated from a small proportion of vascular ulcers, amputation sites and surgical vascular repair sites.

No Acinetobacter spp. were isolated from decubitus ulcers although 23 per cent yielded beta-haemolytic streptococci and 31 per cent yielded anaerobes.

Association of the isolation of potential pathogens with the rate of healing of wounds from PVD patients:

No potential pathogens were isolated from 45 of the 137 wounds studied from patients in the PVD unit. Staph.aureus was isolated either alone (12 wounds) or in combination with other species (23 wounds) from 35 wounds. These 35 wounds took significantly longer ($\chi^2 = 6.26$, $p < 0.05$) to heal compared to wounds from which no potential pathogens were isolated. Similarly, 55 wounds from which coliforms were isolated (20 where coliforms were isolated alone and 35 where coliforms were isolated together with other species of potential pathogens) took significantly longer ($\chi^2 = 9.08$, $p < 0.01$) to heal than potential pathogen-free wounds. In contrast, there was no significant difference ($\chi^2 = 2.39$, $p > 0.05$) in the healing time of the 24 wounds from which Pseudomonas spp. were isolated (5 containing Pseudomonas spp. alone, 19 possessing Pseudomonas spp. and other potential pathogens) compared to potential pathogen-free wounds.

Ninety-two wounds yielded at least one of the above species of potential pathogen and/or Acinetobacter spp., Strep.faecalis, beta-haemolytic streptococci and anaerobes. These wounds took significantly longer (χ^2 = 13.73, $p < 0.001$) to heal than the 45 wounds which did not contain any potential pathogens. Thus there is a clear association between the isolation of potential pathogens (excluding Pseudomonas spp.) and a prolonged healing time.

Topical Therapy:

A large proportion of the wounds studied were being treated with topical agents including hypochlorite solution (Milton), hydrogen peroxide, povidine-iodine (Betadine), chlorhexidine gluconate (Hibitane), trypsin (Trypure), dextranomer (Debrisan), and a number of other agents. With the exception of Milton, none of these topical agents were applied to more than 10 per cent of the wounds studied. Table 10 shows the incidence of isolation of potential pathogens from each specimen in the presence and absence of Milton as a topical antimicrobial agent. Milton had been applied topically to the wound before the collection of 35 per cent of specimens.

TABLE 10: **The effect of topical therapy with Milton on the isolation of potential pathogens from 163 wounds.**

	TOPICAL THERAPY		
	No. of Wounds Treated with Milton	No. of Wounds not Treated with Milton	
Coliforms isolated	69	98	χ^2 4.37
No coliforms isolated	53	124	$p < 0.05$
Pseudomonas spp. isolated	29	64	χ^2 0.78
No Pseudomonas spp. isolated	93	158	$p > 0.25$
Acinetobacter spp. isolated	2	4	
No Acinetobacter spp. isolated	120	218	NA
Staph.aureus isolated	28	43	χ^2 0.42
No Staph.aureus isolated	94	179	$p > 0.5$
Strep.faecalis isolated	13	16	
No Strep.faecalis isolated	109	206	NA
Beta-haemolytic streptococci isolated	3	20	
No beta-haemolytic streptococci isolated	119	202	NA
Anaerobes isolated	7	13	
No anaerobes isolated	115	209	NA

NA = not applicable.

Acinetobacter spp. were only isolated from 6 (2%) of the 163 wounds studied, Strep.faecalis from 29 (8%), beta-haemolytic streptococci from 23 (7%) and anaerobes from 20 (6%). As the numbers of specimens from which these organisms were isolated was small and appeared to be unrelated to the use of Milton, they were not analysed further.

Coliforms were isolated from 167 (49%), Pseudomonas spp. from 93 (27%) and Staph.aureus from 71 (21%) of all wounds. Statistical analysis demonstrates that there was no significant difference in the isolation rate of Pseudomonas spp. ($p > 0.25$) and Staph.aureus ($p > 0.5$) in the presence and absence of Milton as a topical antimicrobial agent. However, there was an increase ($p < 0.05$) in the numbers of specimens from which coliforms were isolated when Milton was applied topically to the wound prior to sampling, compared to those from wounds to which no Milton was applied.

Systemic antimicrobial therapy:

To assess the influence of systemic antimicrobial agents on the microbial flora of individual wounds, all antimicrobial therapy administered during the 14 days prior to wound sampling was analysed. Systemic antimicrobial agents prescribed included cephalosporins, flucloxacillin,

metronidazole, penicillin V, penicillin G, amoxycillin, ampicillin, augmentin, gentamicin, erythromycin, septrin, tetracycline, naladixic acid and fucidin. Except for cephalosporins, flucloxacillin, metronidazole and penicillin V, the other antimicrobial agents were prescribed for fewer than 10 per cent of patients and the effects of these agents individually were not considered further.

The number of wounds from which different species of potential pathogens were isolated and correlation with administration of cephalosporin, flucloxacillin, metronidazole or penicillin V systemic therapy is shown in Table 11.

There was a significant reduction in the numbers of specimens from which coliforms were isolated when cephalosporins ($p < 0.05$), flucloxacillin ($p < 0.001$) and penicillin V ($p < 0.01$) had been prescribed for a period before specimen collection but no significant difference when metronidazole ($p > 0.5$) was administered (Table 12).

Pseudomonas spp. were isolated from significantly fewer wounds when cephalosporins ($p < 0.001$), flucloxacillin ($p < 0.05$) and metronidazole ($p < 0.05$) were prescribed but not when penicillin V ($p > 0.75$) was administered.

TABLE 11: **The isolation of potential pathogens in the presence and absence of systemic antimicrobial therapy with cephalosporins, flucloxacillin, metronidazole and penicillin V.**

	SYSTEMIC THERAPY								
	Cephalosporins		Flucloxacillin		Metronidazole		Penicillin V		Total Specimens
	+	-	+	-	+	-	+	-	
Coliforms isolated	44	123	26	141	36	131	9	158	167
No coliforms isolated	70	107	57	120	43	134	30	147	177
Pseudomonas spp. isolated	14	79	14	79	12	81	8	85	93
No Pseudomonas spp. isolated	100	151	69	182	67	184	31	220	251
Acinetobacter spp. isolated	2	4	4	2	3	3	3	3	6
No Acinetobacter spp. isolated	112	226	79	259	76	262	36	302	339
Staph.aureus isolated	19	52	14	57	20	51	6	65	71
No Staph.aureus isolated	95	178	69	204	59	214	33	240	273
Strep.faecalis isolated	20	9	12	17	8	21	6	23	29
No Strep.faecalis isolated	94	221	71	244	71	244	33	282	315
Beta-haemolytic streptococci isolated	3	20	2	21	1	22	1	22	23
No beta-haemolytic streptococci isolated	111	210	81	240	78	243	38	283	321
Anaerobes isolated	4	16	5	15	4	16	1	19	20
No anaerobes isolated	110	214	78	246	75	249	38	286	324

+ = Systemic therapy administered
- = Systemic therapy not administered

TABLE 12: **Correlation of systemic antimicrobial therapy with the isolation of coliforms, Pseudomonas spp., and Staph.aureus from 344 wound specimens.**

		Cephalosporins	Flucloxacillin	Metronidazole	Penicillin V
Coliforms	χ^2	6.18	12.10	0.23	10.3
	p	< 0.05	< 0.001	> 0.5	< 0.01
Pseudomonas spp.	χ^2	17.71	5.07	6.54	0.61
	p	< 0.001	< 0.05	< 0.05	> 0.75
Staph.aureus	χ^2	1.30	0.67	1.02	4.24
	p	> 0.25	> 0.25	> 0.25	< 0.05

There was no significant reduction in the numbers of specimens from which Staph.aureus were isolated when cephalosporins ($p > 0.25$), flucloxacillin ($p > 0.25$) and metronidazole ($p > 0.25$) was administered. However, there was a significant reduction ($p < 0.05$) in the isolation incidence of Staph.aureus isolation when penicillin V was prescribed.

Topical and systemic therapy:

There was no significant difference in the numbers of specimens from which Staph.aureus was isolated in the presence or absence of topical therapy with Milton ($p > 0.5$) (Table 10) or any systemic therapy ($p > 0.25$) (Table 13). There was a significant increase in the numbers of specimens from which coliforms were isolated when topical therapy with Milton ($p < 0.05$) was administered but no significant difference when systemic therapy was prescribed ($p > 0.1$). In contrast there was no significant difference in the numbers of specimens from which Pseudomonas spp. were isolated when topical therapy with Milton was applied ($p > 0.25$). However, there was a significant reduction when systemic therapy ($p < 0.05$) was administered.

TABLE 13: **Isolation incidence of potential pathogens in the presence and absence of any systemic antimicrobial therapy.**

	SYSTEMIC THERAPY		
	No. of Patients Treated	No. of Patients Not Treated	
Coliforms isolated	102	65	χ^2 2.00
No coliforms isolated	122	55	$p > 0.10$
Pseudomonas spp. isolated	51	42	χ^2 5.32
No Pseudomonas spp. isolated	173	78	$p < 0.05$
Acinetobacter spp. isolated	5	1	NA
No Acinetobacter spp. isolated	219	119	
Staph.aureus isolated	42	29	χ^2 1.09
No Staph.aureus isolated	182	91	$p > 0.25$
Strep.faecalis isolated	23	6	NA
No Strep.faecalis isolated	201	114	
Beta-haemolytic streptococci isolated	8	15	NA
No beta-haemolytic streptococci isolated	216	105	
Anaerobes isolated	14	6	NA
No anaerobes isolated	210	114	

NA = not applicable

Neither topical nor systemic antimicrobial therapy, separately or in combination, had any significant effect on the numbers of specimens from which Staph.aureus ($p > 0.25$) was isolated as shown in Table 14. There was however, a statistically significant effect on the incidence of isolation of both coliforms ($p < 0.05$) and Pseudomonas spp. ($p < 0.05$).

Isolation of potential pathogens from vascular ulcers and surgical wounds subsequent to amputation:

Three of the patients with vascular ulcers underwent either toe or partial foot amputations after collection of specimens from vascular ulcers. In each case the same organisms were isolated from the ulcer before amputation and from the amputation site after surgery. From one patient, the organism was Acinetobacter spp. Pseudomonas spp. and Staph.aureus were isolated from a second patient's wounds before and after surgery. Klebsiella spp., Proteus spp., Citrobacter spp. and Pseudomonas spp. were isolated from the third patient's vascular ulcer and the amputation site after surgery.

Potential pathogens isolated from multiple sites in the same patient:

Twenty eight patients studied possessed multiple wound

TABLE 14: **Effects of topical therapy with Milton and systemic therapy on the isolation of potential pathogens from 344 wound specimens**

	Topical -ve Systemic -ve	Topical +ve Systemic -ve	Topical -ve Systemic +ve	Topical +ve Systemic +ve	χ^2 df_3
Coliforms isolated	45	20	53	49	12.1
No coliforms isolated	36	19	88	34	$p < 0.05$
Pseudomonas spp. isolated	32	10	32	19	8.5
No Pseudomonas spp. isolated	49	29	109	64	$p < 0.05$
Acinetobacter spp. isolated	1	0	3	2	
No Acinetobacter spp. isolated	80	39	138	81	NA
Staph.aureus isolated	17	12	29	13	3.6
No Staph.aureus isolated	64	27	112	70	$p > 0.25$
Strep.faecalis isolated	3	3	12	11	
No Strep.faecalis isolated	78	36	129	72	NA
Beta-haemolytic streptococci isolated	14	1	6	2	
No beta-haemolytic streptococci isolated	67	38	135	81	NA
Anaerobes isolated	4	2	8	6	
No anaerobes isolated	77	37	133	77	NA

NA = not applicable, df_3 = three degrees of freedom.

sites as shown in Table 5.

Fifteen patients had two or more wound sites each of which were of the same group, i.e. vascular ulcers, amputation sites, surgical vascular repair sites, or decubitus ulcers. These 15 patients can be divided into three groups according to the potential pathogens isolated.

The first group consists of:- one patient from whom no potential pathogens were isolated from either of two sites; two patients from whom potential pathogens were isolated from one site but no organisms were isolated from the second site; and one patient from whom the same potential pathogens were isolated from two sites but the third site gave negative results.

The second group consists of 5 patients (4 with 2 wounds, 1 with 3 wounds) where the same species of potential pathogens were isolated from multiple sites in the same patient.

From patients in the third group the same species of potential pathogen was isolated from all sites in the same patient but in addition, at least one different species of potential pathogen was isolated from one wound site e.g. in patient No.10 Pseudomonas spp. was isolated from both vascular

ulcers whereas Staph.aureus was only isolated from one site. Patient No.143 possessed 8 decubitus ulcers as shown in Table 15. Pseudomonas spp. were isolated from all sites. Klebsiella spp. were isolated from 7 sites, Proteus spp. from 4 sites and Citrobacter spp. and Enterobacter spp. were both isolated from two ulcers. In addition, anaerobic organisms were isolated from three wounds.

Thirteen patients possessed multiple wound sites of which at least two were of different wound groups. None of these patients yielded exactly the same potential pathogens from each wound. No potential pathogens were isolated from two sites in one patient. Five patients possessed one wound which yielded at least one species of potential pathogen and one which did not yield any. From three of the remaining patients the same species of potential pathogen was isolated from each wound in the same patient. Additional species were also present in some wounds, e.g. patient No.52 possessed three wounds, Staph.aureus was isolated from all three wounds and Serratia spp. from two sites. One of these sites also harboured Strep.faecalis.

Four patients possessed multiple wound sites which did not yield the same species of potential pathogen, i.e. patient No.92 possessed two sites, one of which yielded Staph.aureus

TABLE 15: **Isolation of potential pathogens from 8 decubitus ulcers from patient No. 143**

	Decubitus Ulcer No.							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Proteus spp.	+	+	+	+	-	-	-	-
Klebsiella spp.	-	+	+	+	+	+	+	+
Citrobacter spp.	-	+	-	+	-	-	-	-
Enterobacter spp.	-	-	-	+	-	-	-	+
Pseudomonas spp.	+	+	+	+	+	+	+	+
Anaerobes	+	+	+	-	-	-	-	-

and the other Strep.faecalis. Patient No.36 possessed 5 wound sites: 4 sites yielded Strep.faecalis. Three of these 4 sites and the additional fifth yielded Proteus spp., two yielded beta-haemolytic streptococci, and one contained E.coli.

Individual wounds: correlation of rate of healing, wound grade and antimicrobial therapy with potential pathogens isolated:

The following cases illustrate individual patients and in particular the effects antimicrobial therapy had on the healing of the wound.

Patient No.28 had a partial foot amputation before specimen collection. Eight specimens were collected and with the exception of the first specimen when only Staph.aureus was isolated, all subsequent specimens yielded both Staph.aureus and Enterobacter spp. This patient was being treated intermittently with both Milton topically and erythromycin and/or metronidazole systemically. Gradual, but not complete healing of the wound was apparent.

Patient No.35 yielded E.coli and Strep.faecalis from an above-knee amputation site on 4 occasions studied. Despite systemic treatment with cephalexin and gentamicin the wound healed very slowly but still yielded the same potential pathogens on all occasions sampled.

Similar results were obtained from many other patients where neither topical nor systemic therapy appeared to influence the isolation of potential pathogens from the individual wounds but gradual healing was evident over a period of weeks. In a number of patients there was, however, evidence of changes in organisms isolated from the wound when topical and/or systemic therapy was administered.

Patient No.12 had a surgical vascular repair site which was of grade III on the first two occasions sampled (see Fig. III). The patient was not receiving topical therapy but flucloxacillin, cefuroxime and metronidazole had been administered systemically before initial specimen collection. E.coli and Klebsiella spp. were isolated from the wound on both occasions. On the third and fourth occasion sampled, the wound had improved to grade II; systemic therapy was reintroduced using gentamicin and metronidazole. No coliforms were isolated but Staph.aureus was isolated on both occasions. From the fifth specimen no potential pathogens were isolated. By this time antimicrobial therapy had stopped; however, the wound remained of grade II.

From a few individual patients there was some evidence that suggested the effectiveness of antimicrobial therapy. Pseudomonas spp. and Staph.aureus were isolated from a vascular

FIGURE III: **Correlation between grade of wound, systemic antimicrobial therapy and potential pathogens isolated from a surgical vascular repair site from patient No.12 on 5 occasions sampled.**

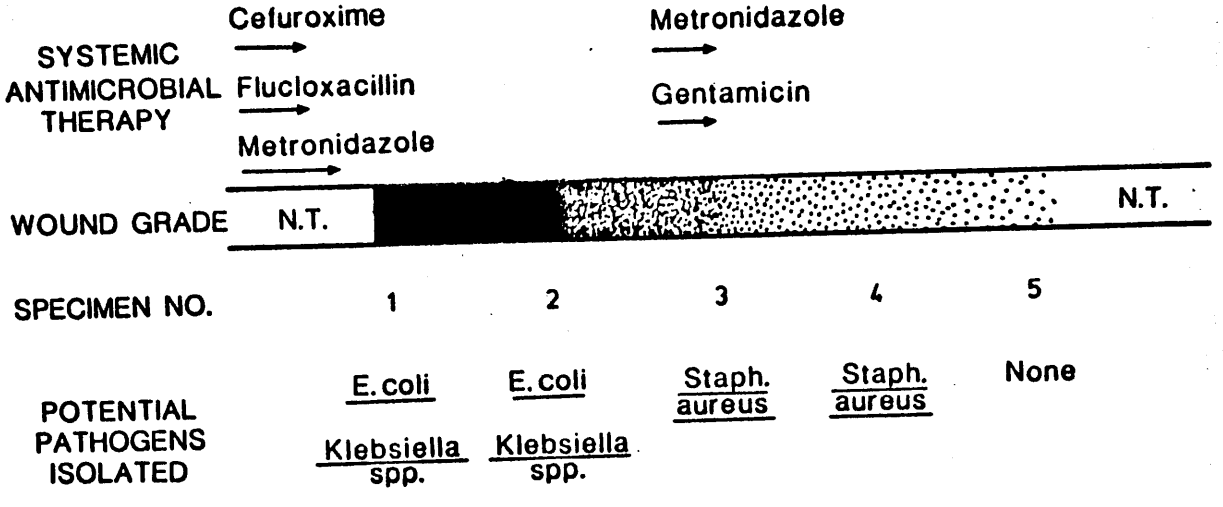
NT = not tested

 = Grade III

 = Transition between Grade III and II

 = Grade II

FIGURE III


SYSTEMIC ANTIMICROBIAL THERAPY
Cefuroxime
Flucloxacillin
Metronidazole
Metronidazole
Gentamicin
WOUND GRADE
N.T.
N.T.
SPECIMEN NO.
1
2
3
4
5
POTENTIAL PATHOGENS ISOLATED
E.coli
Klebsiella spp.
E.coli
Klebsiella spp.
Staph. aureus
Staph. aureus
None

ulcer from patient No.89 and from the surgical wound following partial foot amputation. On the occasion following treatment, both topically and systemically with gentamicin, no potential pathogens were isolated from the wound.

Development of resistance:

A number of species of potential pathogen are known to express resistance in the presence of specific antimicrobial agents. This expression of resistance was observed in two patients studied.

A surgical wound from patient No.100 was sampled only once after the patient had been treated systemically with gentamicin. The only species of potential pathogen isolated was E.coli: however, two sensitivity variants were isolated. The first was sensitive to all antimicrobial agents tested. The second variant was partially resistant to gentamicin, kanamycin, streptomycin, tobramycin and amikacin.

Patient No.139 had 4 decubitus ulcers. In addition to Pseudomonas spp., Staph.aureus and beta-haemolytic streptococci being isolated from some of the wounds, on a number of occasions E.coli, Prot.vulgaris and Prot.mirabilis were also isolated. During the two weeks before systemic therapy with naladixic acid was started E.coli and

Prot.vulgaris were isolated from the wounds. All coliforms isolated were sensitive to naladixic acid. From the specimens collected after systemic therapy with naladixic acid, all E.coli isolated from all 4 wounds were resistant to naladixic acid. Prot.vulgaris was still isolated from two wounds and remained sensitive to naladixic acid. Prot.mirabilis was also isolated from all 4 wounds after the commencement of naladixic acid systemic therapy. All isolates were resistant to the drug.

Plasmid distribution in coliforms isolated:

Plasmid-mediated resistance to antimicrobial agents contributes significantly to resistance amongst many bacterial species together with resistance due to chromosomal mutations. The transmissible nature of many plasmids, their dissemination and the consequent spread of any resistance determinants encoded by them, can play an important role in the overall resistance profile of taxonomic groups of bacteria. Therefore, an investigation into the distribution, conjugal ability and resistance markers specified by plasmids in the coliforms collected was studied.

Although coliforms were isolated from 81 of the 163 wounds studied they were often isolated from the same wound on more than one occasion. In addition, more than one species of coliform was isolated from many of the wounds studied. Thus, 168 specimens yielded coliforms and the number of distinct isolates from each specimen is shown in Table 16. These results include the isolation of two or more morphological types or sensitivity variants of one species from the same specimen. A mean of 1.63 (range 1-5) coliforms was isolated from coliform-containing specimens.

In addition to collecting wound swabs from all patients, 91 rectal swabs were also obtained from 39 patients. With the exception of one specimen which yielded no coliforms, the numbers of distinct isolates from each specimen are also show in in Table 16. The mean number of isolates per coliform-containing rectal swab was 3.10 (range 1 - 7).

When coliforms with the same morphological appearance and antibiotic sensitivity pattern were isolated from both a wound and rectal swab from the same patient they were considered as separate isolates until their respective plasmid profiles had been considered.

TABLE 16: **Number of distinct coliform isolates from 168 wound swabs and 90 rectal swabs which yielded at least one coliform per specimen.**

No. of Distinct Coliform Isolates per Specimen	No. of Wound Swabs	No.of Rectal Swabs
1	93	12
2	51	21
3	19	26
4	3	15
5	2	11
6	-	3
7	-	2

Of the 553 coliforms isolated, 250 were E.coli and 303 belonged to other enteric genera (OEG) as shown in Table 17. The most common genus within the OEG was Proteus spp. followed by Klebsiella spp., Enterobacter spp. and Citrobacter spp. Providencia spp., Serratia spp., Hafnia spp. and Yersinia spp. were isolated in small numbers. Since the numbers of each individual genus within OEG were small, they were combined for the purposes of investigating the distribution of plasmids, plasmid mobility and resistance transfer. Comparisons of all OEG were then made with E.coli. Multiple isolates either from two or more wounds in the same patient, multiple specimens from the same wound or multiple rectal swabs from any patient, were excluded for the construction of plasmid frequency distributions.

Table 18 shows the numbers of coliforms studied after exclusion of multiple isolates. Figures IVa and IVb show the distribution of plasmids in E.coli and OEG respectively. Only 13 per cent of the 135 E.coli isolates studied did not possess plasmids. The mean number of plasmids per isolate was 2.13 (range 0-7). Sixty-four per cent of the 144 OEG isolates did not possess any plasmids. This was significantly more (χ^2 = 74.88, $p < 0.001$) than in E.coli. The mean number of plasmids per OEG isolate was 0.56 (range 0-4).

TABLE 17: **Numbers of *E.coli* and other enteric genera isolated from wound and rectal swabs**

Species Isolated	Wound Swabs	Rectal Swabs	Total
E.coli	76	174	250
Proteus spp.	103	35	138
Klebsiella spp.	25	32	57
Enterobacter spp.	33	19	52
Citrobacter spp.	19	12	31
Providencia spp.	9	3	11
Serratia spp.	8	-	8
Hafnia spp.	1	3	4
Yersinia spp.	-	1	1

TABLE 18: Numbers of sensitive and resistant coliforms studied after the exclusion of multiple isolates

Species isolated	Total Isolates	Sensitive Isolates	Resistant Isolates
E.coli	135	62	73
Proteus spp.	63	51	12
Klebsiella spp.	29	22	7
Enterobacter spp.	27	14	13
Citrobacter spp.	11	8	3
Providencia spp.	5	4	1
Serratia spp.	5	5	-
Hafnia spp.	3	3	-
Yersinia spp.	1	1	-
Combined OEG	144	108	36

OEG = enteric genera other than *E.coli*

FIGURE IV: **Distribution of plasmids in -**

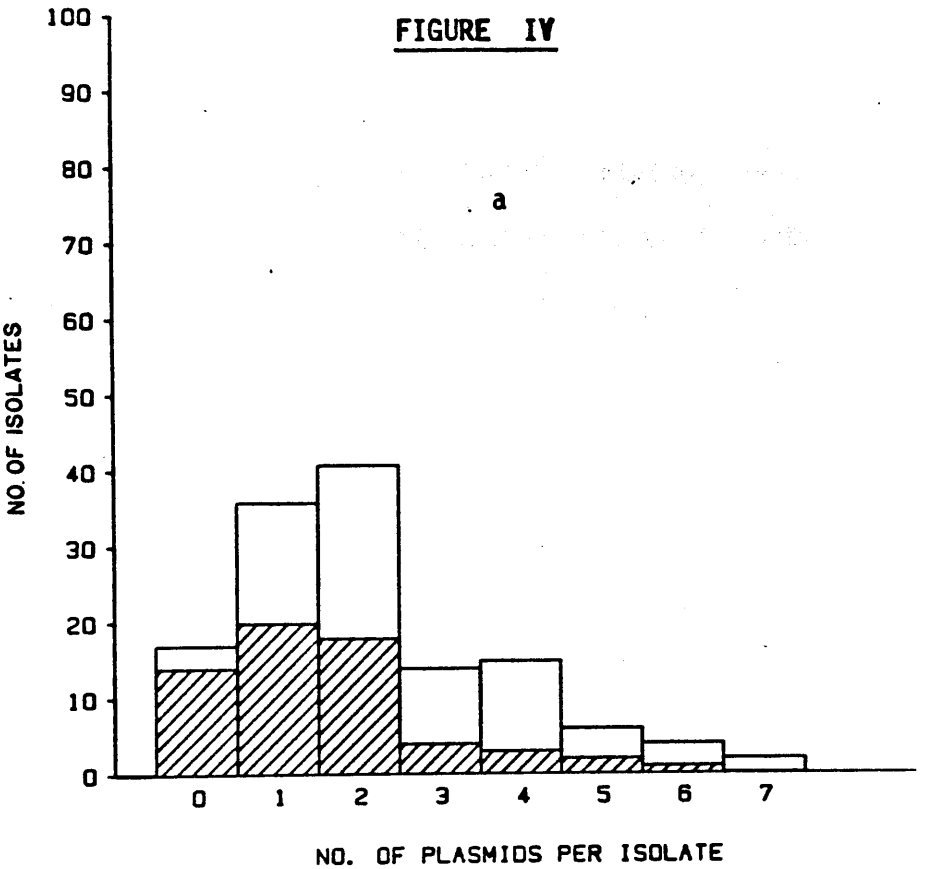
(a) 135 *E.coli* isolates

(b) 144 isolates of other enteric genera


sensitive isolates


resistant isolates

FIGURE IV


a
NO. OF ISOLATES
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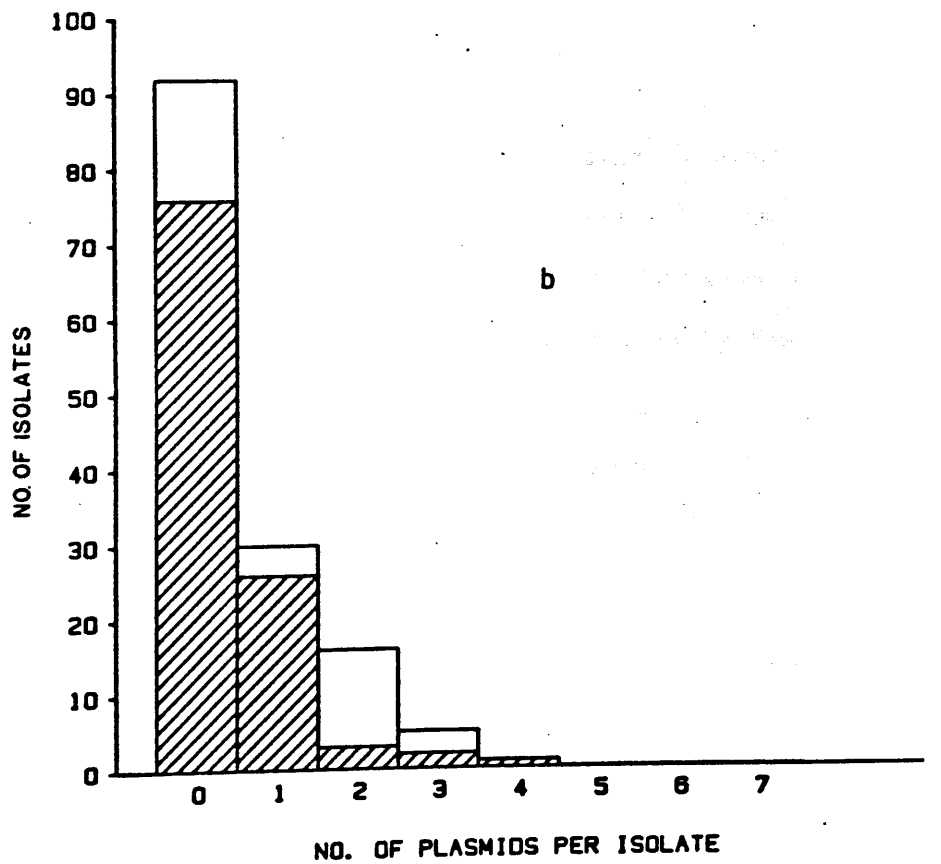
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7
NO. OF PLASMIDS PER ISOLATE


b
NO. OF ISOLATES
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7
NO. OF PLASMIDS PER ISOLATE

The coliforms were further divided into 4 sub-populations on the basis of susceptibility to antimicrobial agents as follows:

(a) sensitive E.coli

(b) resistant E.coli

(c) sensitive OEG

(d) resistant OEG.

Categorisation was according to genus and presence of resistance determinants other than intrinsic resistance. Seventy-three (54%) of the E.coli isolates possessed at least one of the following antimicrobial resistance markers (Table 18, Fig. IVa):- ampicillin-carbenicillin, tetracycline, kanamycin, streptomycin, gentamicin-tobramycin-amikacin, chloramphenicol, sulphamethoxazole and trimethoprim.

Sixty-two (46%) of the E.coli isolates were sensitive to these antimicrobial agents. Significantly fewer (χ^2 = 23.54, $p < 0.001$) of the 144 OEG isolates carried resistance markers (25%) other than intrinsic, compared to E.coli (Table 18, Fig. IVb). One hundred and eight (75%) OEG isolates possessed no resistance markers, other than those that were

intrinsic to all the species. The distribution of plasmids in the four coliform sub-populations were as shown in Table 19 and Figures Va, Vb, VIa and VIb.

The mean number of plasmids per isolate was lower in sensitive E.coli (mean 1.55) than resistant E.coli (mean 2.63). However, the distribution of plasmids was similar in both sub-populations. Sensitive OEG possessed a mean of 0.39 plasmids per isolate and resistant OEG a mean of 1.08. Both the mean and the maximum number of plasmids present were lower in these two sub-populations compared to either sensitive or resistant E.coli.

Determination of best recipient strains:

Twelve E.coli strains were tested for their ability to act as recipient strains for 4 plasmids, RP4, R1, 7679 and 8080a, transferred separately from E.coli K12 donors. All 4 plasmids were transferred into 5 strains. Of these 5 strains, three possessed at least one, and up to 4, different resistance determinants. Of the remaining two, GRI 42782-2 and E.coli K12 J62-2, the former possessed two plasmids whereas the latter did not harbour any plasmids. Therefore E.coli K12 J62-2 was used as a recipient for future conjugation experiments with clinical E.coli isolates as potential donors.

TABLE 19: **Distribution of plasmids in 135 E.coli (62 sensitive, 73 resistant) and 144 OEG (108 sensitive, 36 resistant) including the percentages of plasmid-free strains, strains with only Tra^- plasmids and strains with at least one $Tra^+ R^-$ or $Tra^+ R^+$ plasmid present**

	No. of Isolates	Range	Mean	Plasmid-free No. (%)	Tra^- No. (%)	Tra^+R^- No. (%)	Tra^+R^+ No. (%)	Tra^+ No. (%)
E.coli	135	0-7	2.13	17 (13)	32 (24)	33 (24)	53 (39)	86 (63)
Sensitive E.coli	62	0-6	1.55	14 (23)	20 (32)	28 (45)	- -	28 (45)
Resistant E.coli	73	0-7	2.63	3 (4)	12 (16)	5 (7)	53 (73)	58 (80)
OEG	144	0-4	0.56	92 (64)	25 (17)	22 (15)	5 (4)	27 (19)
Sensitive OEG	108	0-4	0.39	76 (70)	19 (18)	13 (12)	- -	13 (12)
Resistant OEG	36	0-3	1.08	16 (45)	6 (16)	9 (25)	5 (14)	14 (39)

Tra^- = non-conjugative plasmid
$Tra^+ R^-$ = conjugative plasmid not associated with resistance
$Tra^+ R^+$ = conjugative resistance plasmid
Tra^+ = conjugative plasmid
OEG = enteric genera other than E.coli

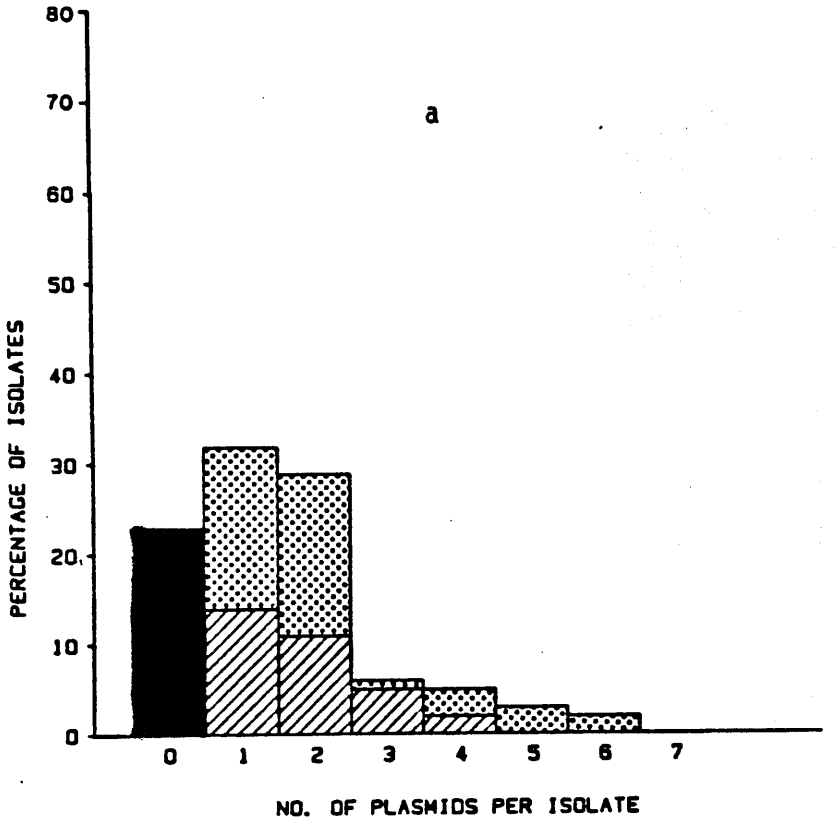
Footnote:- see page 154 et seq.

FIGURE V: **Distribution of plasmids in -**

(a) **62 sensitive E.coli isolates**

(b) **108 sensitive isolates of other enteric genera**

FIGURE V


a
PERCENTAGE OF ISOLATES
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7
NO. OF PLASMIDS PER ISOLATE

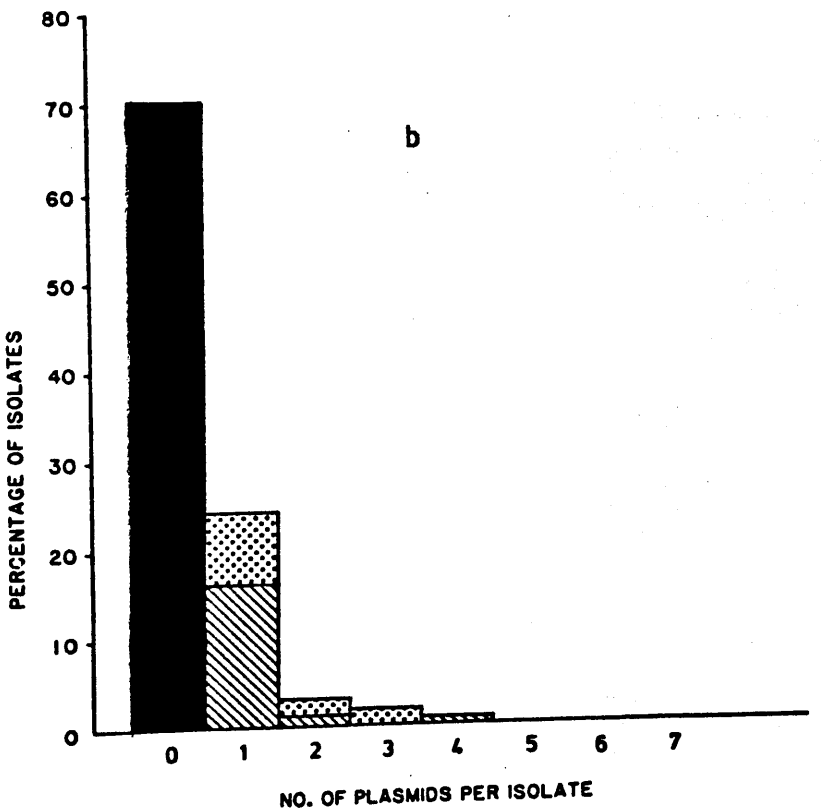

b
PERCENTAGE OF ISOLATES
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7
NO. OF PLASMIDS PER ISOLATE

FIGURE VI: **Distribution of plasmids in -**

(a) 73 resistant *E.coli* isolates

(b) 36 resistant isolaes of other enteric genera

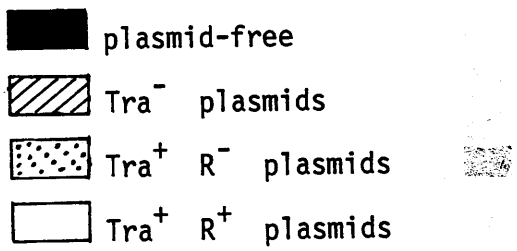
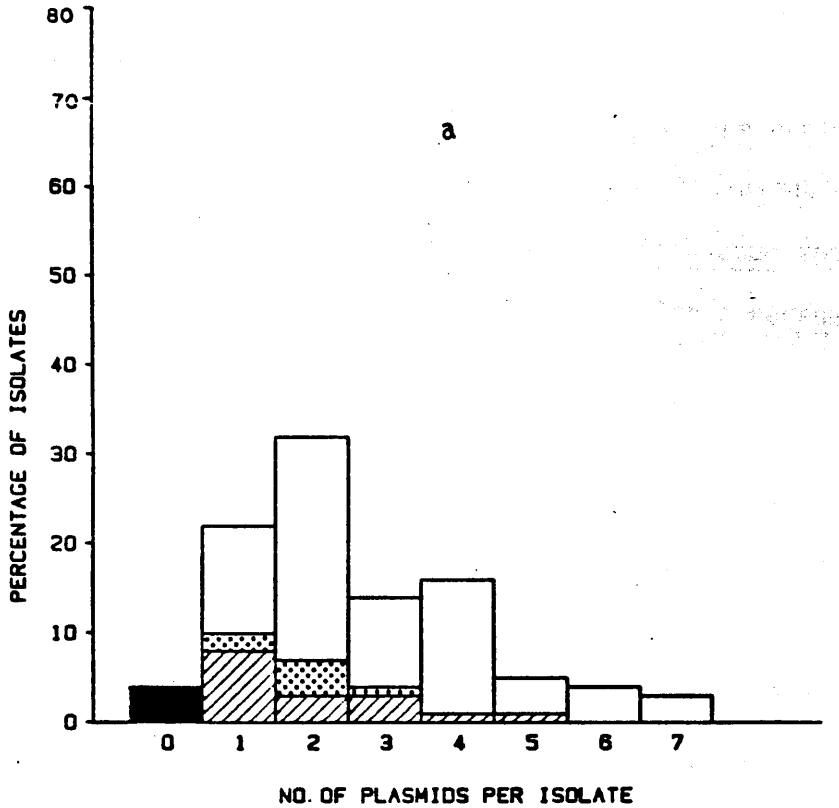


FIGURE VI


a
PERCENTAGE OF ISOLATES
80
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50
40
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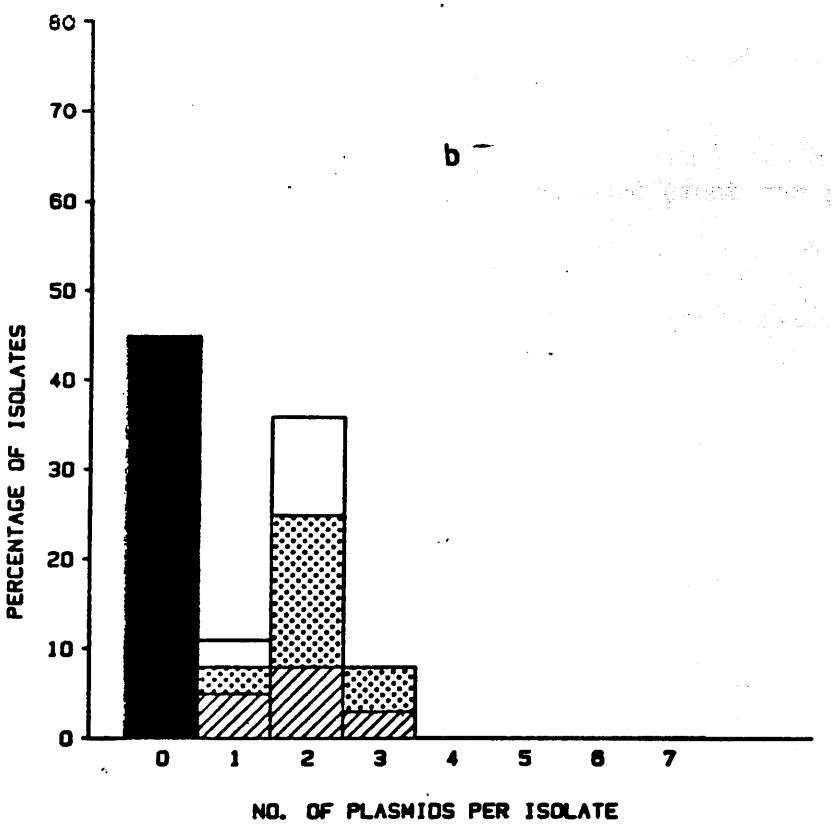
20
10
0
0 1 2 3 4 5 6 7
NO. OF PLASMIDS PER ISOLATE


b
PERCENTAGE OF ISOLATES
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10
0
0 1 2 3 4 5 6 7
NO. OF PLASMIDS PER ISOLATE

Ent.aerogenes NCTC 10006, containing separately the 4 plasmids, was used as a donor to assess the recipient ability of Enterobacter spp., Klebsiella spp., Citrobacter spp. and Hafnia alvei. The transfer of R1 into Kleb.aerogenes NCTC 8172 or Cit.freundii NCTC 9750 was unsuccessful and therefore both of these strains harbouring RP4, 7679 and 8080a and E.coli K12 J53-1 containing R1 were used in conjunction with the Ent.aerogenes donor to assess recipient ability of these genera.

Only two strains of Ent.aerogenes were tested for their recipient ability as 45 strains had been previously studied in this department (Platt and Sommerville, 1981). Both strains tested acted as recipients from all of the potential donors but as Ent.aerogenes NCTC 10006-2 harboured one plasmid whereas Ent.aerogenes GRI 9880-2 harboured none, the latter strain was chosen as the best recipient for further conjugation experiments.

Three of 10 Klebsiella spp. received the plasmids RP4, 7679 and 8080a from each of at least two of the Ent.aerogenes NCTC 10006, Kleb.aerogenes NCTC 8172 and Cit.freundii NCTC 9750 donors. Plasmid R1 was only transferred into two of these three isolates from the Ent.aerogenes NCTC 10006 donor, not from the E.coli K12 donor. Of these three strains one which

acted as a recipient for R1 and which contained one plasmid, Kleb.aerogenes NCTC 8172-2, was chosen as the most suitable recipient for further conjugation experiments.

Of the 11 Citrobacter spp. isolates tested for recipient ability two gave almost identical results. The first acted as a recipient for all 4 plasmids harboured by Ent.aerogenes NCTC 10006, three plasmids from Kleb.aerogenes NCTC 8172 and Cit.freundii NCTC 9750 donors but not plasmid R1 from E.coli K12. The second isolate, Cit.freundii GRI 182-2, acquired R1 from E.coli K12 in addition to all plasmids from each of the other donors. As this second strain did not possess any plasmids it was selected as the most suitable recipient.

Haf.alvei were generally poorer recipients than E.coli, Enterobacter spp., Klebsiella spp. or Citrobacter spp. for the plasmids tested. None of the 11 strains tested acted as recipients for all 4 plasmids. Three strains acted as recipients for three plasmids, two for RP4, 7679 and 8080a, the third for RP4, 7679 and R1. This latter strain, Haf.alvei GRI 38882-2, was selected as the most suitable recipient.

A clinical isolate of Prot.mirabilis containing separately the 4 plasmids, was used as a donor to screen for an efficient Proteus spp. recipient. None of the 11

Prot.mirabilis, 11 Prot.morganii or 5 Prot.vulgaris strains tested for recipient ability acted as recipients for all 4 plasmids. Only 1 Prot.mirabilis strain, GRI 17381-2, accepted three plasmids, RP4, R1 and 7679. Seven of the 11 Prot.morganii acted as recipients for plasmids RP4, 7679 and 8080a and one of these, GRI 16182-2 which did not harbour any plasmids, was selected as the most suitable recipient. Only one of the Prot.vulgaris isolates, GRI 21181-2, received plasmids RP4, 7679 and 8080a but not R1.

During conjugation experiments with clinical OEG isolates only 5 were found with transferable resistance determinants. Two of these clinical isolates were Ent.cloacae, both of which transferred resistance determinants to E.coli K12 J62-2, Kleb.aerogenes NCTC 8172-2, Cit.freundii GRI 182-2 and Haf.alvei GRI 38882-2. Transfer to Ent.aerogenes GRI 9880-2 was not attempted. Two Klebsiella spp. were isolated which possessed transferable resistance determinants, both transferred R-plasmids to E.coli K12 J62-2, Ent.aerogenes, GRI 9880-2, Cit.freundii GRI 182-2 and Haf.alvei GRI 38882-2. Transfer to Kleb.aerogenes NCTC 8172-2 was not attempted. One Prot.vulgaris was detected which possessed transferable resistance determinants. The resistance determinants were successfuly transferred into E.coli K12 J53-

2, Ent.aerogenes GRI 9880-2, Haf.alvei GRI 38882-2, Prot.mirabilis GRI 17381-2 and Prot.morganii GRI 16181-2 but not Kleb.aerogenes NCTC 8172-2, Cit.freundii GRI 182-2. Transfer to Prot.vulgaris was not attempted.

On no occasion was transfer found into an OEG recipient without transfer into E.coli K12. As the E.coli K12 strains used in the study possessed no resistance determinants other than naladixic acid or rifampicin resistance, it was decided to use only these strains (or Ent.aerogenes GRI 9880-2 in instances when clinical E.coli isolates produced colicins to which E.coli K12 was sensitive) as a recipient for further conjugation experiments to determine the presence of Tra^+ R^- plasmids and Tra^- plasmids which could be mobilised by RP4 as the three plasmids (pHH1310a, R300B and RP4) involved in these experiments, were readily transferred to E.coli K12.

Conjugative plasmids:

All plasmid-containing coliform isolates, irrespective of resistance, were tested for their ability to transfer plasmids to the recipient isolates listed in the Materials and Methods.

The proportion of E.coli (63%) which possessed at least one Tra^+ plasmid was greater than OEG (19%), (Figures VIIa and VIIb). Furthermore, when sensitive and resistant sub-populations were considered separately, differences were found in the number of plasmid free-isolates. The conjugal ability and resistance determinants transferred by plasmids from isolates in the different sub-populations also varied.

The proportion of plasmid-free isolates was significantly higher ($p < 0.01$) in sensitive E.coli (23% compared to the resistant sub-populations (4%) (Tables 19 and 20). Moreover, there were significantly more ($p < 0.01$) resistant E.coli which possessed at least one Tra^+ plasmid (80%) and fewer which harboured only Tra^- plasmids (16%) compared to sensitive E.coli (14 and 32% respectively).

In many instances when Tra^+ plasmids were detected and electrophoresis of the transconjugant DNA was performed, more than one plasmid was shown to have been transferred. Using the methods described, it was not possible to distinguish between separate transfer events into the same recipient and mobilisation of Tra^- plasmids by a Tra^+ plasmid. Therefore, many instances permit only the limited conclusion that at least one plasmid was conjugative. However, the more likely interpretation will be presumed, that when

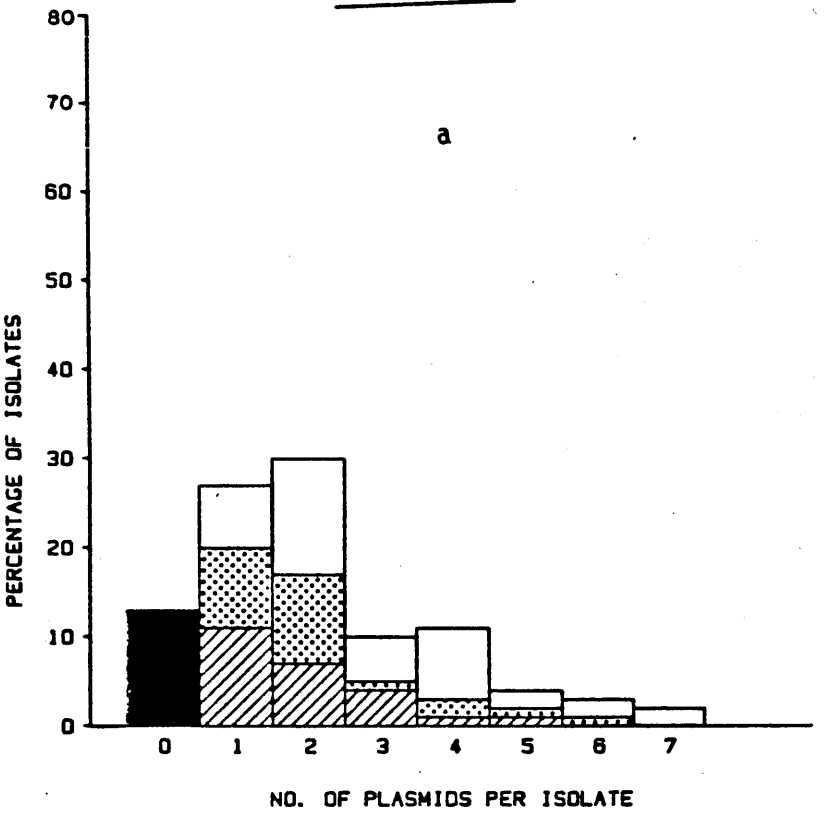
FIGURE VII: **Distribution and mobility of plasmids in -**

(a) 135 *E.coli* isolates

(b) 144 isolates of other enteric genera

key:- see figure VI, page 150.

FIGURE VII


a
PERCENTAGE OF ISOLATES
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70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7
NO. OF PLASMIDS PER ISOLATE

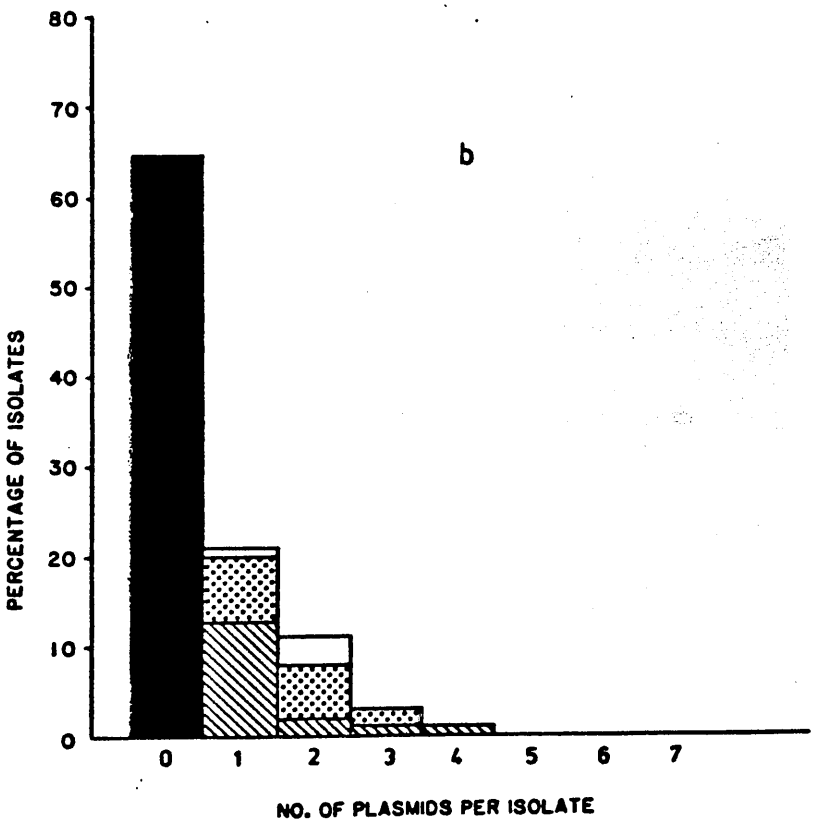

b
PERCENTAGE OF ISOLATES
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7
NO. OF PLASMIDS PER ISOLATE

TABLE 20: **Comparison between sensitive and resistant sub-populations of coliforms and plasmid groups harboured by them.**

	Sensitive E.coli	Resistant E.coli	Sensitive OEG	Resistant OEG	χ^2	p
Plasmid-free	14	3	-	-	8.78	< 0.01
Plasmid-containing	48	70	-	-		
Tra^-	20	12	-	-	7.47	< 0.01
Tra^+	28	58	-	-		
Plasmid-free	-	-	76	16	6.78	< 0.01
Plasmid-containing	-	-	32	20		
Tra^-	-	-	19	6	3.16	> 0.05
Tra^+	-	-	13	14		
Plasmid-free	14	-	76	-	34.22	< 0.001
Plasmid-containing	48	-	32	-		
Tra^-	20	-	19	-	1.75	> 0.05
Tra^+	28	-	13	-		

Plasmid-free	-	3	-	16	24.52	< 0.001
Plasmid-containing	-	70	-	20		
Tra^-_+	-	12	-	6	0.09	> 0.05
Tra^+	-	58	-	14		
Tra^+_+ R^-_+	-	5	-	9	18.9	< 0.001
Tra^+ R^+	-	53	-	5		

OEG = enteric genera other than E.coli

Tra^- = non-conjugative plasmid

Tra^+ = conjugative plasmid

Tra^+ R^+ = conjugative resistance plasmid

Tra^+ R^- = conjugative plasmid not associated with resistance.

resistance determinants have been transferred, they were encoded by a Tra^+ R^+ plasmid rather than by a Tra^- R^+ plasmid which was mobilised by a Tra^+ R^- plasmid.

In the sensitive E.coli sub-population 45 per cent of isolates possessed at least one Tra^+ R^- plasmid. In contrast, Tra^+ R^+ plasmids were detected in 73 per cent of resistant E.coli and Tra^+ R^- plasmids in only 7 per cent. However, if a Tra^+ R^+ plasmid was detected in a resistant isolate using the methods described, one could not demonstrate the presence of a Tra^+ R^- plasmid in the same isolate. The value of 7 per cent resistant E.coli possessing at least one Tra^+ R^- plasmid is likely therefore to have been underestimated.

Similar results were found when comparing both OEG sub-populations as were found in E.coli. Significantly more ($p < 0.01$) sensitive OEG were plasmid-free (70%) compared to resistant OEG (45%). However, there was no significant difference ($p > 0.05$) in the proportion of resistant OEG which possessed at least one Tra^+ plasmid (39%) and those which harboured only Tra^- plasmids (16%) compared to the sensitive sub-population (12 and 18% respectively). The proportion of resistant isolates containing at least one Tra^+

R^+ plasmid was lower (14%) than in resistant E.coli (73%). Detectable Tra^+ R^- plasmids were found in 25 per cent and 12 per cent of resistant and sensitive OEG respectively.

A comparison of sensitive E.coli and OEG showed that the latter sub-population possessed significantly more ($p < 0.001$) plasmid-free isolates than the former group. There was however, no significant difference ($p > 0.05$) in the number of Tra^+ and Tra^- containing isolates within the two sensitive sub-populations.

Similarly, when comparing resistant E.coli and OEG there were significantly fewer ($p < 0.001$) plasmid-free isolates in resistant E.coli compared to resistant OEG but no significant difference ($p > 0.05$) in the number of isolates possessing Tra^+ plasmids and those harbouring only Tra^- plasmids.

Within the limitations of the methods used, it was found that there was a significantly lower proportion ($p < 0.001$) of resistant E.coli isolates which contained Tra^+ R^- plasmids (7%) and a higher proportion which possessed Tra^+ R^+ plasmids (73%) compared to resistant OEG (2% and 14% respectively).

Comparison of sub-populations after exclusion of plasmid-free isolates:

Exclusion of plasmid-free E.coli (Table 21, Figures VIIIa, IXa, and Xa) from the distribution did not have a marked effect in changing the percentages of different types of plasmids found. In E.coli the total number of isolates containing at least one Tra^+ plasmid increased from 63 to 73 per cent when plasmid-free isolates were excluded. Similarly, the percentage of Tra^+ plasmids increased from 45 to 58 per cent in sensitive E.coli and from 80 to 82 per cent in resistant E.coli. More pronounced differences were however, seen in the distribution of plasmids in OEG after exclusion of plasmid-free isolates (Table 21, Figs. VIIIb, IXb and Xb). Tra^+ plasmid-containing coliforms had increased from 19 per cent in all OEG to 52 per cent in plasmid-containing OEG. The percentage of Tra^+ plasmids in sensitive OEG had increased from 12 to 41 per cent and the increase in resistant OEG was from 39 to 70 per cent in plasmid-containing resistant OEG.

Exclusion of plasmid-free isolates demonstrates that the distribution of Tra^+ plasmids was very similar in sensitive E.coli (58%) compared to sensitive OEG (41%) and in resistant E.coli (82%) compared to resistant OEG (70%). Major differences lay in the association of transferability with

TABLE 21: Distribution of plasmids in plasmid-containing coliform isolates

	No. of Isolates	Range	Mean	Tra^- No. (%)	Tra^+ R^- No. (%)	Tra^+ R^+ No. (%)	Tra^+ No. (%)
E.coli	118	1-7	2.44	32 (27)	33 (28)	53 (45)	86 (73)
Sensitive *E.coli*	48	1-6	2.00	20 (42)	28 (58)	- -	28 (58)
Resistant *E.coli*	70	1-7	2.74	12 (18)	5 (6)	53 (76)	58 (82)
OEG	52	1-4	1.56	25 (48)	22 (42)	5 (10)	27 (52)
Sensitive OEG	32	1-4	1.31	19 (59)	13 (41)	- -	13 (41)
Resistant OEG	20	1-3	1.95	6 (30)	9 (45)	5 (25)	14 (70)

Tra^- = non-conjugative plasmid
Tra^+ R^- = conjugative plasmid not associated with resistance
Tra^+ R^+ = conjugative resistance plasmid
Tra^+ = conjugative plasmid
OEG = enteric genera other than *E.coli*

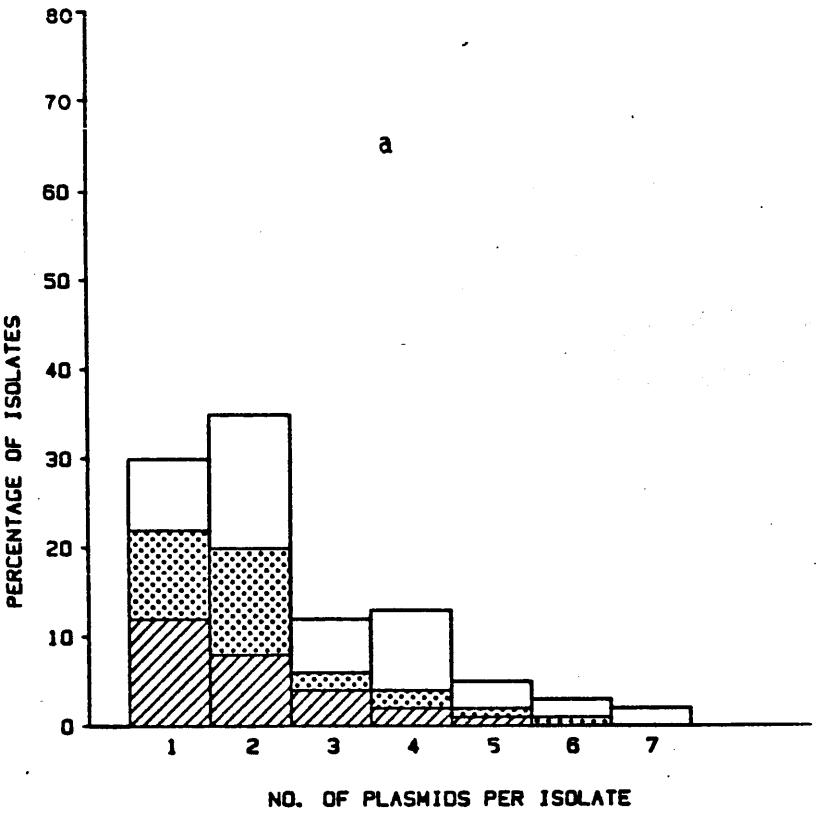
FIGURE VIII: **Distribution of plasmids in -**

(a) **118 plasmid-containing *E.coli***

(b) **52 plasmid-containing isolates of other enteric genera**

Key:- see Figure VI, page 150.

FIGURE VIII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PERCENTAGE OF ISOLATES
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10
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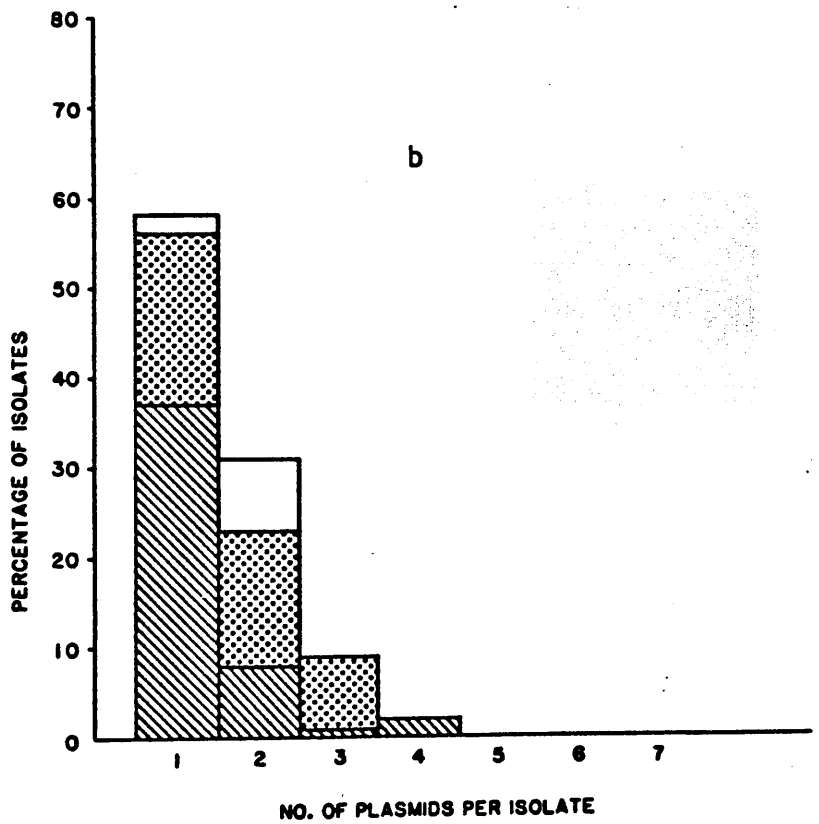
30
40
50
60
70
80
1
2
3
4
5
6
7
NO. OF PLASMIDS PER ISOLATE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PERCENTAGE OF ISOLATES
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10
20
30
40
50
60
70
80
1
2
3
4
5
6
7
NO. OF PLASMIDS PER ISOLATE

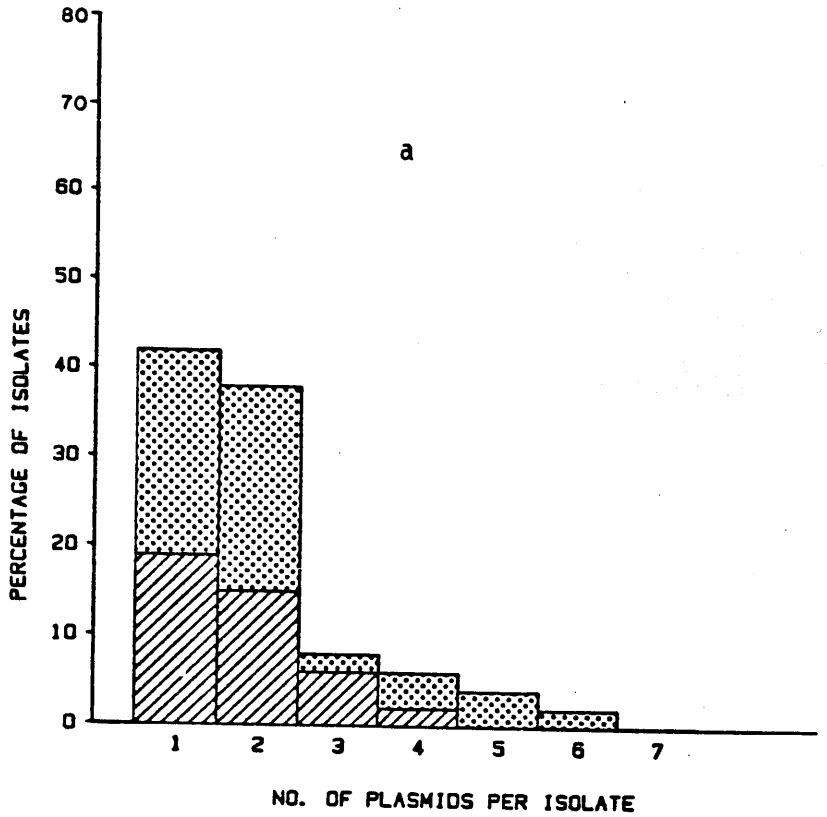
FIGURE IX: **Distribution of plasmids in -**

(a) **48 sensitive plasmid-containing *E.coli* isolates.**

(b) **32 sensitive plasmid-containing isolates of other enteric genera**

Key:- see Figure VI, page 150

FIGURE IX


a
PERCENTAGE OF ISOLATES
80
70
60
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30
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1
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7
NO. OF PLASMIDS PER ISOLATE

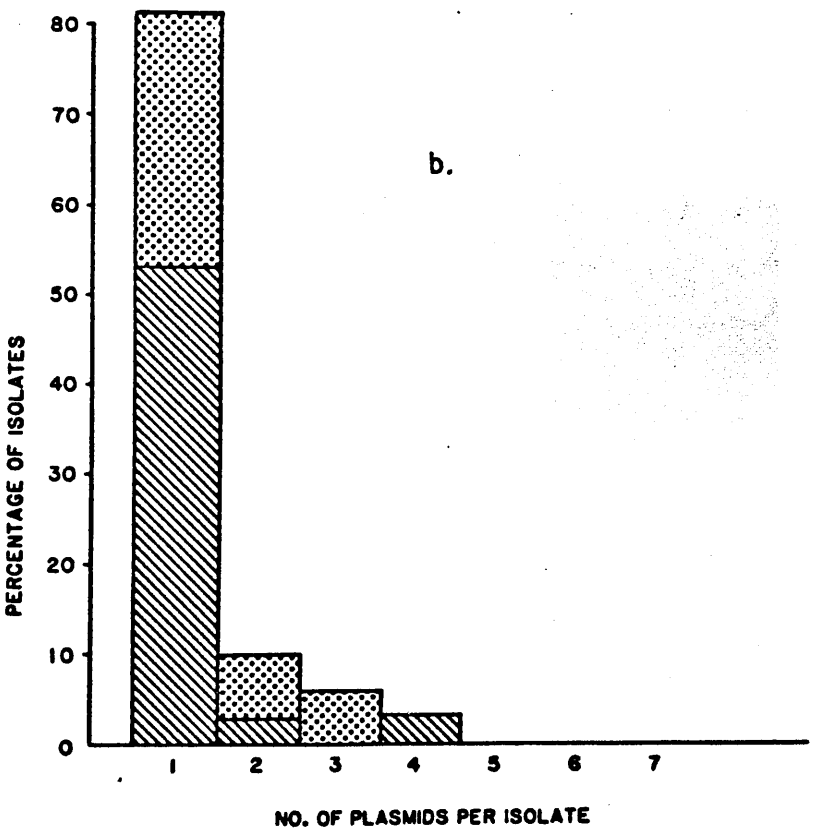

b.
PERCENTAGE OF ISOLATES
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
NO. OF PLASMIDS PER ISOLATE

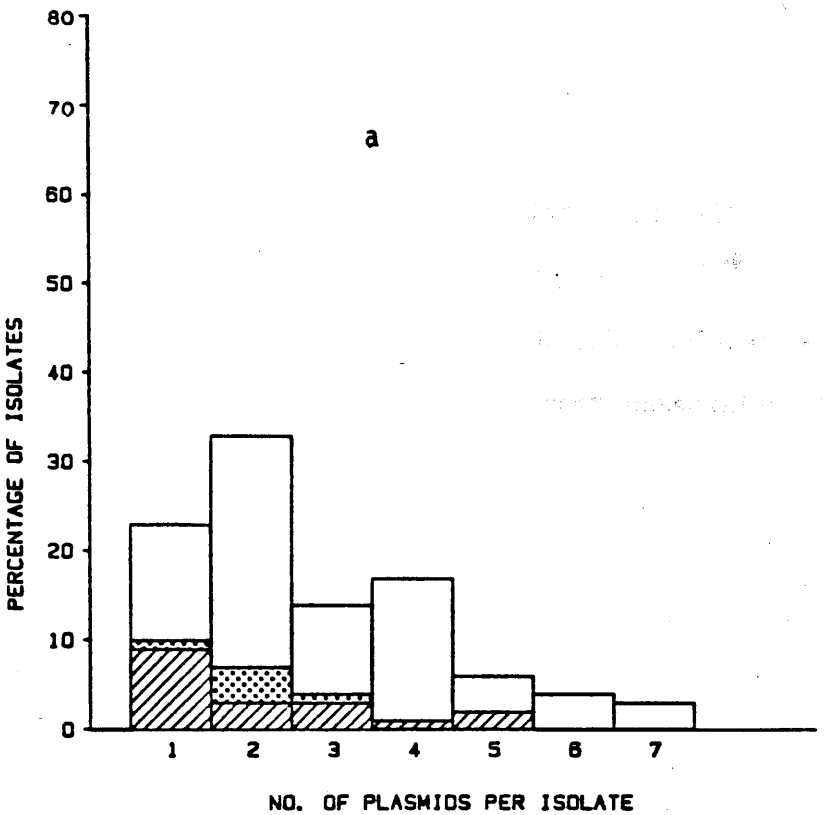
FIGURE X: Distribution of plasmids in -

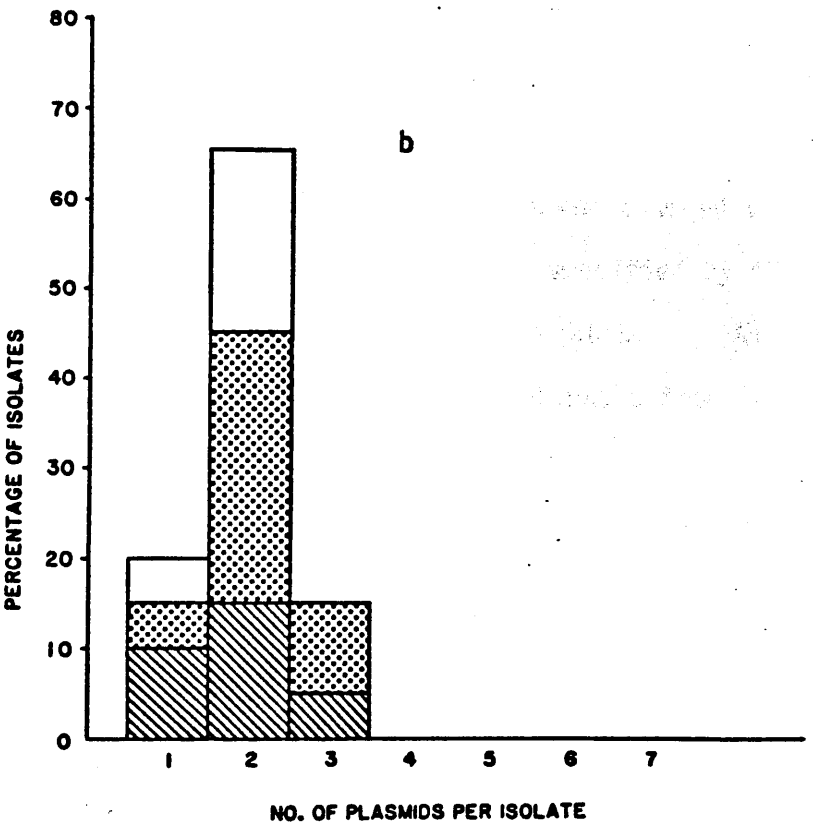
(a) 70 resistant plasmid-containing E.coli isolates.

(b) 20 resistant plasmid-containing isolates of other enteric genera

Key:- see Figure VI, page 150.

FIGURE X


a
PERCENTAGE OF ISOLATES
0
10
20
30
40
50
60
70
80
1
2
3
4
5
6
7
NO. OF PLASMIDS PER ISOLATE


b
PERCENTAGE OF ISOLATES
0
10
20
30
40
50
60
70
80
1
2
3
4
5
6
7
NO. OF PLASMIDS PER ISOLATE

resistance in the resistant sub-populations. In resistant E.coli only 6 per cent of the isolates contained Tra^+ R^- plasmids with 76 per cent possessing Tra^+ R^+ plasmids. Forty-five per cent of the resistant OEG isolates contained Tra^+ R^- plasmids with only 25 per cent possessing Tra^+ R^+ plasmids.

Transferable resistance:

The coliforms studied which possessed Tra^+ R^+ plasmids transferred a minimum of one and a maximum of 7 resistance markers to a recipient coliform. Table 22 shows the patterns of resistance transferred and the number of isolates which transferred these markers. In some instances additional resistance determinants were present in the clinical isolate but were not transferred.

The most common resistance marker encoded by Tra^+ R^+ plasmids was ampicillin (Ap) which was specified by 47 (81%) of the Tra^+ R^+ plasmid containing isolates. As a single resistance marker ampicillin was transferable from 18 (31%) of the isolates and in combination with at least one other resistance marker in 29 (50%) of isolates carrying Tra^+ R^+

TABLE 22: **Resistance determinants specified by Tra^+ R^+ plasmids harboured by 58 coliforms.**

Resistances Transferred	No. of Strains	%
Ap	18	31
Sm	3	5
Ap Tc	11	19
Tc Su	1	2
Tc Tp	2	3
Sm Su	1	2
Ap Km Sm	1	2
Ap Sm Su	10	17
Tc Sm Su	2	3
Km Sm Su	1	2
Ap Tc Km Su	1	2
Ap Km Sm Su	1	2
Ap Sm Su Tp	3	5
Tc Sm Su Tp	1	2
Ap Tc Km Su Tp Cm	1	2
Ap Tc Km Sm Su Tp Cm	1	2

Ap = ampicillin Tc = tetracycline
Km = kanamycin Sm = streptomycin
Su = sulphamethoxazole Tp = trimethoprim
Cm = chloramphenicol

plasmids. Ampicillin resistance was linked to all 6 of the other resistances which were found to be mediated by Tra^+ R^+ plasmids.

Twenty-four isolates (41%) contained Tra^+ R^+ plasmids which encoded streptomycin (Sm) resistance. Transferable sulphamethoxazole (Su) resistance was also present in 20 of these 24 streptomycin-resistant isolates. Of the 20 isolates which specified tetracycline (Tc) resistance, 14 also mediated ampicillin resistance and 7 of the 20 also specified sulphamethoxazole resistance.

In all instances when Tra^+ R^+ plasmids were detected by the transfer of resistances to a sensitive recipient strain, the presence of plasmid DNA in the transconjugant was confirmed by agarose gel electrophoresis. In one instance a Tra^+ R^+ plasmid from a clinical Prot.vulgaris was found to be unstable in the E.coli K12 recipient. After storage for one year, 90 per cent of transconjugant clones had lost their resistance markers and were plasmid-free. The original Prot.vulgaris stock culture however, had retained both its resistance determinants and plasmid.

Transfer of plasmids not associated with resistance:

Fifty-five coliforms were found to contain Tra^+ R^- plasmids capable of mobilising small Tra^- R^+ plasmids (see Fig. I). The Tra^+ R^- plasmids were found in:-

33 *E.coli*

10 *Proteus* spp.

8 *Enterobacter* spp.

3 *Klebsiella* spp.

1 *Citrobacter* spp.

From 49 coliforms the Tra^+ R^- plasmids were capable of mobilising both, pHH1310a and R300B, Tra^- R^+ plasmids. The remaining 6 Tra^+ R^- plasmids mobilised pHH1310a but did not mobilise R300B. No Tra^+ R^- plasmids were detected which only mobilised R300B.

A total of 10 *Proteus* spp. were found to harbour Tra^+ R^- plasmids as both Tra^- R^+ plasmids pHH1310a and R300B, were mobilised into the *E.coli* K12 recipients. However, in these 10 cases, no plasmid DNA was detected in the transconjugants in addition to the mobilised Tra^- R^+

plasmids. This result was reproducible and attempts to re-mobilise the Tra^- R^+ plasmids from the transconjugants were unsuccessful.

Mobilisation of cryptic plasmids:

In addition to determining whether or not coliforms contained Tra^+ plasmids, the ability of the plasmid RP4 to mobilise Tra^- plasmids was tested. All isolates which gave negative results in conjugation experiments and which contained plasmids were investigated. Mobilisation of resistance from clinical isolates was attempted in addition to the selection of RP4 resistance markers. No mobilisation of resistance was found. However, from three isolates plasmids were mobilised by RP4 but these possessed no resistance markers.

Non-transferrable resistance:

One of the features of the results was that although 73 per cent of resistant E.coli possessed Tra^+ R^+ plasmids, only 14 per cent of resistant OEG contained Tra^+ R^+ plasmids. After exclusion of transferable resistance determinants from the 73 resistant E.coli isolates, 40 (55%) contained at least one resistance marker which was not transferred. Thirty-three (92%) of the resistant OEG contained at least one resistance

marker which was not transferable by the methods described. This indicates that not only are there differences in the numbers of Tra^+ R^+ plasmids found in E.coli compared to OEG but that the proportion of resistances not encoded by Tra^+ R^+ plasmids were also different in both populations.

Inhibition of E.coli K12 growth:

During experiments to mobilise indigenous Tra^- plasmids with RP4 in which the transfer of RP4 alone was used to control the system, it was found that a number of clinical E.coli isolates completely inhibited the transfer of RP4 to the recipient strain. All E.coli which exhibited this phenomenon were subsequently shown to produce factors (probably colicins) which inhibited the growth of the E.coli K12 strains previously used as recipients.

Transfer experiments to determine the presence of Tra^+ R^+ and Tra^+ R^- plasmids were therefore repeated using recipient strains (including intermediate strains in the case of Tra^+ R^- plasmids), which were not affected by the putative colicins produced by the clinical E.coli. RP4 mobilisation experiments were also repeated using a resistant RP4-containing strain and recipient.

Of the 18 isolates producing factors which inhibited the growth of E.coli K12, 4 possessed Tra^+ R^+ plasmids and 5 contained Tra^+ R^- plasmids. Three of these transconjugants acquired the ability to produce colicins to which E.coli K12 was sensitive.

Pseudomonas spp. and Acinetobacter spp.:

In addition to coliforms, 113 Pseudomonas spp. were also collected from specimens from 30 patients. No plasmids were detected amongst these isolates and they were not studied further.

Six Acinetobacter spp. were isolated from 4 patients. As 4 of these did not survive storage the remaining two isolates were not further studied.

The analysis of plasmids from clinical isolates and transconjugants using plasmid profiles and restriction enzyme fingerprinting:

The analysis of plasmid profiles and fingerprints was carried out to define further a number of features of clinical isolates and transconjugants suggested by previous results. A plasmid profile comprises the number and approximate molecular weight of plasmids harboured by a clinical isolate or

transconjugant. Plasmid fingerprints are characteristic fragmentation patterns of DNA generated by the digestion of plasmids with restriction endonucleases.

Details of the patient, source, resistance determinants and approximate size of plasmids present in each distinct clinical isolate are shown in Table 23 together with transferable resistance determinants and/or plasmids. Using the strategy described in detail in the 'Materials and Methods' and summarised in Figure XI, the initial fragmentation pattern (PstI) from all clinical isolates and transconjugants when present, are shown in Figure XII. Further fingerprints with the pairs of enzymes EcoRI and HindIII, PstI and SmaI, or Bspl286 and AvaII from the plasmids from clinical isolates and transconjugants are as shown in Figures XIII, XIV and XV respectively.

Variation in estimates of the number and size of restriction fragments from an individual fingerprint were dependent on a number of factors:-

TABLE 23: **Resistance determinants specified by 279 distinct coliform isolates (and transconjugants when present) and plasmids harboured by these strains.**

(code) = organism identity code
(see Materials and Methods, page 83, and Table 2)

W = wound isolate
R = rectal isolate
CI = clinical isolate
TCT = transconjugant

Ap = ampicillin
Tc = tetracycline
Km = kanamycin
Sm = streptomycin
su = sulphamethoxazole
Cm = chloramphenicol
Tp = trimethoprim.

* numeral in parenthesis indicates number of plasmids detected.

TABLE 23:

Organism (Code)	Patient Number	Specimen	Source/ Derivative	Resistance Profile Ap	Tc	Km	Sm	Su	Cm	Tp	Plasmid Profile (Molecular Weight x 10^6)		
E.coli (56AATa)	56	W	CI	Ap			Sm	Su			46		
			TCT-1	Ap							46		
E.coli (56AA2b)	56	W	CI	Ap	Tc		Sm	Su			46		
			TCT-1	Ap							46		
E.coli (56bAA1b)	56	W	CI	Ap			Sm	Su			75	46	
			TCT-1	Ap								46	
E.coli (80RAA2)	80	R	CI	Ap				Su			70	62	36
			TCT-1	Ap								62	36
E.coli (80RAA4a)	80	R	CI	Ap	Tc		Sm	Su			70	15	
			TCT-1	Ap							70		
E.coli (94RAA4)	94	R	CI	Ap			Sm	Su			85	10	< 10(1)*
			TCT-1	Ap							85		
E.coli (105RAA1a)	105	R	CI	Ap							60	15	< 10(3)
			TCT-1	Ap							60		< 10(3)
E.coli (107AA1)	107	W	CI	Ap							62	60	< 10(2)
			TCT-1	Ap								60	

E.coli (T07RAA2a)	107	R	CI TCT-1	Ap Ap		62	60 60	< 10(2)	
E.coli (T08RAA1)	107	R	CI TCT-1	Ap Ap		62	46 46	36	20 < 10(3) < 10(3)
E.coli (T09RAA3)	109	R	CI TCT-1	Ap Ap		46 46			
E.coli (T09RAA3a)	109	R	CI TCT-1	Ap Ap		85	62 62	< 10(2)	
E.coli (T38AA1)	138	W	CI TCT-1	Ap Ap		75 75	46 46		
E.coli (T38RAA1)	138	R	CI TCT-1	Ap Ap		75 75	46 46		
E.coli (T38RAA3)	138	R	CI TCT-1	Ap Ap	Sm Su Cm Tp	70	55 55	< 10(1) < 10(1)	
E.coli (T41AA1)	141	W	CI TCT-1	Ap Ap	Sm Su Cm Tp	70	55 55	< 10(1) < 10(1)	
E.coli (T41RAA1)	141	R	CI TCT-1	Ap Ap	Sm Su Cm Tp	70	55 55	< 10(1) < 10(1)	
E.coli (T41RAA1a)	141	R	CI TCT-1	Ap Ap		70	55 55	< 10(2) < 10(2)	

E.coli (86RAA1)	86	R	CI			Sm		62	46	40	36	<10(3)
			TCT-1			Sm		62				
E.coli (94RAA1)	94	R	CI			Sm	Su	62	36	15	10	<10(2)
			TCT-1			Sm		62				<10(1)
E.coli (96RAA1a)	96	R	CI		Tc	Sm		62	46			
			TCT-1			Sm			46			
E.coli (57AAT)	57	W	CI	Ap	Tc	Sm	Su	62				
			TCT-1	Ap	Tc			62				
E.coli (75ABT)	75	W	CI	Ap	Tc	Sm	Su	70				
			TCT-1	Ap	Tc			70				
E.coli (75AA2a)	75	W	CI	Ap	Tc			70				
			TCT-1	Ap	Tc			70				
E.coli (106AA1)	106	W	CI	Ap	Tc			85	50	15	<10(1)	
			TCT-1	Ap					50			
			TCT-2	Ap	Tc			85	50			
E.coli (106AA1a)	106	W	CI	Ap	Tc			85	<10(1)			
			TCT-1	Ap	Tc			85	<10(1)			
E.coli (106RAA1)	106	R	CI	Ap	Tc			85	<10(1)			
			TCT-1	Ap	Tc			85	<10(1)			
E.coli (107RAA1a)	107	R	CI	Ap	Tc	Sm	Su	85	46	15	<10(3)	
			TCT-1	Ap	Tc			85	46			

E.coli (107RAA3d)	107	R	CI	Ap	Tc				70	55	15	10
			TCT-1	Ap	Tc				70	55		
E.coli (139AA2a)	139	W	CI	Ap	Tc	Sm	Su	Tp	85			
			TCT-1	Ap	Tc				85			
E.coli (144AA2)	144	W	CI	Ap	Tc				85	< 10(1)		
			TCT-1	Ap	Tc				85	< 10(1)		
E.coli (144RAA2)	144	R	CI	Ap	Tc				85	< 10(1)		
			TCT-1	Ap	Tc				85	< 10(1)		
E.coli (96RAA2a)	96	R	CI		Tc		Su		62	15		
			TCT-1		Tc		Su		62	15		
E.coli (105RAA1)	105	R	CI			Sm	Su		62	15	10	< 10(2)
			TCT-1			Sm	Su		62		10	< 10(2)
Kleb.pneumoniae (101RCD1)	101	R	CI		Tc	Sm	Su	Tp	62	36		
			TCT-1		Tc			Tp		36		
Kleb.oxytoca (105CH1)	105	W	CI		Tc	Sm	Su	Tp	62	36		
			TCT-1		Tc			Tp		36		
E.coli (107AA1a)	107	W	CI	Ap	Km	Sm	Su		85	< 10(1)		
			TCT-1	Ap	Km	Sm			85	< 10(1)		
E.coli (16AA3)	16	W	CI	Ap		Sm	Su		62	46	15	< 10(1)
			TCT-1	Ap		Sm	Su		62	46		
E.coli (32AA2)	32	W	CI	Ap		Sm	Su		85	62		
			TCT-1	Ap		Sm	Su			62		

E.coli (35AAT)	35	W	CI	Ap		Sm Su	62				
			TCT-1	Ap		Sm Su	62				
E.coli (36ABT)	36	W	CI	Ap		Sm Su	55				
			TCT-1	Ap		Sm Su	55				
E.coli (79RAA1)	79	R	CI	Ap		Sm Su	62	36			
			TCT-1	Ap		Sm Su	62				
E.coli (81RAB1)	81	R	CI	Ap		Sm Su	70	55	<10(2)		
			TCT-1	Ap		Sm Su		55			
E.coli (89RAA3)	89	R	CI	Ap		Sm Su	85	55			
			TCT-1	Ap		Sm Su	85	55			
E.coli (90RAA3)	90	R	CI	Ap		Sm Su	70	15	<10(1)		
			TCT-1	Ap		Sm Su	70				
			TCT-2	Ap		Sm Su	70	15	<10(1)		
			TCT-3	Ap			70		<10(1)		
E.coli (105RAA2a)	105	R	CI	Ap		Sm Su	62	60	15	10	<10(2)
			TCT-1			Sm Su	62				<10(2)
			TCT-2	Ap			62			10	<10(2)
			TCT-3			Sm Su	62			10	<10(2)
E.coli (109RAA1)	109	R	CI	Ap		Sm Su	62	60	50	<10(1)	
			TCT-1	Ap		Sm Su	62			<10(1)	
E.coli (80RAA4b)	80	R	CI		Tc	Sm Su	70	15			
			TCT-1		Tc	Sm Su	70				

Prot.vulgaris (139BC1)	139	W	CI		Tc		Sm	Su		Tp	62			
			TCT-1		Tc		Sm	Su			62			
E.coli (105RAA7a)	105	R	CI	Ap	Tc	Km	Sm	Su	Cm	Tp	85	62	10	< 10(1)
			TCT-1			Km	Sm	Su				62	10	< 10(1)
			TCT-2				Sm	Su				62	10	
E.coli (88RAA1b)	88	R	CI	Ap	Tc	Km	Sm	Su			55	46	36	< 10(2)
			TCT-1	Ap	Tc	Km		Su				46		< 10(2)
			TCT-2		Tc	Km						46		< 10(2)
E.coli (107RAA1)	107	R	CI	Ap		Km	Sm	Su			85	46	<10(1)	
			TCT-1	Ap								46		
			TCT-2	Ap		Km	Sm				85		<10(1)	
			TCT-3	Ap			Sm	Su			85		<10(1)	
E.coli (109RAA2b)	109	R	CI	Ap			Sm	Su	Cm	Tp	62	46	20	< 10(1)
			TCT-1	Ap							62			
			TCT-2				Sm	Su			62			< 10(1)
			TCT-3	Ap			Sm	Su			62			< 10(1)
			TCT-4	Ap						Tp	62			
Ent.cloacae (141JC1)	141	W	CI	Ap			Sm	Su		Tp	46	10		
			TCT-1	Ap			Sm	Su		Tp	46			
Ent.cloacae (143RJC1)	143	R	CI	Ap			Sm	Su		Tp	46	10		
			TCT-1	Ap			Sm	Su		Tp	46			
E.coli (101RAA1)	101	R	CI		Tc		Sm	Su		Tp	62	36		
			TCT-1		Tc		Sm	Su				36		
			TCT-2		Tc		Sm	Su		Tp		36		
			TCT-3		Tc					Tp		36		

E.coli (T39aAA1)	139	W	CI	Ap	Tc	Km	Sm	Su	Cm	Tp	85	62		
			TCT-1			Km				Tp		62		
			TCT-2	Ap	Tc			Su	Cm		85			
			TCT-3	Ap	Tc	Km			Cm	Tp	85			
			TCT-4			Km		Su		Tp		62		
E.coli (T39RAA1)	139	R	CI	Ap	Tc	Km	Sm	Su	Cm	Tp	85	62		
			TCT-1	Ap	Tc				Cm	Tp	85			
			TCT-2			Km				Tp		62		
			TCT-3				Sm	Su		Tp		62		
			TCT-4							Tp	85			
E.coli (67AAT)	67	W	CI								55			
			TCT-1								55			
E.coli (84RAA1)	84	R	CI								46	40		
			TCT-1								46			
E.coli (86RAA2)	86	R	CI								62	36	30	<10(3)
			TCT-1								62	36		
E.coli (87RAA1)	87	R	CI								70	40		
			TCT-1								70	40		
E.coli (87RAA1a)	87	R	CI								90	46		
			TCT-1									46		
E.coli (87RAA2a)	87	R	CI								70	62	46	
			TCT-1										46	

E.coli (88RAA1)	88	R	CI TCT-1			Sm	Su	90 90	< 10(1)
E.coli (88RAA1a)	88	R	CI TCT-1					46 46	36 < 10(3)
E.coli (89RAA1)	89	R	CI TCT-1		Tc	Sm	Su	36 36	< 10(1)
E.coli (89RAB4)	89	R	CI TCT-1					70	46 46
E.coli (90RAA1)	90	R	CI TCT-1					46 46	15 < 10(2)
E.coli (91RAA1)	91	R	CI TCT-1		Ap	Sm		90	62 10 62
E.coli (91RAA1a)	91	R	CI TCT-1					36 36	
E.coli (91RAA1b)	91	R	CI TCT-1					85 85	
E.coli (92RAA1)	92	R	CI TCT-1					85 85	10 10
E.coli (96RAA1)	96	R	CI TCT-1					85	46 46

E.coli (96RAA2)	96	R	CI TCT-1		Sm	46 46		
E.coli (97RAA1b)	97	R	CI TCT-1			70 70		
E.coli (98RAA1a)	98	R	CI TCT-1			62 62	46 46	< 10(3)
E.coli (99AAT)	99	W	CI TCT-1			46 46		
E.coli (99RAA1)	99	R	CI TCT-1			46 46		
E.coli (100AA1a)	100	W	CI TCT-1			70 70		
E.coli (100RAA1)	100	R	CI TCT-1			70 70		
E.coli (102RAA1)	102	R	CI TCT-1	Tc		85 85	15	
E.coli (103RAA2)	103	R	CI TCT-1			62 62	60	
E.coli (105RAA6)	105	R	CI TCT-1			46 46	< 10(1)	

E.coli (107RAA3b)	107	R	CI TCT-1		46 46	15	< 10(2)	
E.coli (140AA1)	140	W	CI TCT-1		85 85			
E.coli (140RAA1a)	140	R	CI TCT-1		85 85			
E.coli (144RAB2)	144	R	CI TCT-1		85	46 46		
E.coli (145AA2a)	145	W	CI TCT-1		85	46 46		
E.coli (145RAA1)	145	R	CI TCT-1		85	46 46		
E.coli (145RAA1a)	145	R	CI TCT-1		46 46			
Ent.cloacae (28aJC2)	28	W	CI TCT-1	Sm Su Cm	70 70	<10(1)		
Ent.cloacae (79RJC1)	79	R	CI TCT-1		70 70	10		
Kleb.pneumoniae (82RCD2)	82	R	CI TCT-1		85	62	46 46	

Cit.freundii (82RRA3)	82	R	CI TCT-1			90	62 62	
Ent.cloacae (89RJC5)	89	R	CI TCT-1		Su	70	62 62	
Ent.cloacae (92RJC4)	92	R	CI TCT-1			85 85	< 10(2) < 10(2)	
Ent.cloacae (94RJC1)	94	R	CI TCT-1	Ap		46	36 36	<10(1) <10(1)
Ent.cloacae (102aJC2)	102	W	CI TCT-1	Ap		46 46	36	<10(1)
Ent.cloacae (102RJC2)	102	R	CI TCT-1	Ap		62	46 46	
Kleb.pneumoniae (143cCD2)	143	W	CI TCT-1			62 62		
Kleb.pneumoniae (143RCD2)	143	R	CI TCT-1			62 62		
Ent.agglomerans (143cJE1)	143	W	CI TCT-1			62 62		
Prot.morganii (1BB1)	1	W	CI TCT-1			46 46		

Prot.mirabilis (T5BAT)	15	W	CI TCT-1	Tc	Km		Su	Tp	55 55	
Prot.morganii (83RBBT)	83	R	CI TCT-1						46 46	
Prot.mirabilis (T07BAT)	107	W	CI TCT-1	Tc		Sm	Su	Tp	36 36	15
Prot.mirabilis (T07RBAT)	107	R	CI TCT-1	Tc		Sm	Su	Tp	36 36	15
Prot.mirabilis (T08RBAT)	108	R	CI TCT-1	Tc		Sm	Su	Tp	36 36	15
Prot.mirabilis (T40BAT)	140	W	CI TCT-1						46 46	
Prot.mirabilis (T40RBAT)	140	R	CI TCT-1						46 46	
Prot.mirabilis (T41BAT)	141	W	CI TCT-1						46 46	
Prot.mirabilis (T41RBAT)	141	R	CI TCT-1						46 46	
E.coli (T2AAT)	12	W	CI						15	

E.coli (69AAT)	69	W	CI							85		
E.coli (75AA2)	75	W	CI				Sm	Su		62		
E.coli (79RAA1a)	79	R	CI							85		
E.coli (80RAA1)	80	R	CI							70	62	
E.coli (80RAA3)	80	R	CI							70	< 10(1)	
E.coli (82RAB1)	82	R	CI		Tc					85	< 10(1)	
E.coli (82RAA2)	82	R	CI							< 10(1)		
E.coli (82RAB3)	82	R	CI	Ap	Tc	Km	Sm	Su	Cm	75		
E.coli (83RAA1a)	83	R	CI							70		
E.coli (83RAA1b)	83	R	CI							20		
E.coli (83RAB1)	83	R	CI							70	55	

E.coli (85RAA1)	85	R	CI							10	<10(1)		
E.coli (86RAB1)	86	R	CI	Ap	Tc	Km	Sm	Su	Cm	85			
E.coli (89RAA4)	89	R	CI							90	10		
E.coli (93RAA2)	93	R	CI							95	46		
E.coli (93RAA3)	93	R	CI							95	62	46	
E.coli (95RAA1a)	95	R	CI							62	<10(1)		
E.coli (97RAA1a)	97	R	CI							70	10	<10(1)	
E.coli (98RAA1)	98	R	CI							62	15	<10(1)	
E.coli (103RAA2a)	103	R	CI							36			
E.coli (105RAA1b)	105	R	CI	Ap						46	20	<10(2)	

E.coli (T06RAA1a)	106	R	CI	Ap	Sm Su	70	10		
E.coli (T07AA4)	107	W	CI			85			
E.coli (T07RAA3c)	107	R	CI			< 10(1)			
E.coli (T08RAA1a)	108	R	CI	Ap		46	36	25	< 10(2)
E.coli (T09AB8)	109	W	CI	Ap Tc		85			
E.coli (T09RAB4)	109	R	CI	Ap Tc		85			
E.coli (T39AA3)	139	W	CI	Ap	Sm Su Cm Tp	70	< 10(2)		
E.coli (T39RAA2)	139	R	CI	Ap	Sm Su Cm Tp	70	< 10(2)		
E.coli (T41AB1)	141	W	CI	Ap Tc		85			
E.coli (T42RAA1)	142	R	CI			85	< 10(3)		

Hafnia alvei (18JH1)	18	W	CI			46	
Ent.cloacae (22JC1)	22	W	CI			70	
Ent.cloacae (39JC1)	39	W	CI			70	
Kleb.pneumoniae (80RCD4)	80	R	CI	Ap		36	
Ent.cloacae (89RJC5a)	89	R	CI		Su	85	<10(2)
Cit.freundii (93RA1)	93	W	CI			<10(1)	
Ent.cloacae (93JC1)	93	W	CI			<10(1)	
Ent.cloacae (93RJC1)	93	R	CI			62	
Ent.cloacae (103RJC1)	103	R	CI	Ap		62	<10(1)
Kleb.pneumoniae (103RCD2)	103	R	CI			85	
Ent.cloacae (107RJC2)	107	R	CI			62	<10(3)

Kleb.pneumoniae (138RCD1)	138	R	CI				46	
Hafnia alvei (138RJH1)	138	R	CI				< 10(1)	
Hafnia alvei (138RJH2)	138	R	CI				36	<10(1)
Ent.cloacae (141RJC1)	141	R	CI				46	
Prot.morganii (17BB1)	17	W	CI				36	
Prot.vulgaris (74BC1)	74	W	CI				46	
Prot.morganii (80BB2)	80	W	CI				62	
Prot.morganii (80RBB5)	80	R	CI				62	
Prot.morganii (90RBB2)	90	R	CI				55	
Prot.mirabilis (105BA1)	105	W	CI	Ap	Tc	Km	46	

Prot.morganii (138BB1)	138	W	CI							46	
Prot.morganii (138RBB1)	138	R	CI							46	
Prot.mirabilis (144BA1)	144	W	CI	Ap	Tc	Km	Sm	Su	Tp	46	36
Prot.mirabilis (144RBA1)	144	R	CI	Ap	Tc	Km	Sm	Su	Tp	46	36
E.coli (41AA3)	41	W	CI								
E.coli (56AA1b)	56	W	CI		Tc		Sm	Su			
E.coli (64AA2)	64	W	CI								
E.coli (83RAA1)	83	R	CI								
E.coli (90RAA2a)	90	R	CI								
E.coli (92RAA4)	92	R	CI								

E.coli (93RAA2a)	93	R	CI			
E.coli (94RAB1)	94	R	CI			
E.coli (95RAA1)	95	R	CI			
E.coli (97RAA1)	97	R	CI			
E.coli (99RAA1a)	99	R	CI			
E.coli (104RAA1)	104	R	CI			
E.coli (109AA4)	109	W	CI			
E.coli (109RAA2a)	109	R	CI			
E.coli (140RAA1)	140	R	CI	Ap	Sm	Su
E.coli (142RAA1a)	142	R	CI		Sm	Su

E.coli (145AA1)	145	W	CI
Ser.marcescens (9KA1)	9	W	CI
Ser.marcescens (10aKA1)	10	W	CI
Kleb.pneumoniae (12CD1)	12	W	CI
Cit.freundii (17RA1)	17	W	CI
Kleb.pneumoniae (18CD1)	18	W	CI
Ent.cloacae (18JC1)	18	W	CI
Prot.mirabilis (19BA1)	19	W	CI
Kleb.oxytoca (29CH1)	29	W	CI
Prot.mirabilis (31BA1)	31	W	CI

Prot.mirabilis (32BA4)	32	W	CI
Prot.mirabilis (36bBA3)	36	W	CI
Prot.mirabilis (37BA1)	37	W	CI
Ent.cloacae (40JC2)	40	W	CI
Cit.freundii (41RA1)	41	W	CI
Prot.morganii (48BB2)	48	W	CI
Ser.liquifaciens (52KB1)	52	W	CI
Prot.morganii (54BB1)	54	W	CI
Prot.mirabilis (57BA1)	57	W	CI
Prot.morganii (57BB2)	57	W	CI

Kleb.pneumoniae (57CD2)	57	W	CI						
Prot.mirabilis (59BA1)	59	W	CI						
Kleb.oxytoca (63CH2)	63	W	CI						
Ser.marcescens (69KA1)	69	W	CI						
Prot.mirabilis (70BA1)	70	W	CI						
Prot.morganii (70BB1)	70	W	CI	Tc	Sm	Su	Cm	Tp	
Ent.cloacae (70JC1)	70	W	CI		Sm				
Prot.vulgaris (74BC2)	74	W	CI						
Prot.vulgaris (75BC1)	75	W	CI						
Ent.cloacae (76JC1)	76	W	CI						

Ser.marcescens (77KA1)	77	W	CI	
Prot.mirabilis (79RBA1)	79	R	CI	
Prot.mirabilis (80BA1)	80	W	CI	
Prot.mirabilis (80RBA1)	80	R	CI	
Kleb.oxytoca (80RCH2)	80	R	CI	Ap
Prot.morganii (82BB1)	82	W	CI	
Prot.mirabilis (82RBA1)	82	R	CI	
Prot.morganii (82RBB1)	82	R	CI	
Kleb.oxytoca (82RCH2)	82	R	CI	
Kleb.pneumoniae (83RCD1)	83	R	CI	

Kleb.pneumoniae (84RCD1)	83	R	CI	
Prov.stuartii (84RDA1)	84	R	CI	
Prot.mirabilis (85BA1)	85	W	CI	
Kleb.pneumoniae (86RCD1)	86	R	CI	
Ent.cloacae (87RJC1)	87	R	CI	
Prot.morganii (88RBB1)	88	R	CI	
Kleb.pneumoniae (90RCD3)	90	R	CI	
Kleb.pneumoniae (93CD3)	93	W	CI	Ap
Cit.freundii (93RRA1)	93	R	CI	Ap
Yers.ruckeri (93RPA1)	93	R	CI	

Cit.freundii (93RRA4)	93	R	CI			
Kleb.oxytoca (94CH1)	94	W	CI	Ap		
Prot.vulgaris (94BC1)	94	W	CI			
Cit.freundii (94RA2)	94	W	CI			
Prot.vulgaris (94RBC1)	94	R	CI			
Kleb.pneumoniae (94RCD1)	94	R	CI			
Kleb.pneumoniae (95RCD1)	95	R	CI			
Kleb.pneumoniae (96RCD2)	96	R	CI			
Ent.agglomerans (96RJE2)	96	R	CI		Tc	Cm
Prot.mirabilis (97RBA1)	97	R	CI			

Kleb.pneumoniae (98RCD1)	98	R	CI			
Kleb.pneumoniae (99RCD2)	99	R	CI			
Ent.cloacae (99RJC2)	99	R	CI	Ap		
Ent.cloacae (99RJC2a)	99	R	CI			
Ent.cloacae (100cJC1)	100	W	CI		Sm	Su
Prot.mirabilis (102BA2)	102	W	CI			
Cit.freundii (102aRA1)	102	W	CI			
Prot.mirabilis (102RBA1)	102	R	CI			
Kleb.oxytoca (102RCH1)	102	R	CI	Ap		
Cit.freundii (102RRA1)	102	R	CI	Ap		

Prot.morganii (105BB1)	105	W	CI					
Prov.stuartii (105DA1)	105	W	CI		Tc	Sm	Cm	
Prot.vulgaris (107BC1)	107	W	CI					
Prov.stuartii (107DA3)	107	W	CI					
Prov.stuartii (107RDA1)	107	R	CI					
Prot.morganii (107RBB2)	107	R	CI					
Cit.freundii (107RRA4)	107	R	CI					
Prot.mirabilis (108BA1)	108	W	CI					
Prot.vulgaris (109BC1)	109	W	CI	Ap	Tc	Sm		Tp
Prot.vulgaris (109aBC2)	109	W	CI					

Prov.stuartii (109DA3)	109	W	CI			
Prot.mirabilis (138BA2)	138	W	CI			
Prot.mirabilis (138RBA3)	138	R	CI			
Prot.mirabilis (139BA4)	139	W	CI	Tc	Sm	Tp
Prot.mirabilis (139RBA2)	139	R	CI	Tc	Sm	Tp
Prot.mirabilis (142BA1)	142	W	CI			
Prot.vulgaris (142BC1)	142	W	CI			
Prot.mirabilis (142RBA1)	142	W	CI			
Prot.mirabilis (143BA1)	143	W	CI			
Cit.freundii (143aRA2)	143	W	CI			

Prot.mirabilis (I43RBAI)	143	R	CI
Kleb.pneumoniae (I44CD2)	144	W	CI
Kleb.pneumoniae (I44RCD2)	144	R	CI

FIGURE XI: Summary of the strategy used to determine which restriction enzyme combinations were used to fingerprint plasmid DNA from clinical isolates and transconjugants.

FIGURE XI

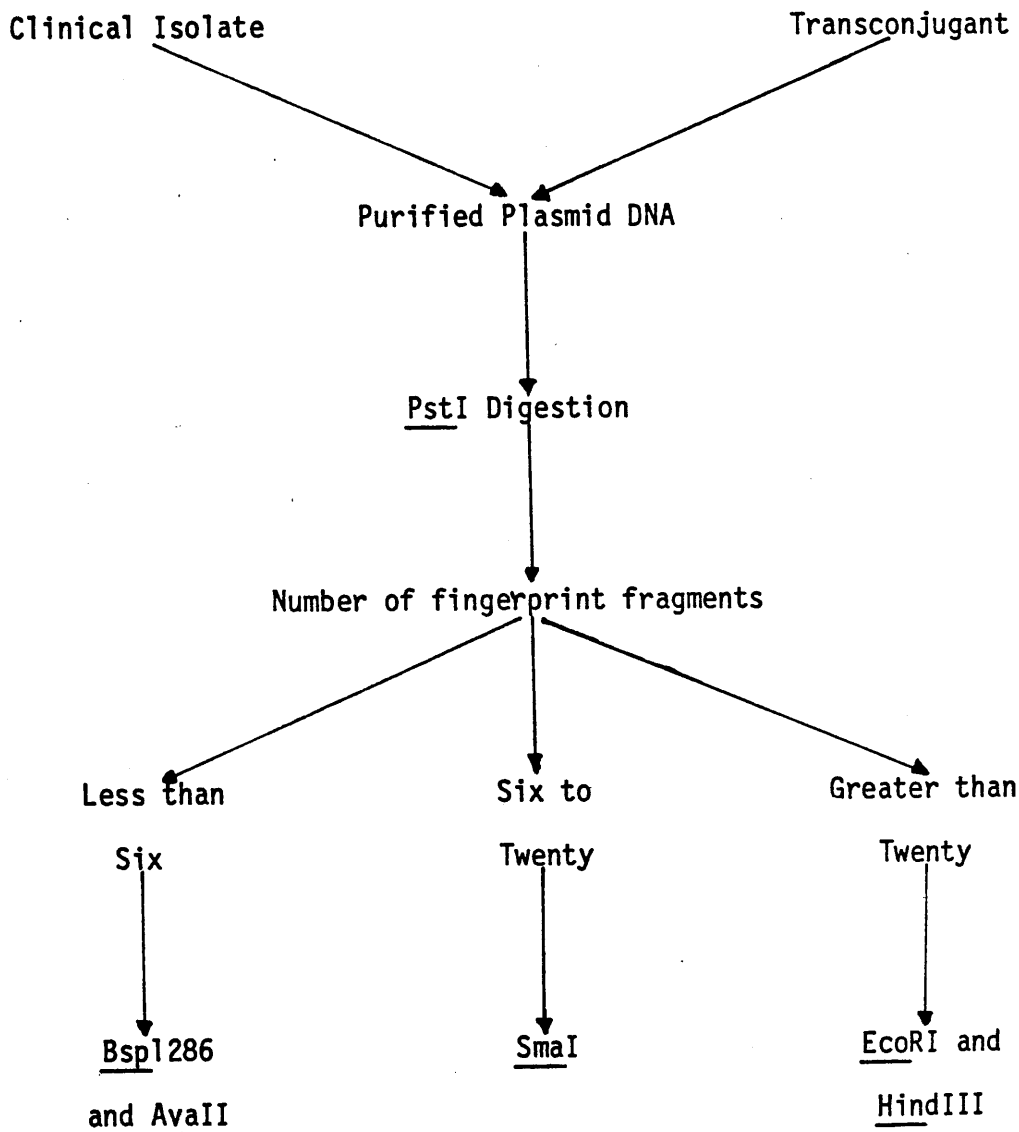
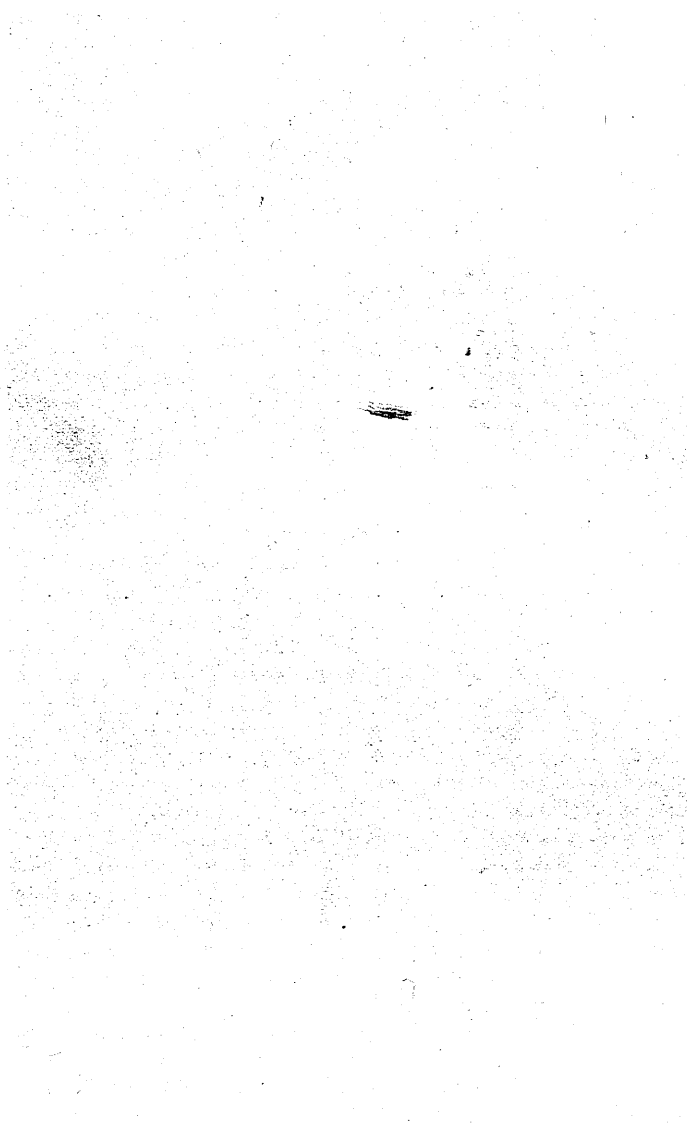
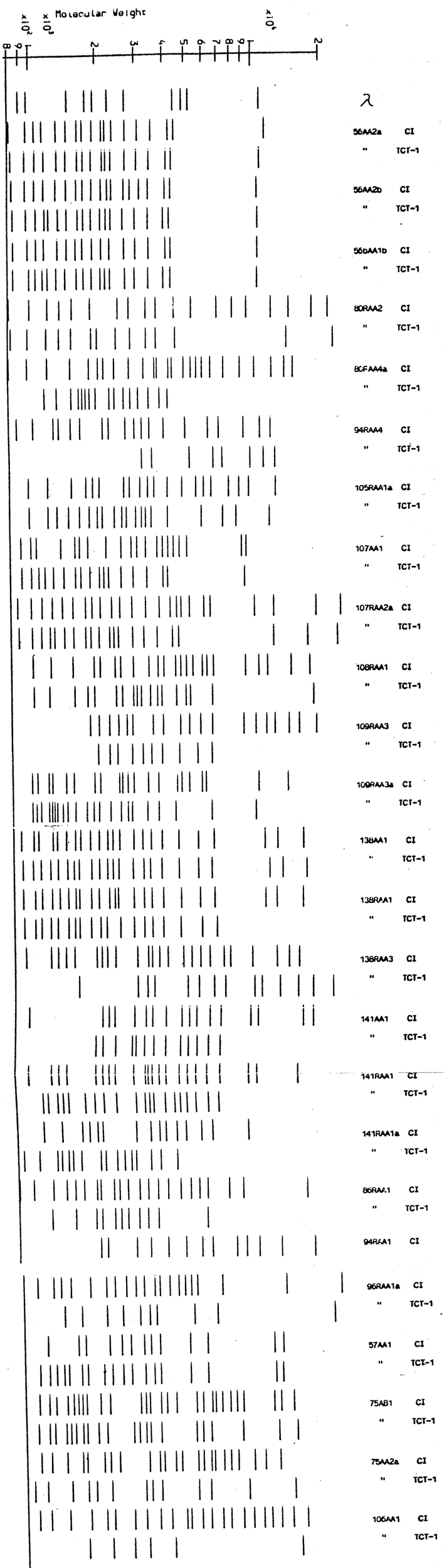

Clinical Isolate
Transconjugant
Purified Plasmid DNA
PstI Digestion
Number of fingerprint fragments
Less than Six
Six to Twenty
Greater than Twenty
Bspl286 and AvaII
SmaI
EcoRI and HindIII

FIGURE XII: PstI fingerprints of plasmid DNA from plasmid-containing clinical isolates and transconjugants.




Molecular Weight
×10²
×10³
×10⁴
λ
56AA2a CI
" TCT-1
56AA2b CI
" TCT-1
56bAA1b CI
" TCT-1
80RAA2 CI
" TCT-1
80FAA4a CI
" TCT-1
94RAA4 CI
" TCT-1
105RAA1a CI
" TCT-1
107AA1 CI
" TCT-1
107RAA2a CI
" TCT-1
108RAA1 CI
" TCT-1
109RAA3 CI
" TCT-1
109RAA3a CI
" TCT-1
138AA1 CI
" TCT-1
138RAA1 CI
" TCT-1
138RAA3 CI
" TCT-1
141AA1 CI
" TCT-1
141RAA1 CI
" TCT-1
141RAA1a CI
" TCT-1
86RAA1 CI
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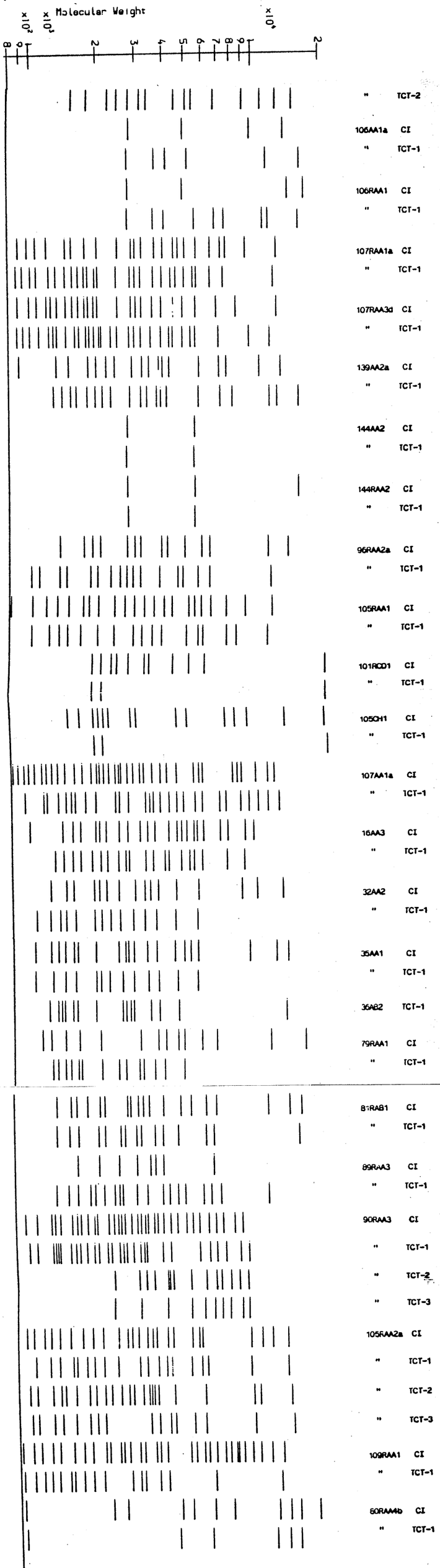
94RAA1 CI
96RAA1a CI
" TCT-1
57AA1 CI
" TCT-1
75AB1 CI
" TCT-1
75AA2a CI
" TCT-1
106AA1 CI
" TCT-1

FIGURE XII


Molecular Weight
×10²
×10³
×10⁴
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2
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4
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6
7
8
9
2
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106AA1a CI
" TCT-1
106RAA1 CI
" TCT-1
107RAA1a CI
" TCT-1
107RAA3d CI
" TCT-1
139AA2a CI
" TCT-1
144AA2 CI
" TCT-1
144RAA2 CI
" TCT-1
96RAA2a CI
" TCT-1
105RAA1 CI
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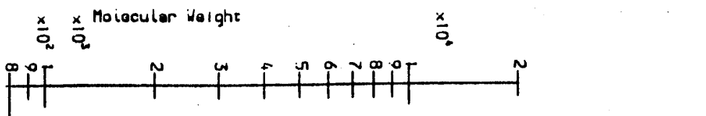
105CH1 CI
" TCT-1
107AA1a CI
" TCT-1
16AA3 CI
" TCT-1
32AA2 CI
" TCT-1
35AA1 CI
" TCT-1
36AB2 TCT-1
79RAA1 CI
" TCT-1
81RAB1 CI
" TCT-1
89RAA3 CI
" TCT-1
90RAA3 CI
" TCT-1
" TCT-2
" TCT-3
105RAA2a CI
" TCT-1
" TCT-2
" TCT-3
109RAA1 CI
" TCT-1
60RAA4b CI
" TCT-1



139BC1 CI
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105RAA7a CI
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" TCT-2
88RAA1b CI
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107RAA1 CI
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109RAA2b CI
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141JC1 CI
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143RJC1 CI
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139aAA1 CI
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" TCT-2
" TCT-3
" TCT-4
pHH1310a
67AA1 CI
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84RAA1 CI
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86RAA2 CI
" TCT-1
87RAA1 CI
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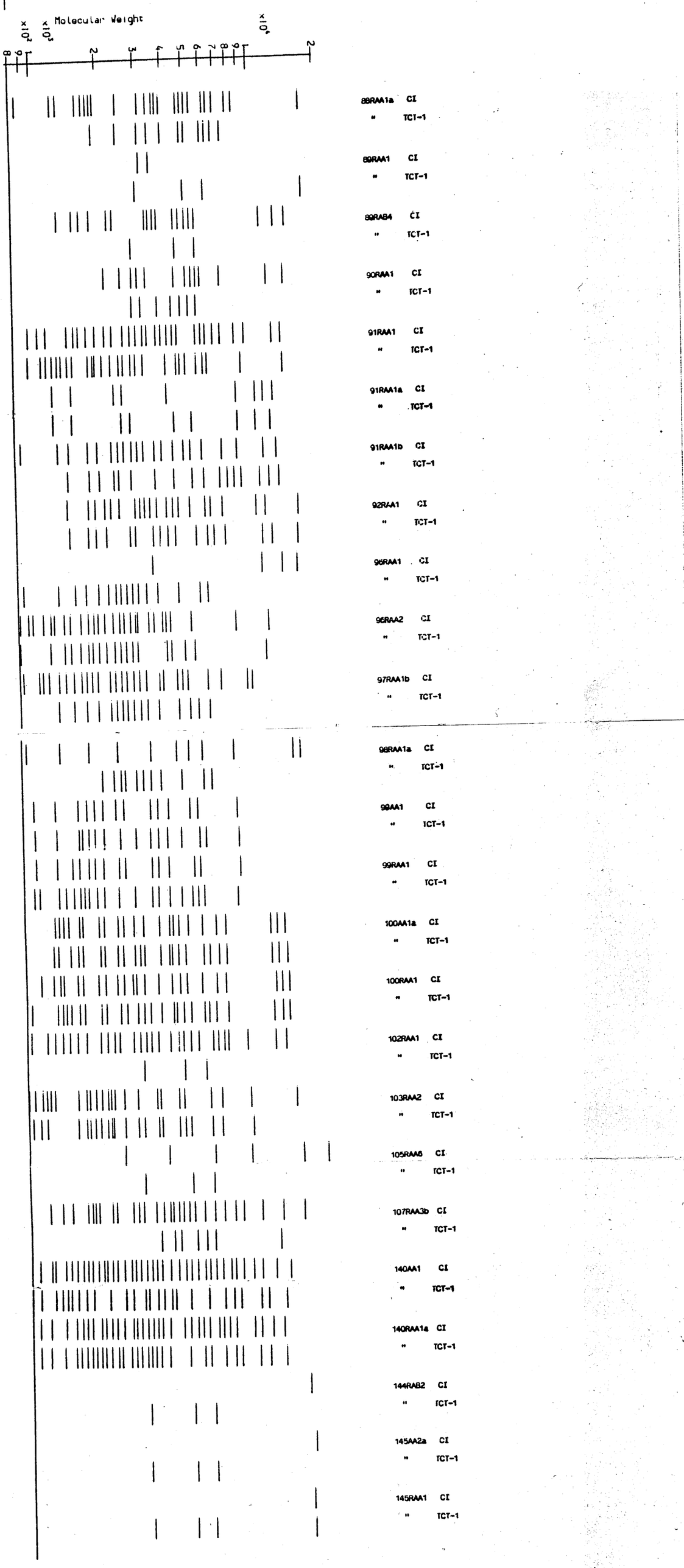
87RAA1a CI
" TCT-1
87RAA2a CI
" TCT-1
88RAA1 CI
" TCT-1


Molecular Weight
×10²
×10³
×10⁴
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2
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4
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6
7
8
9
1
2
88RAA1a CI
" TCT-1
89RAA1 CI
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89RAB4 CI
" TCT-1
90RAA1 CI
" TCT-1
91RAA1 CI
" TCT-1
91RAA1a CI
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91RAA1b CI
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92RAA1 CI
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96RAA1 CI
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96RAA2 CI
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97RAA1b CI
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140AA1 CI
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144RAB2 CI
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145AA2a CI
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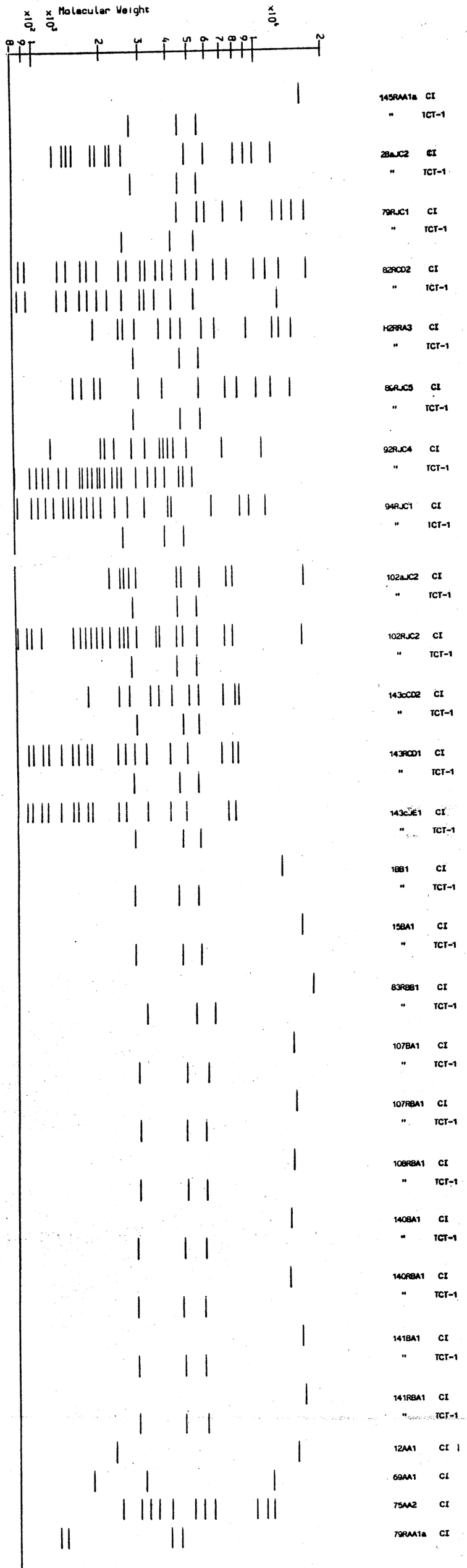
145RAA1 CI
" TCT-1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28aJC2 CI
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79RJC1 CI
" TCT-1
82RCD2 CI
" TCT-1
H2RRA3 CI
" TCT-1
86RJC5 CI
" TCT-1
92RJC4 CI
" TCT-1
94RJC1 CI
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102aJC2 CI
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102RJC2 CI
" TCT-1
143cCD2 CI
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143RCD1 CI
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143cJE1 CI
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158A1 CI
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83RBB1 CI
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107BA1 CI
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140RBA1 CI
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141RBA1 CI
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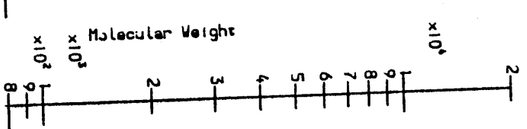
69AA1 CI
75AA2 CI
79RAA1a CI



80RAA1 CI

80RAA3 CI

82RAB1 CI

82RAA2 CI

82RAB3 CI

83RAA1a CI

83RAA1b CI

83RAB1 CI

85RAA1 CI

86RAB1 CI

89RAA4 CI

93RAA2 CI

93RAA3 CI

95RAA1a CI

97RAA1a CI

98RAA1 CI

105RAA1b CI

106RAA1a CI

107AA4 CI

107RAA3c CI

108RAA1a CI

109AB8 CI

109RAB4 CI

139AA3 CI

139RAA2 CI

141AB1 CI

142RAA1 CI

18JH1 CI

22JC1 CI

39JC1 CI

80RCD4 CI

89RJC5a CI

93RA1 CI

93JC1 CI

93RJC1 CI

103RJC1 CI

103RCD2 CI

107RJC2 CI

138RCD1 CI

138RJH1 CI

138RJC2 CI

141RJC1 CI

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74BC1 CI

80BA2 CI

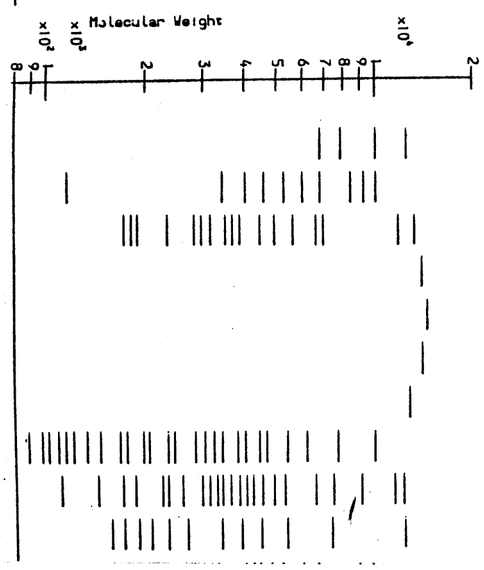
80RBB5 CI

90RBB5 CI

105BA1 CI

138BB1 CI

138RBB1 CI



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83RAA1a CI
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83RAB1 CI
85RAA1 CI
86RAB1 CI
89RAA4 CI
93RAA2 CI
93RAA3 CI
95RAA1a CI
97RAA1a CI
98RAA1 CI
105RAA1b CI
105RAA1a CI
107AA4 CI
107RAA3c CI
108RAA1a CI
109AB8 CI
109RAB4 CI
139AA3 CI
139RAA2 CI
141AB1 CI
142RAA1 CI
18JH1 CI
22JC1 CI
39JC1 CI
80RCD4 CI
89RJC5a CI
93RA1 CI
93JC1 CI
93RJC1 CI
103RJC1 CI
103RCD2 CI
107RJC2 CI
138RCD1 CI
138RJH1 CI
138RJC2 CI
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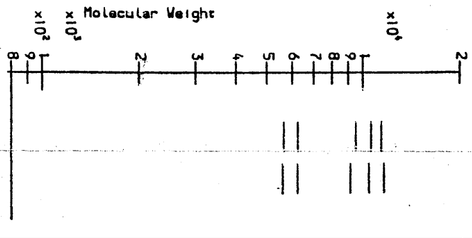
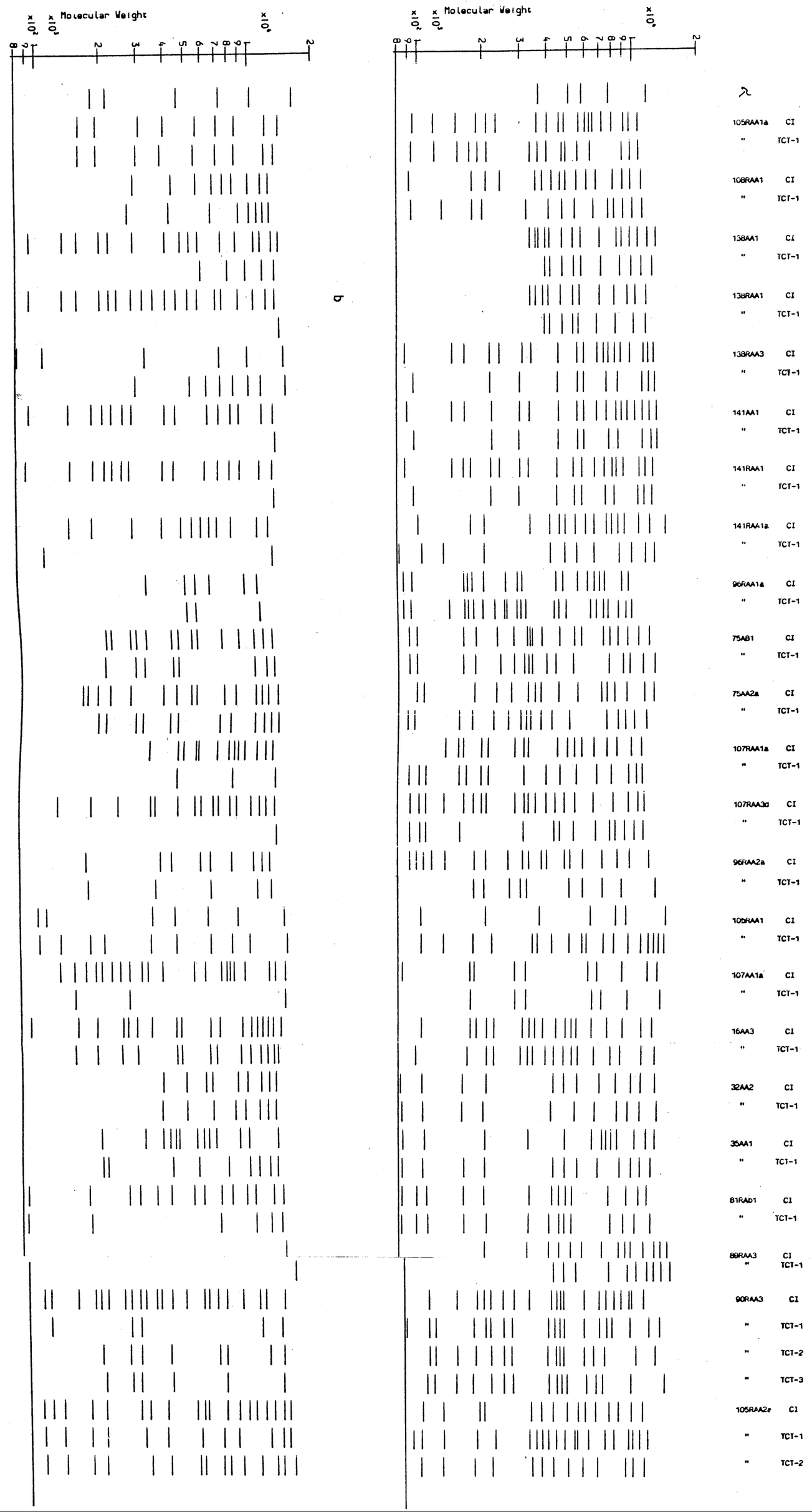


144BA1 CI
144RBA1 CI

FIGURE XIII


Molecular Weight
×10⁴
×10³
×10²
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1
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4
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8
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1
2
a
b
105RAA1a CI
" TCT-1
108RAA1 CI
" TCT-1
138AA1 CI
" TCT-1
138RAA1 CI
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138RAA3 CI
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141AA1 CI
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96RAA1a CI
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107RAA3d CI
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96RAA2a CI
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105RAA1 CI
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107AA1a CI
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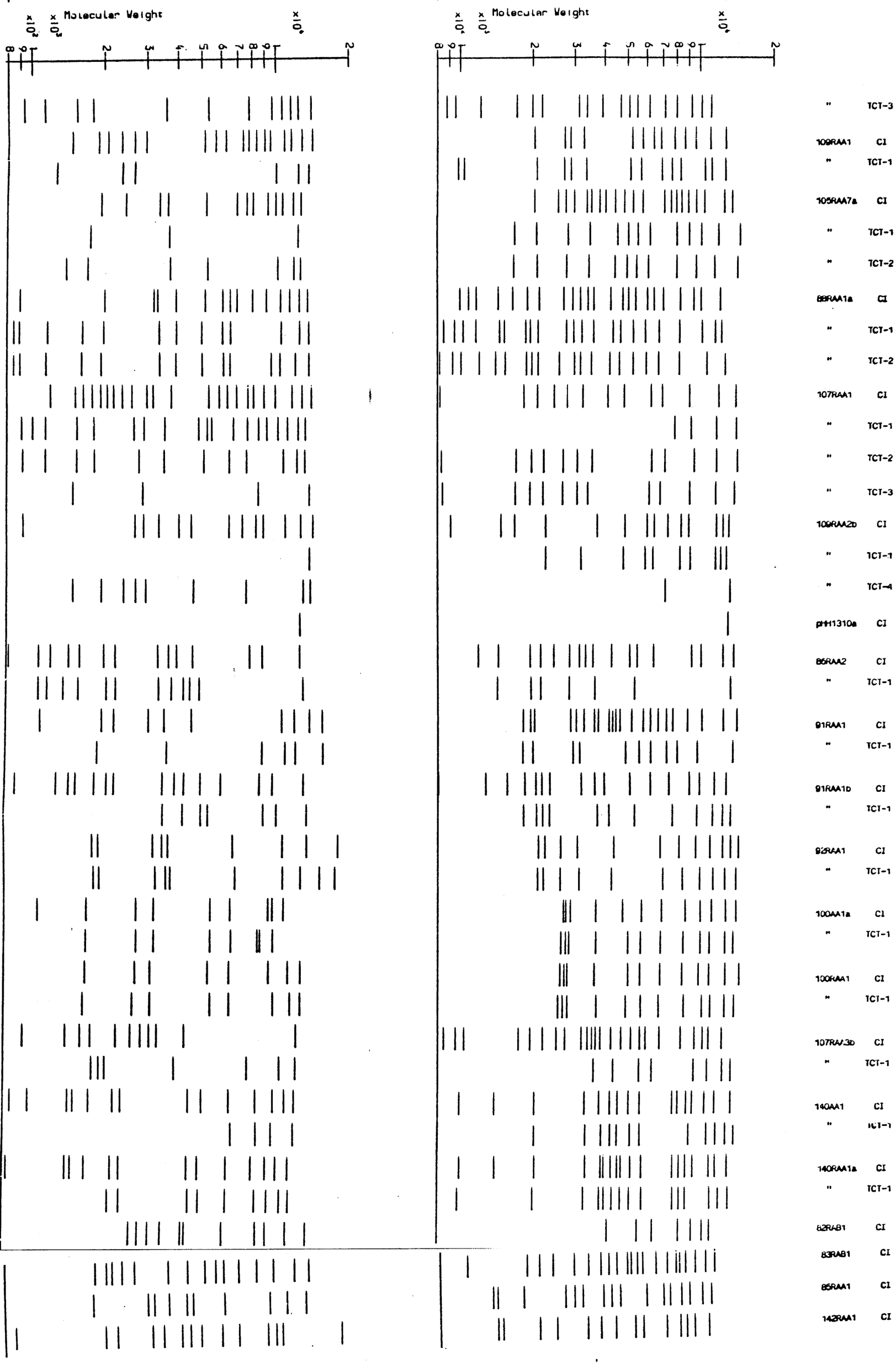
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×10³
×10⁴
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" TCT-2
88RAA1a CI
" TCT-1
" TCT-2
107RAA1 CI
" TCT-1
" TCT-2
" TCT-3
109RAA2b CI
" TCT-1
" TCT-4
pHH1310a CI
86RAA2 CI
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91RAA1 CI
" TCT-1
91RAA1b CI
" TCT-1
92RAA1 CI
" TCT-1
100AA1a CI
" TCT-1
100RAA1 CI
" TCT-1
107RAA3b CI
" TCT-1
140AA1 CI
" TCT-1
140RAA1a CI
" TCT-1
82RAB1 CI
83RAB1 CI
85RAA1 CI
142RAA1 CI

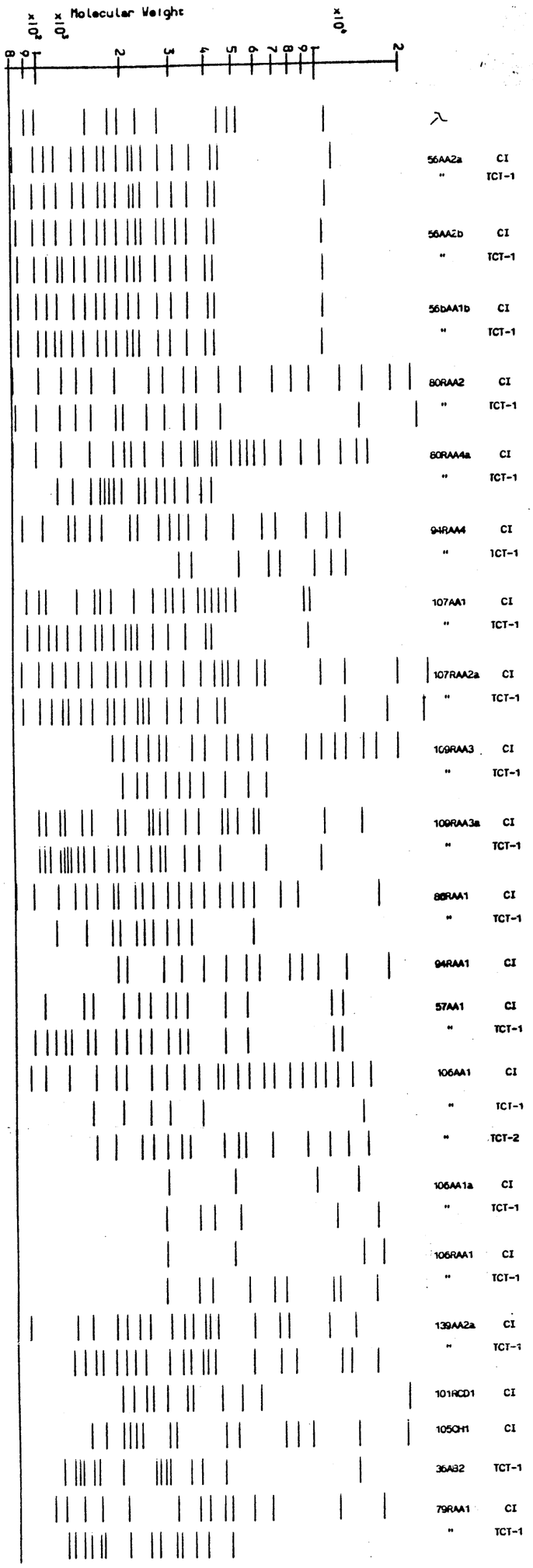
FIGURE XIV: Fingerprints of plasmid DNA from plasmid-containing clinical isolates and transconjugants digested with enzymes:-

(a) PstI

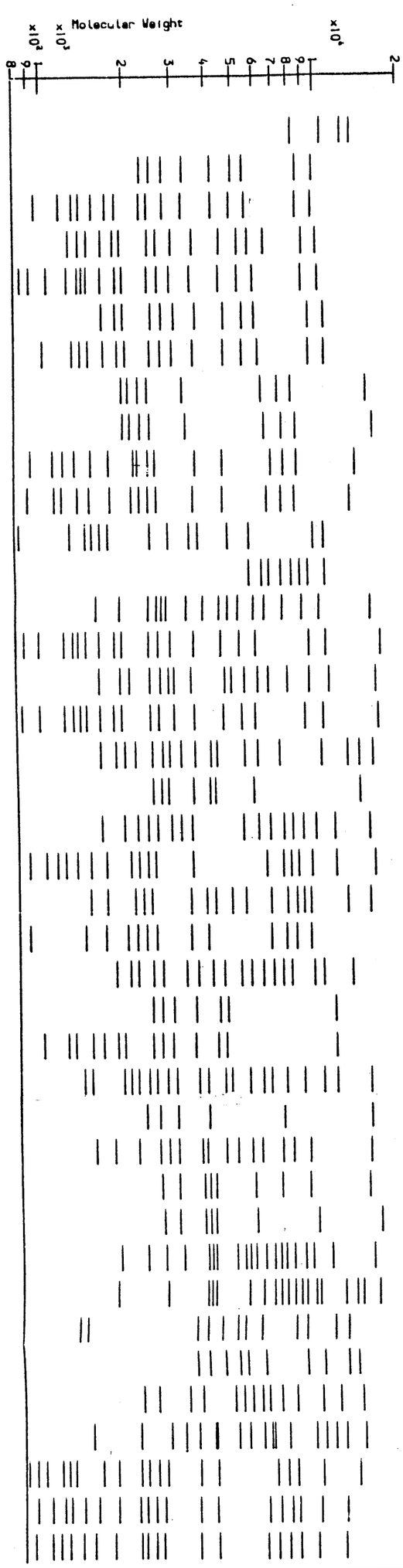
(b) SmaI

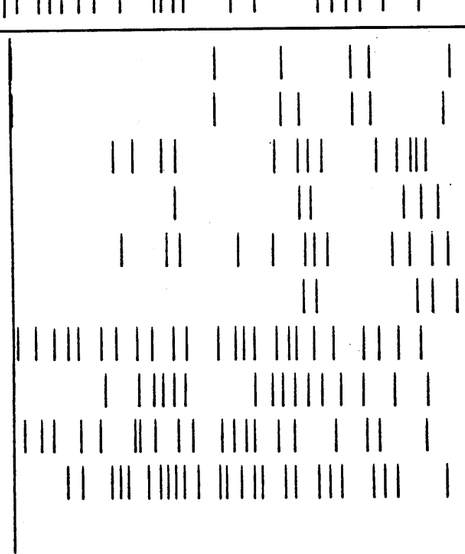
FIGURE XIV

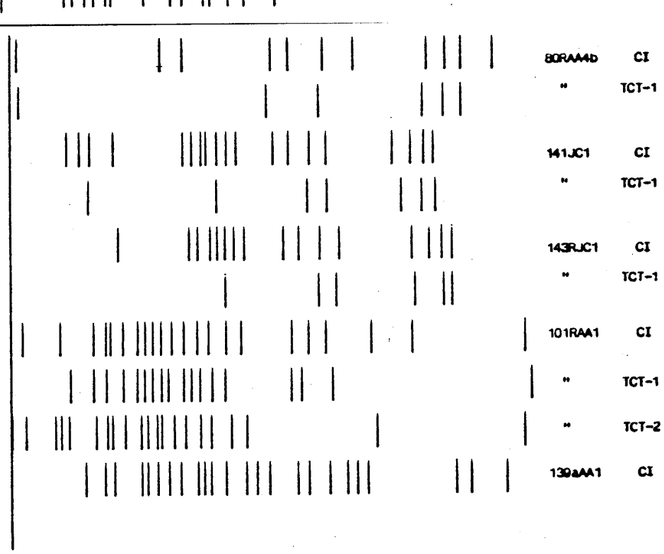
a

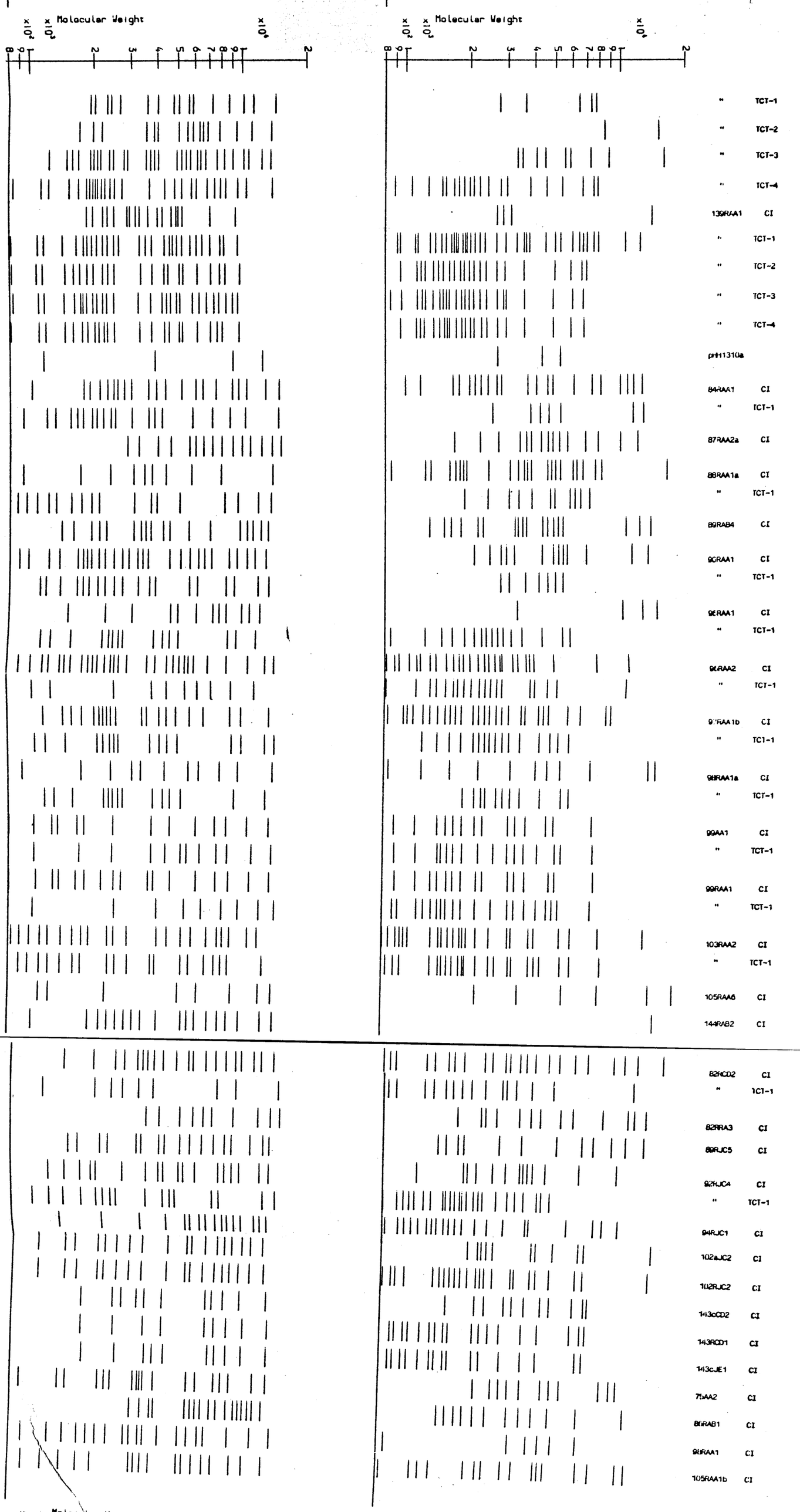


b








Molecular Weight
×10²
×10³
×10⁴
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" TCT-2
" TCT-3
" TCT-4
139RAA1 CI
" TCT-1
" TCT-2
" TCT-3
" TCT-4
pH1310a
84RAA1 CI
" TCT-1
87RAA2a CI
88RAA1a CI
" TCT-1
89RAB4 CI
90RAA1 CI
" TCT-1
91RAA1 CI
" TCT-1
94RAA2 CI
" TCT-1
91RAA1b CI
" TC1-1
98RAA1a CI
" TCT-1
99AA1 CI
" TCT-1
99RAA1 CI
" TCT-1
103RAA2 CI
" TCT-1
105RAA6 CI
144RAB2 CI
82RCD2 CI
" TCT-1
82RRA3 CI
89RJC5 CI
92RJC4 CI
" TCT-1
94RJC1 CI
102aJC2 CI
102RJC2 CI
143cCD2 CI
143RCD1 CI
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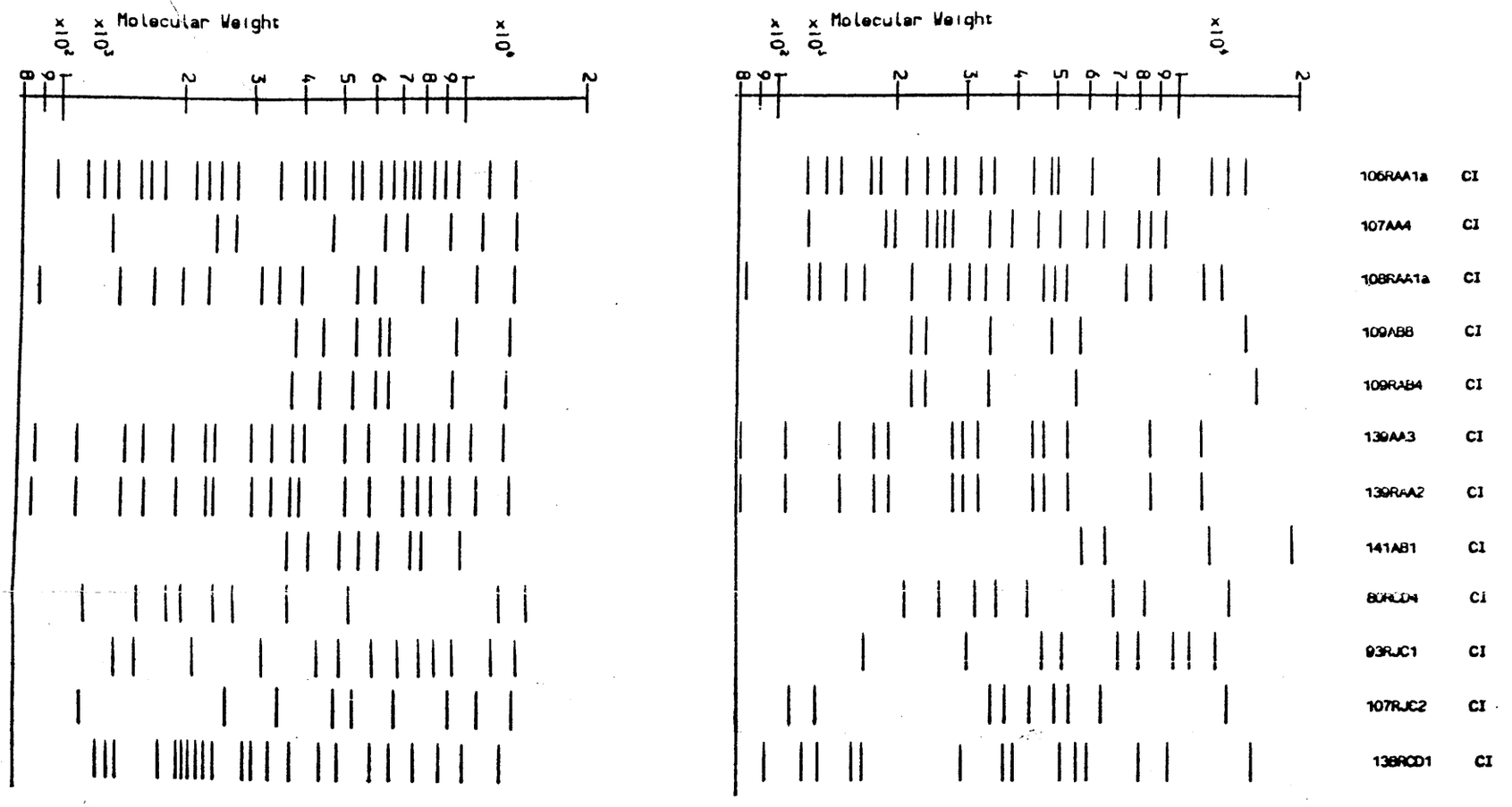
86RAB1 CI
98RAA1 CI
105RAA1b CI


Molecular Weight
×10³
×10⁴
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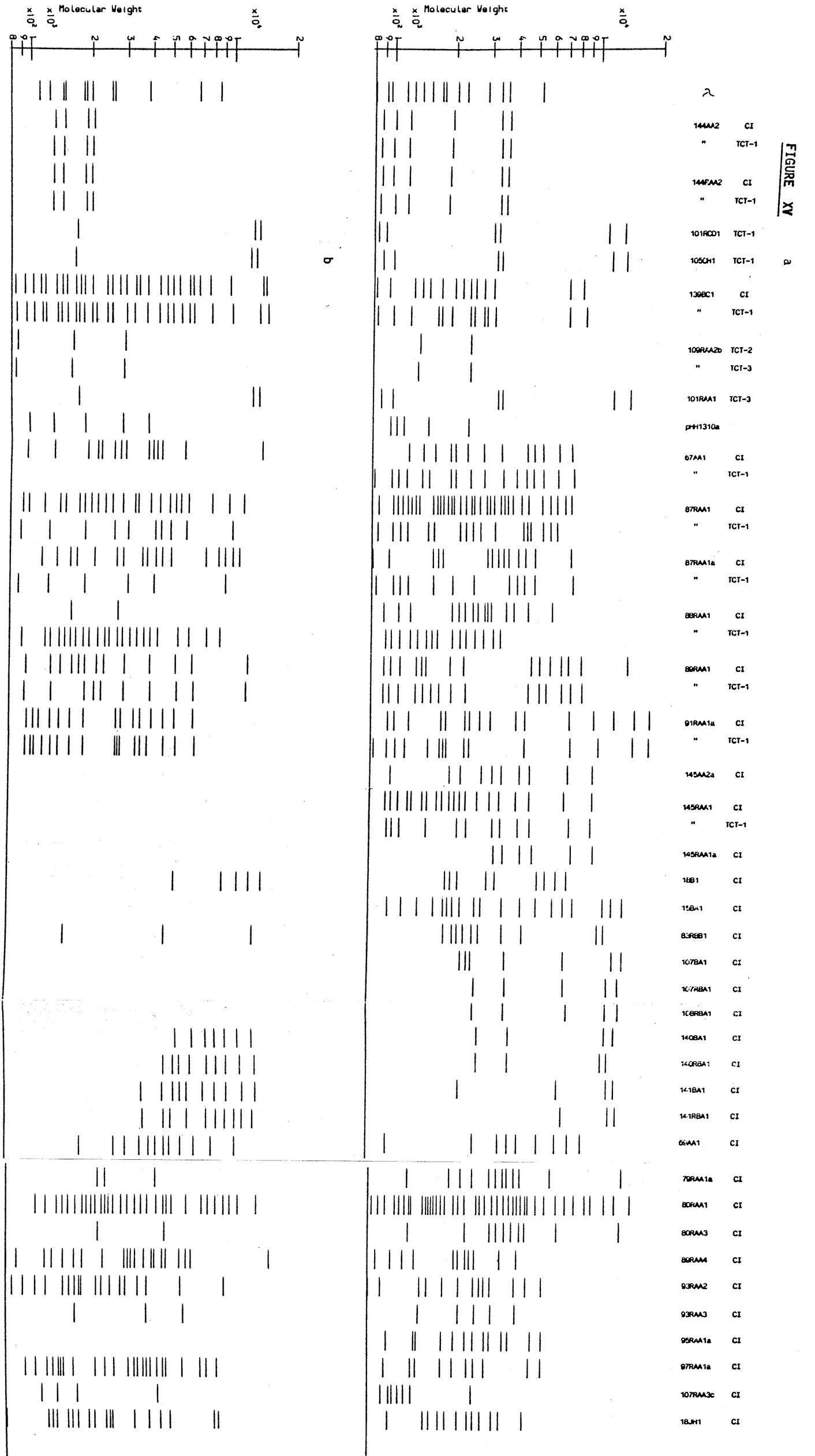
107AA4 CI
108RAA1a CI
109AB8 CI
109RAB4 CI
139AA3 CI
139RAA2 CI
141AB1 CI
93RJC1 CI
107RJC2 CI
138RCD1 CI

FIGURE XV: Fingerprints of plasmid DNA from plasmid-containing clinical isolates and transconjugants digested with enzymes:-

(a) Bspl286

(b) AvaII

FIGURE XV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×10⁴
a
b
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144FAA2 CI
" TCT-1
101RCD1 TCT-1
105CH1 TCT-1
139BC1 CI
" TCT-1
109RAA2b TCT-2
" TCT-3
101RAA1 TCT-3
pH1310a
67AA1 CI
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87RAA1 CI
" TCT-1
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89RAA1 CI
" TCT-1
91RAA1a CI
" TCT-1
145AA2a CI
145RAA1 CI
" TCT-1
145RAA1a CI
15BB1 CI
15BA1 CI
83RBB1 CI
107BA1 CI
107RBA1 CI
108RBA1 CI
140BA1 CI
140RBA1 CI
141BA1 CI
141RBA1 CI
66AA1 CI
79RAA1a CI
80RAA1 CI
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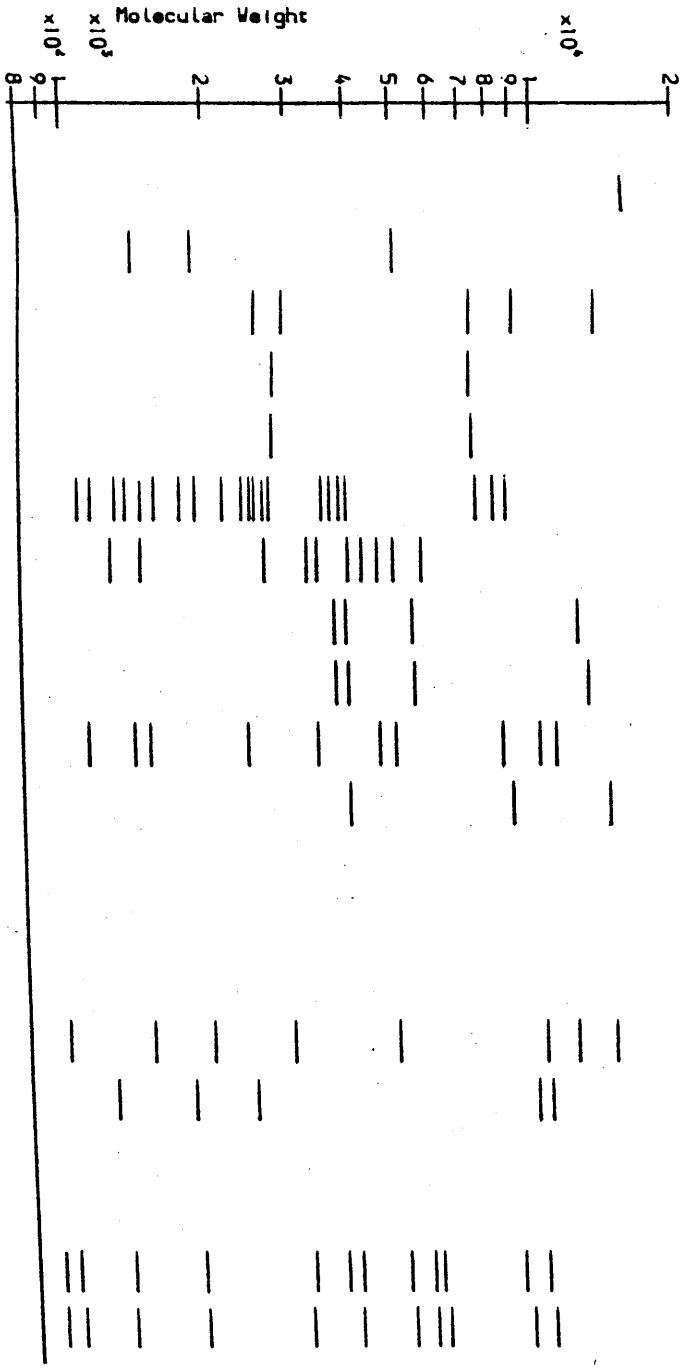
97RAA1a CI
107RAA3c CI
16JH1 CI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×10⁴
×10⁴
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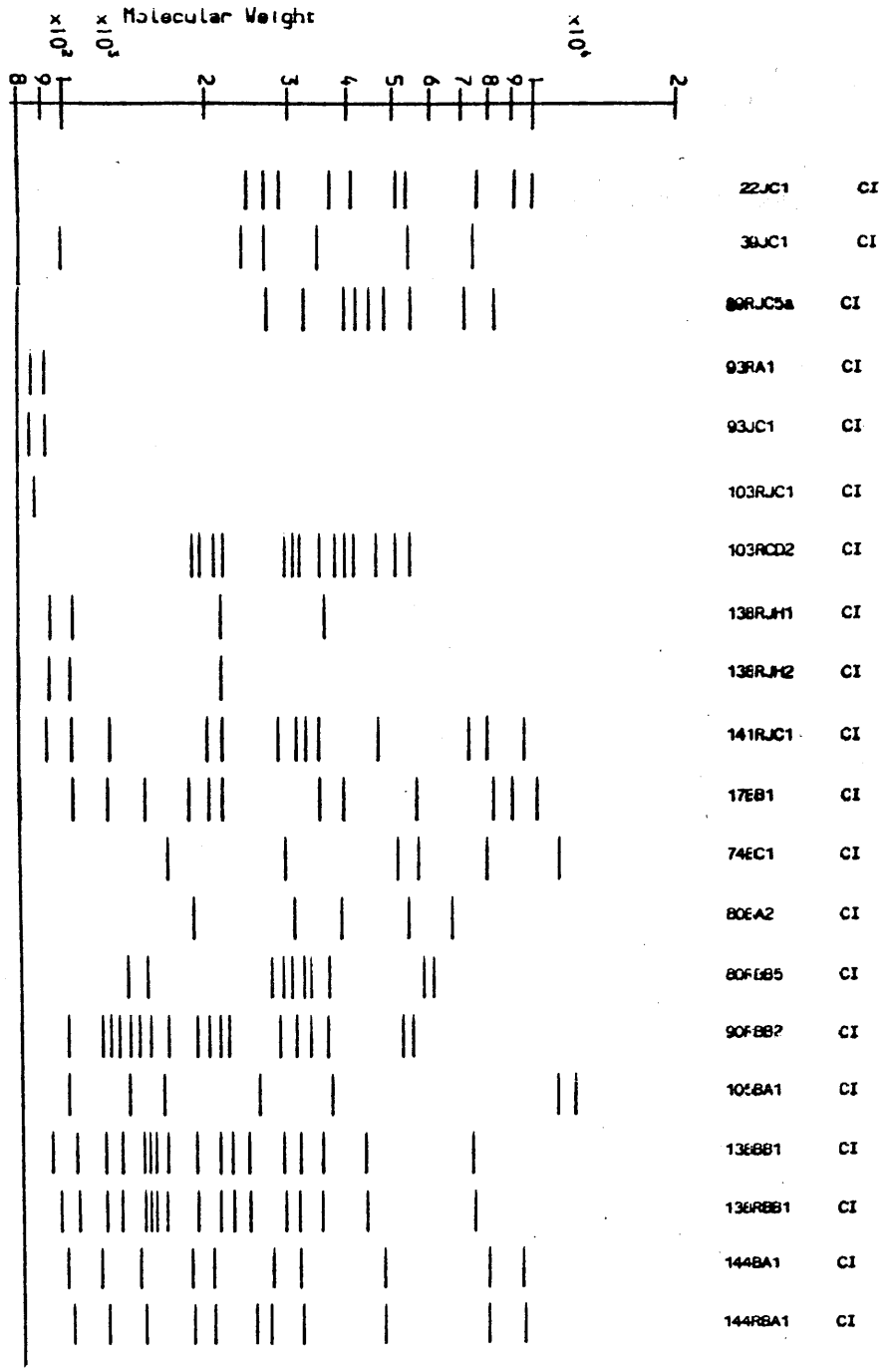
7
8
0
1
2


Molecular Weight
×10⁴
×10⁴
×10⁴
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9
1
2
3
4
5
6
7
8
9
1
2
22JC1 CI
39JC1 CI
89RJC5a CI
93RA1 CI
93JC1 CI
103RJC1 CI
103RCD2 CI
138RJH1 CI
138RJH2 CI
141RJC1 CI
17EB1 CI
74EC1 CI
80EA2 CI
80FBB5 CI
90FBB2 CI
105BA1 CI
136BB1 CI
136RBB1 CI
144BA1 CI
144RBA1 CI

High levels of chromosomal DNA present in any plasmid DNA preparation resulted in higher levels of chromosomal "smearing" and reduced enzyme cutting efficiency of plasmid DNA resulting in poorly visible fingerprint fragments.

Variation in DNA concentration from preparation to preparation led to reduction in visibility of small fragments in low concentration preparations.

Incomplete digestion of plasmid DNA resulting in "partial digests" in which observed "fragments" comprised two or more fragments with one or more internal enzyme recognition sites which had not been cleaved.

The lower limit of detectable fragments varied for each gel dependent on how far or how fast the fragments had migrated and whether some had been lost off the bottom of the gel.

Operator error in the input of presence and position of fragments from photographs when using the digitiser to computerise results.

Plasmid DNA from a single-plasmid-containing clinical isolate was purified, digested with PstI and gel electrophoresis performed on 7 different occasions. The number of fingerprint fragments generated and their mean size are shown in Figure XVI. The cleavage of any enzyme recognition site depends on a number of factors including DNA purity, base sequence adjacent to the cleavage site and amount of both DNA and enzyme present. However, the results obtained on 7 different occasions were very similar with a maximum of 19 fragments between 800 and 15,000 kb being generated. On one occasion a faint band of 2.8 kb was seen which was not present in any other fingerprint and was probably the result of incomplete digestion of two or more smaller fragments. A further three fingerprints contained 19 fragments, each of these showed a faint band of 1.21 to 1.26 kb. The low intensity of this band and its presence in only three fingerprints suggests incomplete cleavage of a specific enzyme recognition site. The remaining 18 bands, present on all 7 occasions fingerprints were obtained, were used as a reference

FIGURE XVI: Fingerprints of a single plasmid-containing clinical isolate which was purified, digested and electrophoresed on 7 different occasions. The mean size, standard error and size range of 18 reference fragments are included.

kb = kilobase

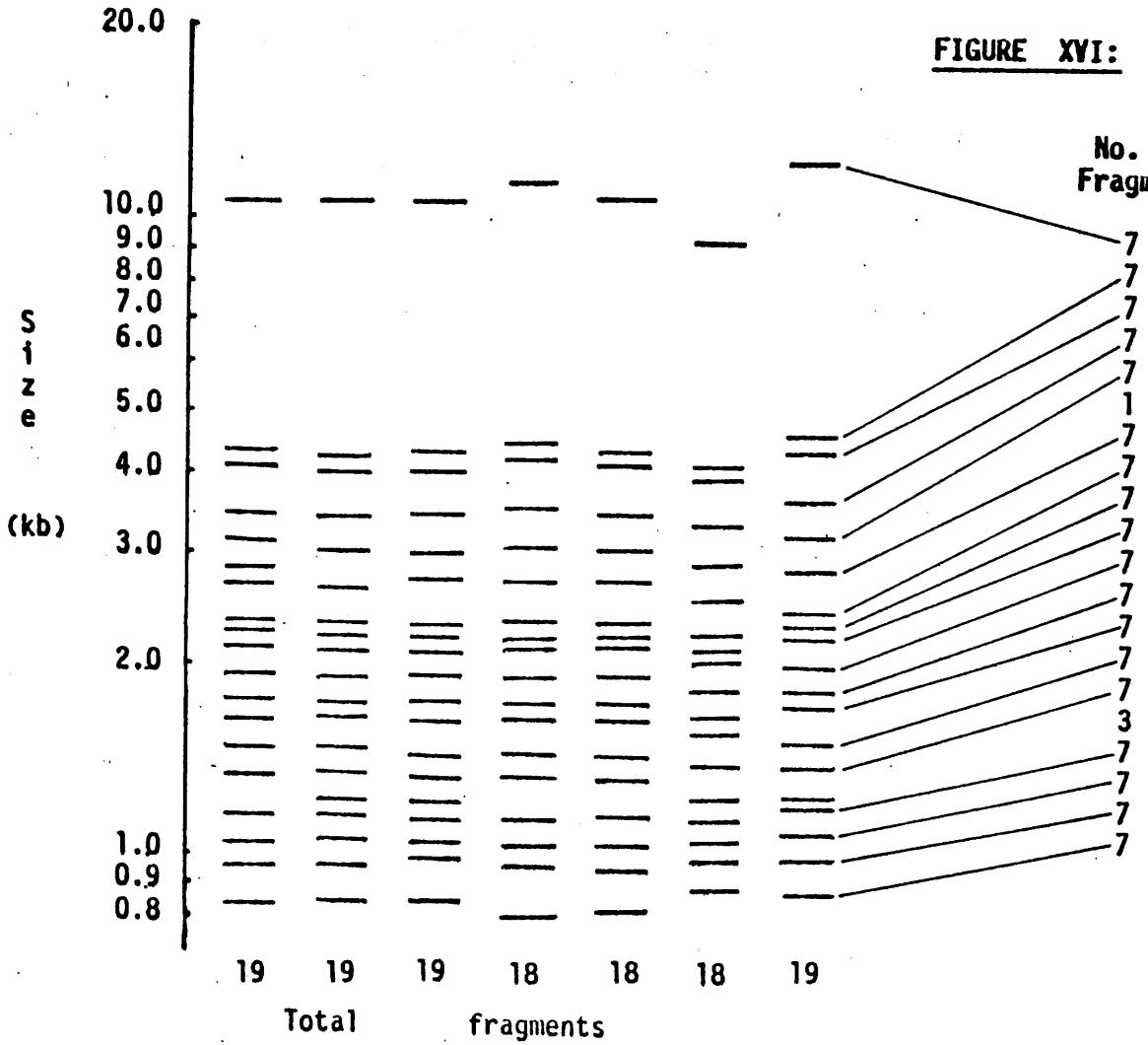


FIGURE XVI:

No. of Fragments	Mean size range ± Standard Error	(range)
7	10.86 ± 0.36	(9.3 - 12.4)
7	4.37 ∓ 0.06	(4.14 - 4.61)
7	4.11 ∓ 0.05	(3.94 - 4.32)
7	3.48 ∓ 0.04	(3.33 - 3.68)
7	3.06 ∓ 0.04	(2.89 - 3.20)
1		
7	2.70 ± 0.03	(2.55 - 2.85)
7	2.35 ∓ 0.02	(2.24 - 2.46)
7	2.23 ∓ 0.02	(2.13 - 2.34)
7	2.13 ∓ 0.02	(2.04 - 2.24)
7	1.92 ∓ 0.02	(1.84 - 2.01)
7	1.76 ∓ 0.02	(1.68 - 1.85)
7	1.66 ∓ 0.02	(1.58 - 1.75)
7	1.47 ∓ 0.02	(1.40 - 1.54)
7	1.34 ∓ 0.02	(1.26 - 1.40)
3		
7	1.17 ± 0.01	(1.21 - 1.15)
7	1.07 ∓ 0.01	(1.06 - 1.10)
7	0.98 ∓ 0.01	(0.96 - 1.00)
7	0.85 ∓ 0.01	(0.81 - 0.90)

to compare the standard error and size of each of these fragments (Fig. XVI). The results obtained show that on no occasion was the standard error greater than 5 per cent of the size of each fragment. Thus, despite the possible sources of inaccuracy in the above method of producing plasmid fingerprints, it was considered to be sufficiently reproducible and accurate to compare plasmid fingerprints from different clinical isolates and transconjugants. To a large extent these sources are recognised with experience and can be eliminated by repeating either DNA purification steps, or digestion and electrophoresis as indicated by the above results.

The same method of purifying plasmid DNA was used for all clinical isolates and transconjugants. A number of features were apparent from the restriction digest analysis of this purified plasmid DNA. Although the majority of plasmids were purified to a state where fingerprint fragments generated were easily seen after agarose gel electrophoresis, a number of exceptions were found.

No DNA was seen on agarose gels after electrophoresis of digested plasmid DNA from clinical isolate 36AB2. When sensitivity tests and agarose gel electrophoresis for detection of plasmid DNA was repeated with this isolate it was found to

have lost both its original resistance determinants and plasmid. This indicates that although the R-plasmid from the clinical isolate was stable in the E.coli K12 transconjugant, it was unstable in the wild type.

Only PstI digests were obtained from 8 unrelated clinical isolates. Four of these produced a number of fragments which were faint as repeated DNA preparations consistently contained a high background fluorescence which rendered the fragment poorly visible. Digestion with additional enzymes gave such poor results that they were not computerised. The remaining 4 isolates did not appear to be cut with PstI and again the quality of the DNA preparation was so poor that no further results were obtained.

No plasmid fingerprint was obtained with any enzyme from one further clinical isolate and a transconjugant from a different isolate. The recipient strain in the latter instance was Ent.aerogenes, results from which were generally poorer than those obtained with E.coli K12 transconjugants. Plasmids from OEG isolates, especially Proteus, generally produced less clear fingerprint results than E.coli. The majority of the clinical E.coli isolates gave unequivocal results with all enzymes used.

The number of fragments generated from PstI digestion of plasmids from each clinical isolate were as shown in Table 24. Plasmid-containing clinical isolates were divided into three groups according to the number of fragments generated. Fifty-eight (35%) produced less than 6 fragments, 63 (37%) yielded between 6 and 20 fragments and 47 (28%) produced more than 20 fragments. Of the clinical isolates that harboured two or more plasmids, 47 per cent produced less than 6 fragments, 62 per cent between 6 and 20 fragments and 81 per cent yielded more than 20 fragments. This trend demonstrates that an increase in the number of plasmids harboured by a clinical isolate was paralleled by an increase in the number of fragments generated by PstI digestion.

Considering clinical isolates that harboured single plasmids, 34 per cent, 40 per cent and 26 per cent of E.coli yielded less than 6, 6 to 20, or more than 20 fingerprint fragments respectively. In contrast, no OEG isolates harbouring a single plasmid produced more than 20 fragments with 65 per cent and 35 per cent yielding less than 6, or 6 to 20 fragments respectively. This indicates that plasmids from E.coli have more PstI restriction sites than plasmids from OEG.

TABLE 24: **The relationship between the number of plasmids harboured and the number of fragments detected after digestion with PstI for 116 and 52 isolates of E.coli and OEG respectively.**

Number of plasmid fragments	Organism	Number of Plasmids						
		1	2	3	4	5	6	7
Less than 6	E.coli	12	16	2	-	-	-	-
	OEG	19	8	1	-	-	-	-
6 to 20	E.coli	14	12	7	5	3	1	-
	OEG	10	8	2	1	-	-	-
Greater than 20	E.coli	9	12	5	10	3	3	2
	OEG	-	1	2	-	-	-	-

OEG = enteric genera other than E.coli

Previously the distribution and characteristics of plasmids were considered overall whereas here the plasmids were investigated in relation to their presence in different isolates from the same patient and in an epidemiological context. To this end plasmid profiles and fingerprints were analysed together to determine the following:-

(a) Whether plasmid profile and fingerprints confirmed the identity of otherwise indistinguishable isolates of the same coliform species from wound and rectal swabs from the same patient.

(b) Whether, and with what frequency, plasmid tranfer occurred in vivo.

(c) The relatedness among conjugative R-plasmids which transferred the same or common resistance determinants.

(d) Whether conjugative plasmids not associated with resistance were related to conjugative R-plasmids.

(e) Comparison of fingerprints of isolates that harbour only non-conjugative plasmids with those plasmids which were not mobilised from isolates which possessed additional conjugative plasmids.

(f) Determine whether the pool of plasmids indigenous to OEG resemble those from E.coli and similarly whether the plasmids of OEG were generally homogeneous or genus-specific.

Correlation of plasmids in isolates from wound and rectal swabs:

In many instances when coliforms of the same genus were isolated from wound and rectal swabs from the same patient the resistance determinants and plasmid profile of both isolates were similar if not identical. To confirm that these were the same organism and to support the conclusion that the wound isolate was a faecal contaminant, fingerprint analysis of plasmid-containing isolates was performed.

Of the 39 patients from whom rectal swabs were obtained coliforms were isolated from wounds in 21 of these patients. At least one species of coliform and up to 5 different types were common to wound and rectal swabs in 19 of

these 21 patients. For example, from patient 107 three morphologically- and/or biochemically-distinct E.coli strains were isolated from wound swabs and 6 distinct E.coli from rectal swabs. Prot.vulgaris, Prot.morganii, Ent.cloacae and Cit.freundii were also isolated from either wound or rectal swabs but not both. Prot.mirabilis and Prov.stuartii were isolated from both wound and rectal swabs. Therefore it was possible that 5 different coliforms were common to wound and rectal swabs in this patient. Overall there was the possibility of 40 pairs of isolates being common to paired wound and rectal swabs in the 21 patients studied.

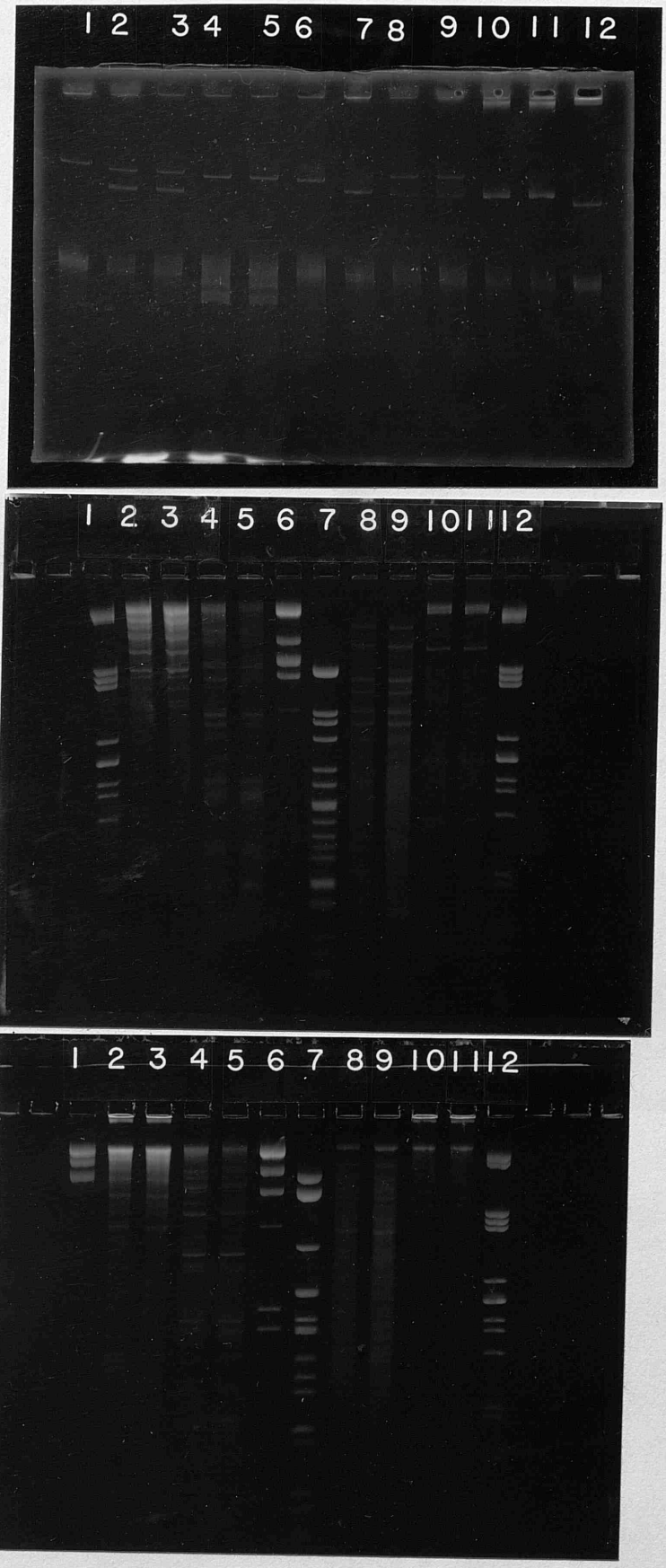
Twelve of these 40 pairs of coliforms did not contain plasmids but 11 were indistinguishable with regard to colony morphology, API 20E code and resistance determinants suggesting that they were the same isolate from both wound and rectal swabs. Nine of the 40 pairs of coliforms were shown to differ with regard to resistance determinants (6) and/or plasmid profile (9). The remaining 19 pairs of coliforms were compared after plasmid restriction enzyme analysis. It would appear from the plasmid fingerprints of these 38 isolates that there were minor differences in the plasmid fingerprints of most of the 19 pairs of coliforms. Figures XVIIa, XVIIb and XVIIc shows 4 examples of these paired coliform isolates. Gel

FIGURE XVII: **Plasmid profile and plasmid fingerprint gels of paired clinical isolates from wound and rectal swabs from 4 different patients.**

Track No.		Gel a (plasmid profile)	Gel b (plasmid digest)	Gel c (plasmid digest)
1	Standard	85 Md	λ PstI	λ SmaI
2	138AA1*	CL	EcoRI	HindIII
3	138RAA1	CL	EcoRI	HindIII
4	139AA3	CL	PstI	SmaI
5	139RAA2	CL	PstI	SmaI
6	Standard	62 Md	λ EcoRI	λ HindIII
7	Standard	46 Md	λ Bstl286	λ AvaII
8	145AA2a	CL	Bspl286	AvaII
9	145RAA1	CL	Bspl286	AvaII
10	141BA1	CL	Bspl286	AvaII
11	141RBA1	CL	bspl286	AvaII
12	Standard	36 Md	λ PstI	λ PstI

Md = megadaltons;
CL = crude lysate;
* code as Table 2, page 83.

FIGURE XVII



electrophoresis of crude lysates (Fig. XVIIa) showed that the plasmid molecular weights were similar for each of the 4 pairs of isolates. Gel electrophoresis of fragments from restriction digests (Figs. XVIIb and XVIIc) show that the fingerprints from these 4 pairs of coliforms were indistinguishable. Within the limitations of the methods used and observations from the original photographs of restriction digests, it would appear that all 19 pairs of coliforms were indistinguishable between wound and rectal isolates.

In summary, a total of 99 E.coli and OEG strains were isolated from rectal swabs from the 21 patients which also yielded coliforms from wound swabs (Table 25). Fifty-nine different coliforms were isolated from the wound swabs. Despite the fact that 32 of the rectal isolates and 25 of those from wounds did not possess any plasmids, 11 pairs of these plasmid-free isolates were otherwise indistinguishable. Of the 32 strains isolated from wound swabs which possessed plasmids, 19 were indistinguishable on the basis of fingerprint results and other characteristics from rectal isolates from the same patient. Thus 19 (59%) of the 32 plasmid-containing isolates from wound swabs were confirmed, on the basis of fingerprint results, as having originated from the patients own faecal flora.

TABLE 25: **Number of distinct coliforms isolated from wound and rectal swabs from 21 patients from whom wounds yielded coliforms and from whom rectal swabs were obtained.**

	Wound Isolates	Rectal Isolates	*Possible Wound & Rectal Isolates	**Confirmed Wound & Rectal Isolates
E.coli	19 (17)	55 (49)	18 (17)	12
Proteus spp.	23 (8)	17 (7)	15 (6)	6
All other coliforms	17 (7)	27 (13)	7 (5)	1
TOTAL	59 (32)	99 (69)	40 (28)	19

() Plasmid-containing isolates.

* Possible isolates common to paired wound and rectal swabs based on indistinguishable colony morphology and API 20E code.

** Confirmed paired wound and rectal isolates on the basis of the above criteria together with matching resistance determinants and indistinguishable plasmid fingerprints.

Plasmid transfer in vivo:

A number of conditions must be fulfilled before plasmid transfer in vivo can be demonstrated. These include that any given specimen must yield at least two distinct coliforms and that at least one must harbour a conjugative plasmid. The potential for plasmid transfer in vivo was calculated separately for wound and rectal coliforms from the same patient as there was the potential for plasmid transfer to occur both in the wound and in the gastrointestinal tract. Although each specimen was assessed separately the in vivo transfer potential was calculated per patient, as the same coliform was often isolated on more than one occasion from the same site. For example, when two coliforms were isolated from the same site on two or more occasions, they were only included once as either donor isolates or potential recipients. Although more than one plasmid was often co-transferred or mobilised from any one donor isolate, on no occasion could this be taken to indicate that two separate plasmids from the donor isolate had conjugative ability. Therefore calculation of the plasmid transfer potential was based on the number of clinical isolates which harboured conjugative plasmids not the number of

plasmids transferred. In 48 sites there was at least one isolate possessing a conjugative plasmid and one or more potential recipients.

These 48 sites comprised wounds from 5 patients, rectal swabs from 23 patients and both wound and rectal swabs from 10 patients. From these 48 sites, 50 isolates were found which possessed conjugative R-plasmids and 46 which harboured conjugative plasmids not associated with resistance. There was a possibility of 151 transfer events involving Tra^+ R^+ plasmids and 129 transfer events involving Tra^+ R^- plasmids. Of the 129 possible transfer events involving Tra^+ R^- plasmids, 25 of the possible recipients were clinical isolates which already possessed Tra^+ R^+ plasmids. Although the transferability of any Tra^+ R^- plasmids could not be demonstrated in these 25 isolates by conjugation, on the basis of fingerprint analysis, none appeared to possess Tra^+ R^- plasmids which were found in clinical isolates from the same patient suggesting that no in vivo transfer had occurred in these isolates. Therefore the combined transfer potential for both Tra^+ R^+ and Tra^+ R^- plasmids was 280 events.

Plasmid transfer *in vivo* was demonstrated on a number of occasions. From patient No. 56, 4 distinct *E.coli* strains were isolated with resistance determinants and plasmid profiles as follows:

56AA1b,	TcSmSu resistant,	no plasmids
56AA2b,	ApTcSmSu resistant,	46Md plasmid
56AA1a,	ApSmSu resistant,	46 Md plasmid
56bAA1b,	ApSmSu resistant,	75 and 46 Md plasmids.

The latter three isolates each possessed transferable Ap resistance associated with the 46 Md plasmid. Since the transferable plasmids were indistinguishable with regard to resistance determinants, molecular weight and fingerprints confirming that they were the same plasmid the observed additional phenotypic differences between the *E.coli* define two distinct potential recipients. Thus it appears that plasmid transfer has occurred *in vivo* on two occasions in this patient, either separately from one isolate to the other two, or sequentially from one isolate to a second which then acted as a donor for the third isolate. It was not possible to define the original donor.

A second example was found where R-plasmid transfer appears to have occurred *in vivo*. On this occasion an identical TcTp (Tp = trimethoprim) R-plasmid was found in two rectal isolates, an *E.coli* and a *Kleb.pneumoniae*, from patient No.101.

Only one example was found where in vivo transfer of a conjugative plasmid not associated with resistance appears to have occurred. This transfer involved the isolation of *Kleb.pneumoniae* and *Ent.agglomerans* from the same wound. Although plasmids from these isolates did not carry any resistance determinants, their molecular weight and fingerprints were identical.

Combining the above results of actual plasmid transfer *in vivo* and plasmid transfer potential, gives an observed ratio of 1:50 (Table 26) for R-plasmid transfer and an observed ratio of 1:129 for transfer of plasmids not associated with resistance. The overall ratio was therefore 1:70 for transfer of any conjugative plasmid irrespective of whether resistance determinants were carried by the conjugative plasmids.

TABLE 26: **Calculation of in vivo transfer potential and transfer rate.**

	No. of Potential Donors	No. of Potential Recipients	Transfer Potential	Transfer Events Detected	In vivo Transfer Rate
Tra^+ R^+	50	104	151	3	1:50
Tra^+ R^-	46	106	129	1	1:129
Combined Tra^+	96	168	280	4	1:70

Tra^+ R^+ = conjugative resistance plasmid

Tra^+ R^- = conjugative plasmid not associated with resistance

Tra^+ = conjugative plasmid

Conjugative R-plasmids:

Ampicillin resistance was the most common transferrable resistance determinant in the clinical isolates studied. Restriction digests showed that a number of these conjugative R-plasmids were related on the basis of common restriction fragments.

Twenty-three transconjugants which acquired Ap resistance (12 which acquired Ap resistance alone and 11 which transferred Ap resistance in combination with at least one other resistance determinant) were fingerprinted with the enzymes PstI and SmaI. The fingerprints of 5 transconjugants which acquired Ap resistance only, and a single plasmid, were identical (Fig. XVIII). Three were derived from clinical isolates obtained from patient 56 and the plasmid from 56AA1a was used as a reference for comparison. A further two from wound and rectal swabs from a second patient (107AA1 and 107RAA2a) were essentially identical.

Comparison of fingerprints from the above Ap-resistance reference plasmid with other Ap-resistant plasmids, showed that although no further transconjugants had identical fingerprints, a number had fragments in common with the transconjugant from 56AA1a (Table 27). All 18 of the Ap-resistant transconjugants had between 4 and 34 fragments in

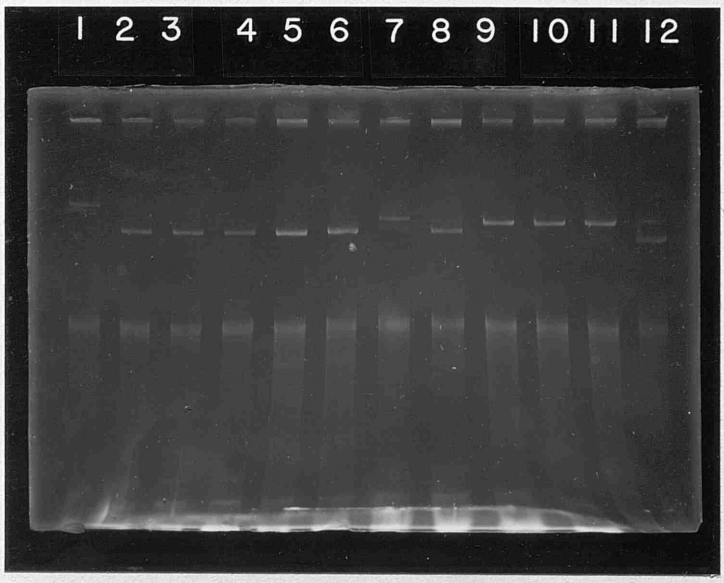
FIGURE XVIII: **Plasmid profile and plasmid fingerprint gels of 8 ampicillin-resistant transconjugants harbouring single plasmids.**

Track No.			Gel a (plasmid profile)	Gel b (plasmid digest)	Gel c (plasmid digest)
1	Standard		85 Md	λ PstI	λ PstI
2	56AA1a*	TCT-1	CL	PstI	SmaI
3	56AA2b	TCT-1	CL	PstI	SmaI
4	56bAA1b	TCT-1	CL	PstI	SmaI
5	107AA1	TCT-1	CL	PstI	SmaI
6	107RAA2a	TCT-1	CL	PstI	SmaI
7	Standard		62 Md	λ PstI	λ SmaI
8	Standard		46 Md	λ EcoRI	λ HindIII
9	138RAA3	TCT-1	CL	EcoRI	HindIII
10	141AA1	TCT-1	CL	EcoRI	HindIII
11	141RAA1	TCT-1	CL	EcoRI	HindIII
12	Standard		36 Md	λ PstI	λ PstI

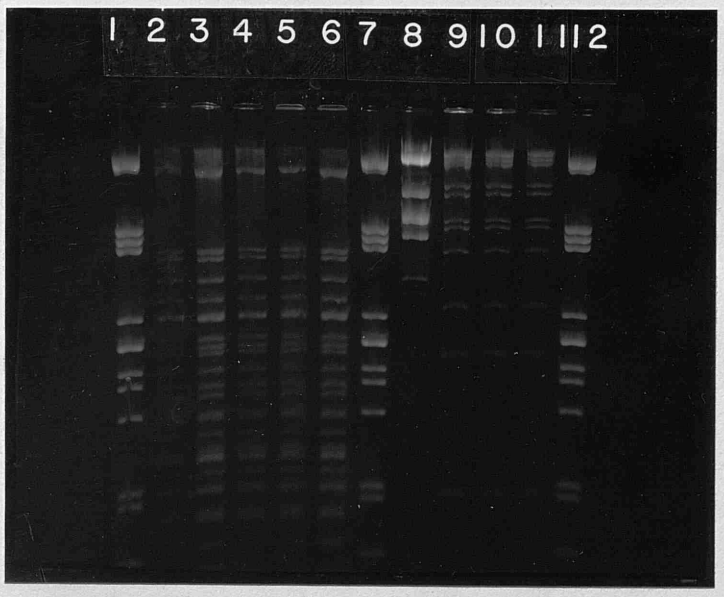
Md = megadaltons;
CL = crude lysate;
TCT = transconjugant;
* code as Table 2, page 83.

FIGURE XVIII

a



b



c

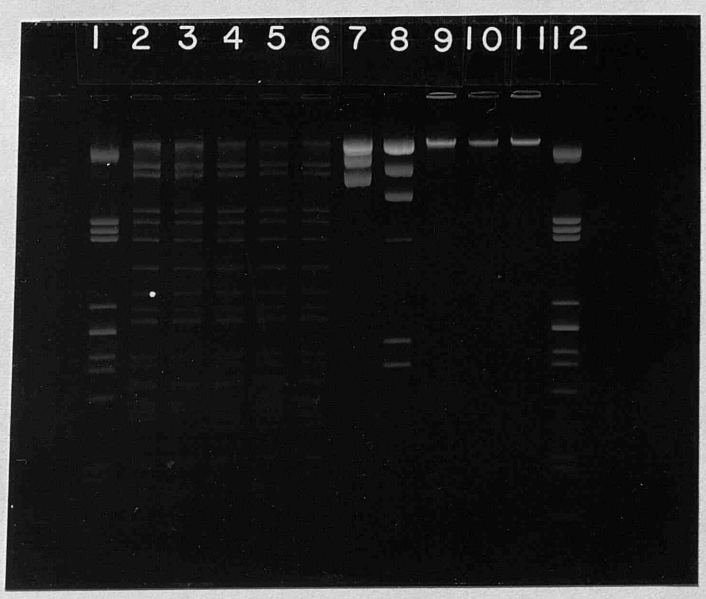


TABLE 27: Indication of relatedness of plasmids in ampicillin-resistant transconjugants based on digestion with PstI and SmaI with 56AA1a as a reference plasmid in E.coli K12.

Transconjugant		Enzyme PstI		Enzyme SmaI	
		No. of Fragments	No. in Common with 56AA1a*	No. of Fragments	No. in Common with 56AA1a
56AA1a	TCT-1	18	18	16	16
56AA2b	TCT-1	19	18	19	16
56bAA1b	TCT-1	19	18	16	16
107AA1	TCT-1	18	17	19	16
107RAA2a	TCT-1	21	18	19	16
80RAA2	TCT-1	14	12	9	4
80RAA4a	TCT-1	16	9	16	8
94RAA4	TCT-1	8	3	8	2
109RAA3	TCT-1	10	5	8	4
109RAA3a	TCT-1	22	13	19	9
57AA1	TCT-1	18	13	14	11
106AA1	TCT-1	6	5	6	4
106AA1	TCT-2	15	8	16	9
106AA1a	TCT-1	6	3	18	3
106RAA1	TCT-1	9	3	19	6
139AA2a	TCT-1	20	7	10	5
36AB2	TCT-1	15	7	19	8
79RAA1	TCT-1	14	5	19	11
141JC1	TCT-1	7	3	6	4
143RJC1	TCT-1	6	2	5	2
139aAA1	TCT-2	2	0	16	4
139aAA1	TCT-3	9	3	30	11
139RAA1	TCT-1	36	10	28	11

TCT = transconjugant: * code as Table 2, page 83.

common with the reference plasmid. However, only 4 transconjugants, each of which possessed a single plasmid (80RAA4a TCT-1, 109RAA3a TCT-1, 57AA1 TCT-1 and 139RAA1 TCT-1), had 50 per cent or more fragments in common with 56AA1a when digested with both enzymes.

Analysis of the fingerprints from transconjugants with common resistance determinants other than Ap, indicated that with the exception of a small number of individual cases few common fragments were evident.

Two Ent.cloacae strains were isolated, one from a wound swab (141JC1) and one from a rectal swab (143RJC1) from a different patient which transferred the same resistance determinants - ApSmSuTp. When digested with enzymes PstI and SmaI no difference was found in the plasmid fingerprints from either the clinical isolates or the resulting transconjugants.

Similar analysis of the Ap-resistant transconjugants digested with enzymes EcoRI and HindIII showed groups of plasmids which had similar fingerprints within the three major transferable resistance patterns (Ap; ApTc; ApSmSu).

Three transconjugants which transferred only Ap-resistance and two plasmids, one from a wound swab (138RAA3) and two which had previously been shown to be the same isolate from a patient's wound and rectal swab (141AA1 and 141RAA1),

had indistinguishable fingerprints (Fig. XIX). Two conjugative ApTc R-plasmids from biochemically distinct E.coli isolates from the same wound (75AB1 and 75AA2a) had similar fingerprints. A third fingerprint pattern was shown to be common to two isolates (32AA2 and 35AA1) which transferred ApSmSu resistance and a single plasmid.

Further comparisons of all Ap-resistant transconjugants digested with EcoRI and HindIII did not show any transconjugants with 50 per cent or more fragments in common. This may have been due to the number of plasmids in the transconjugants. For example, 9 of the Ap-resistant transconjugants digested with EcoRI and HindIII harboured a single plasmid, whereas 20 possessed two or more plasmids. In contrast 19 of the 23 Ap-resistant transconjugants digested with PstI and SmaI possessed only one plasmid with the remaining 4 harbouring two plasmids.

Three Ap-resistant transconjugants were digested with enzymes Bspl286 and AvaII; two of these from wound and rectal swabs in the same patient (144AA2 and 144RAA2) had indistinguishable fingerprints. Fingerprints of the third transconjugant were entirely different.

An isolate of Kleb.pneumoniae from a rectal swab and Kleb.oxytoca from a different patient's wound swab, were both found to transfer TcTp resistance determinants. PstI/SmaI fingerprints of both clinical isolates, each of which harboured two plasmids, were substantially different but contained 6 common fragments. However, resistant transconjugants from both isolates had identical fingerprints with Bspl286 and AvaII and each possessed a single plasmid of the same molecular weight, suggesting that both isolates harboured an identical R-plasmid. A third transconjugant was found which had an identical fingerprint to the above Klebsiella plasmid. This plasmid originated in an E.coli isolate (101RAA1) from the same rectal swab as the Kleb.pneumoniae. In addition to the transfer of TcTp resistance, two further transconjugants were also isolated: one specified TcSmSu resistance, the other carried TcSmSuTp resistance, and all three had a plasmid of approximately 36 Md. Fingerprint analysis (PstI and SmaI) of the clinical E.coli plasmid DNA and plasmid DNA from the TcSmSu and TcSmSuTp transconjugants demonstrated that both transconjugants had fragments in common but that each also possessed additional fragments. Thus it would appear that although all three transconjugants appear to possess the same

molecular weight plasmid, there are differences in the resistance determinants carried by the transconjugants and in the plasmid fingerprints.

A further 9 clinical isolates (106AA1, 90RAA3, 105RAA2a, 105RAA7a, 88RAA1b, 107RAA1, 109RAA2b, 139aAA1 and 139RAA1) were found which transferred more than one pattern of resistance and/or plasmids. In most instances it was not possible to define which plasmids specified the different resistance determinants transferred. However, an attempt was made to determine which resistance determinants were carried on which plasmids from the two of these, i.e. 90RAA3 and 107RAA1.

Clinical isolate 90RAA3 was resistant to ApSmSu and harboured 6 plasmids. Three transconjugants were isolated which differed with respect to resistance determinants and plasmid profiles as shown in Table 28 and Figure XIX. The first transconjugant (TCT-1) harboured only one 80Md plasmid and determined resistance to ApSu and partial Sm (pSm) (disc sensitivity testing demonstrated a significant reduction in normal zone size for E.coli K12, not full resistance). TCT-2 was fully resistant to ApSmSu and harboured three small plasmids, each of <10 Md, in addition to the 80 Md plasmid. TCT-3 was resistant only to Ap and harboured the 80 Md plasmid and two of the three plasmids of <10 Md harboured by TCT-2.

TABLE 28: **Resistance determinants and plasmid profiles of clinical isolates 90RAA3 and 107RAA1 and their transconjugants including the number of fingerprint fragments generated from each strain after digestion separately with EcoRI and HindIII.**

Strain	Resistance Determinants	Plasmid Molecular Weight (Md)		No. of Fingerprint Fragments	
				EcoRI	HindIII
90RAA3* CI	ApSmSu	80,75,	<10(4)	26	16
TCT-1	Ap pSmSu	80		18	8
TCT-2	ApSmSu	80	<10(3)	17	11
TCT-3	Ap	80	<10(2)	16	9
107RAA1 CI	ApKmSmSu	75,50	<10(1)	14	17
TCT-1	Ap	50		5	1
TCT-2	ApKmSm	75	<10(1)	9	10
TCT-3	Ap pKm pSm	75	<10(1)	9	10

CI = clinical isolate, TCT = transconjugant, Md = megadalton

Ap = ampicillin, Sm = streptomycin, pSm = partial streptomycin, Km = kanamycin, pKm = partial kanamycin, Su sulphamethoxazole.

* code as Table 2, page 83.

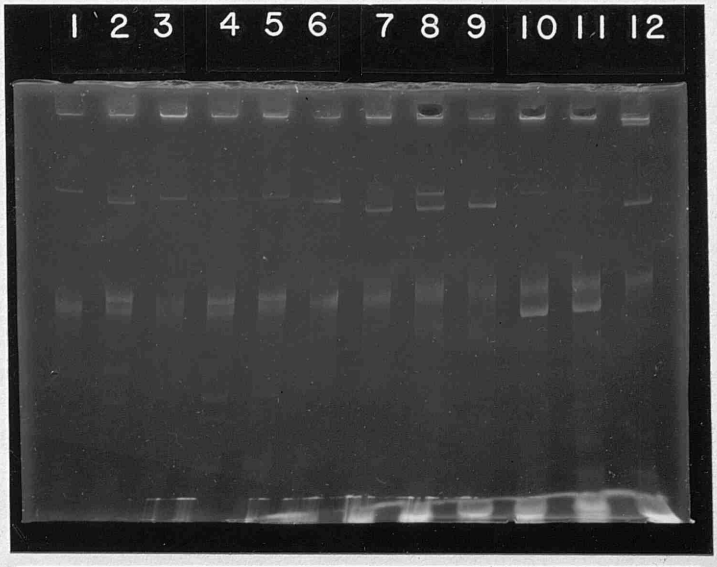
FIGURE XIX: **Plasmid profile and plasmid fingerprint gels of clinical isolates 90RAA3 and 107RAA1 and their transconjugants.**

Track No.			Gel a (plasmid profile)	Gel b (plasmid digest)	Gel c (plasmid digest)
1	Standard		85 Md	λ PstI	λ PstI
2	90RAA3*	CI	CL	EcoRI	HindIII
3	90RAA3	TCT-1	CL	EcoRI	HindIII
4	90RAA3	TCT-2	CL	EcoRI	HindIII
5	90RAA3	TCT-3	CL	EcoRI	HindIII
6	Standard		62 Md	λ EcoRI	λ HindIII
7	Standard		46 Md	λ EcoRI	λ HindIII
8	107RAA1	CI	CL	EcoRI	HindIII
9	107RAA1	TCT-1	CL	EcoRI	HindIII
10	107RAA1	TCT-2	CL	EcoRI	HindIII
11	107RAA1	TCT-3	CL	EcoRI	HindIII
12	Standard		36 Md	λ PstI	λ PstI

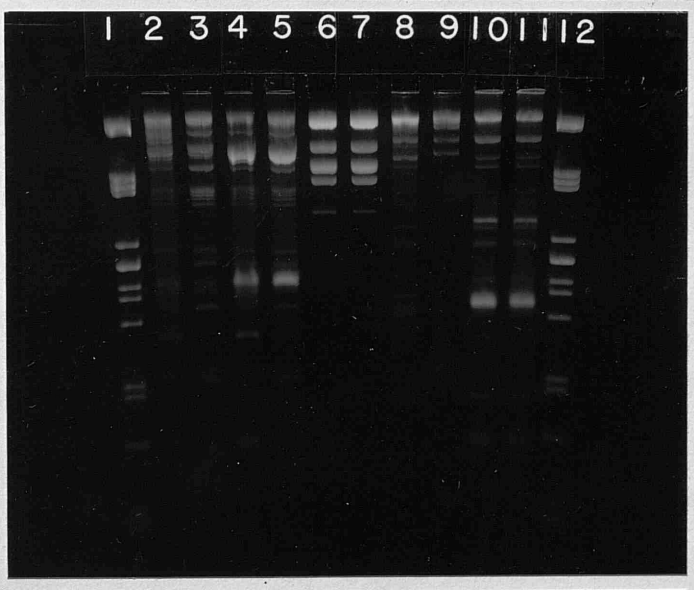
Md = megadaltons;
TCT = transconjugant;
Md = megadalton;
CL = crude lysate;
* code as Table 2, page 83.

FIGURE XIX

a



b



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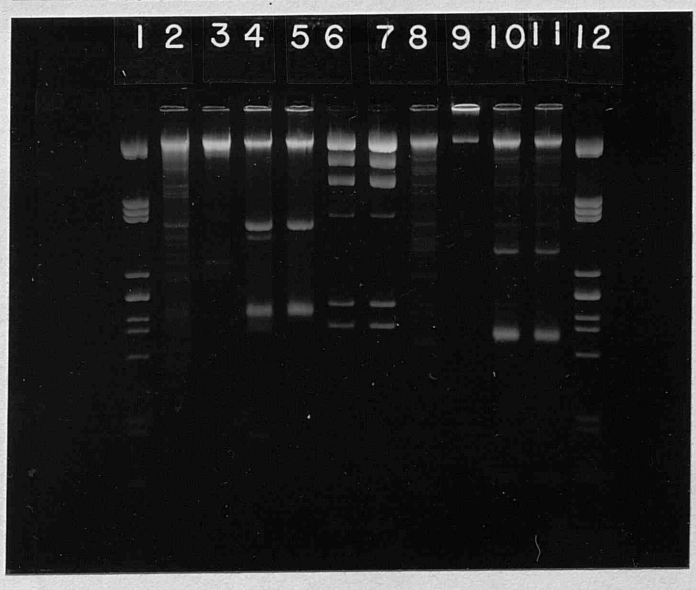

Clinical isolate 107RAA1 harboured three plasmids and was resistant to ApKmSmSu (km = kanamycin). TCT-1 was resistant to Ap and harboured a 50 Md plasmid. TCT-2 and TCT-3 harboured the same plasmids, one of 75 Md and one of < 10 Md, but differed with respect to resistance determinants with TCT-2 being resistant to ApKmSm whereas TCT-3, although resistant to Ap, demonstrated partial resistance to both Km (pKm) and Sm. No difference was seen in the fingerprints of these two transconjugants after restriction digestion with EcoRI and HindIII.

It was hoped that fingerprint analysis, in addition to determining whether there was overall similarity in those plasmids which had resistance determinants in common, would also show if there were common fingerprint fragments to suggest the presence of similar segments of DNA or transposons in the plasmids concerned.

Seventy-eight transconjugants were obtained from the 58 isolates which transferred resistance. Of these, 56 transconjugants were resistant to ampicillin. Forty-three (77%) possessed a single fingerprint fragment in common when digested with PstI. The mean molecular weight of this fragment was 3478 Kb (range 3311-3694). However, 11 of the 22 transconjugants which did not possess ampicillin resistance

also had a fragment within the same molecular weight range. Therefore, the presence of this fragment cannot be attributed to a common sequence responsible for ampicillin resistance.

Analysis of fingerprint fragments from transconjugants possessing other resistance determinants in common, gave similar results in that although a number of fragments appeared to be common to several transconjugants none could be attributed unequivocally to the presence of a specific resistance determinant. Furthermore, analysis of transconjugant fingerprints with different enzymes did not show common fragments despite the presence of common resistance determinants.

Conjugative plasmids not associated with resistance:

Fifty-five clinical isolates were found which possessed plasmids with conjugative ability but which did not carry resistance determinants. Ten were Proteus spp. from which no donor plasmid DNA was seen in the transconjugants. Fingerprints of plasmid DNA from the clinical isolates gave consistently poor results with the purification method described and no AvaII digestion results were obtained for a

number of isolates. Despite this, restriction digests with Bspl286 suggest that although the plasmids were not identical they were closely related.

After attempting restriction analysis with PstI of all clinical isolates and transconjugants with conjugative plasmids not associated with resistance, a further 17 transconjugants were detected which did not appear to contain conjugative plasmids. When this was discovered repeat cell lysis and agarose gel electrophoresis for detection of plasmid DNA was performed with each donor isolate and corresponding transconjugant. Results from this showed that although plasmid DNA was still present in the clinical isolates no DNA corresponding to the donor plasmid was seen in the transconjugants. This suggests that although the plasmids were stable in the clinical isolates, they were either unstable in the E.coli K12 recipients or although they did not transfer themselves, provided requirements enabling the Tra^- R^+ plasmids to transfer. Comparison of fingerprints from the clinical isolates showed that there were similarities between two Ent.cloacae strains (102aJC2 and 102RJC2) isolated from one patient's wound and rectal swabs. A different fingerprint pattern was seen which was common to two Kleb.pneumoniae isolates (143cCD2 and 143RCD2) from a second patient's wound

and rectal swabs. An Ent.agglomerans strain harbouring an identical plasmid (143cJE1) was also isolated from a wound swab from this patient. Apart from these isolates no obvious similarities were found among the plasmids from unrelated clinical isolates which possessed conjugative plasmids not associated with resistance but which were unstable in E.coli K12 recipients. Ten of the 17 isolates which possessed conjugative plasmids unstable in E.coli K12, were Klebsiella spp., Enterobacter spp. or Citrobacter spp. Only one Kleb.pneumoniae and one Cit.freundii isolate possessed conjugative plasmids not associated with resistance which were stable in E.coli K12. In contrast 22 E.coli isolates possessed conjugative plasmids which were stable in E.coli K12 and 4 were stable in Ent.aerogenes. Six E.coli plasmids were unstable in E.coli K12 and a further one was unstable in Ent.aerogenes

Only 28 of the original 55 transconjugants resulting from the transfer of Tra^+ R^- plasmids were still found to harbour the conjugative plasmid when restriction endonuclease digestion was performed. Of these, after the exclusion of common isolates from wound and rectal swabs from any given patient, only three (96RAA1, 97RAA1b and 98RAA1a) appeared to be closely related.

Fifty-five isolates harboured conjugative plasmids not associated with resistance and mobilised the plasmid pHH1310a, which contributed fragments to the transconjugant fingerprints. Forty-nine of these 55 Tra^+ R^- plasmids also mobilised R300B. Six isolates mobilised plasmid pHH1310a but not plasmid R300B. The fingerprints of the plasmids from the above 6 clinical isolates were different.

Fingerprints from transconjugants which contained conjugative R-plasmids were compared to those that possessed conjugative plasmids not associated with resistance in an attempt to find similarities in the plasmids carried. In many instances both groups of plasmids shared a number of fragments which indicates a degree of relatedness. However, no examples were found where 50 per cent or more of the fragments from a Tra^+ R^- plasmid were also found in a Tra^+ R^+ plasmid.

Non-conjugative plasmids:

The assessment of similarities between non-conjugative plasmids and those present in clinical isolates which were neither transferred nor mobilised into recipient strains during conjugation was difficult and generally unsuccessful. This may be due in part to the method used

which involved subtraction of the transconjugant fingerprint from clinical isolates which possessed conjugative plasmids, the remaining fingerprint fragments were compared to fingerprints from clinical isolates which possessed only non-conjugative plasmids.

After exclusion of plasmids from wound and rectal isolates from the same patient, only two non-conjugative plasmids were found which had similar fingerprints. These, as previously described, were from Cit.freundii (93RA1) and Ent.cloacae (93JC1) isolates from the same wound. Both isolates possessed plasmids which were mobilised by RP4. Bsp 1286 digests yielded two fragments both of which were common to each plasmid: AvaII digestion similarly yielded two common fragments and PstI 4 common fragments. Thus, no differences were detected in the two plasmids. A third isolate of Ent.cloacae from this patient's rectal swab was found which possessed a different, but related, non-conjugative plasmid which was also mobilised by RP4. This strain was isolated from a rectal swab from the same patient (93RJC1). PstI digestion showed that both the plasmids from the wound isolates produced 4 fragments common to the plasmid from the rectal isolate which produced 5 additional fragments. These findings

are consistent with the molecular weights of the plasmids; <10 Md (wound isolates) and approximately 36 Md (rectal isolate).

Plasmids in E.coli and other enteric genera:

Fingerprint analysis of the plasmids from the 118 E.coli which possessed plasmids, demonstrated that in only a few specific cases were identical plasmids recognised in two or more different clinical isolates. Comparisons of the PstI fingerprints, irrespective of the number of plasmids carried by the individual isolates, suggested that there was considerable variation in the fingerprints of plasmids from E.coli and the evidence from the use of other enzymes supports this conclusion (Table 24). Not only was there considerable variation between the fingerprints of conjugative R-plasmids, conjugative plasmids not associated with resistance and non-conjugative plasmids but also within each plasmid group, considerable variation was found.

Twenty-one Proteus spp. were isolated which harboured plasmids. Fourteen of these had no more than a single cleavage site for PstI. The remaining 7, although they produced a number of fragments with PstI, yielded only linearised or uncut DNA when digested with SmaI. Therefore,

Bspl286 and AvaII digestion was carried out with all of the Proteus spp. plasmids. AvaII digestion was not always successful and only Bspl286 results were compared further. There was a substantial number of fragments common to many of the plasmids from these clinical isolates. These results, together with PstI and SmaI results, indicate that there was a considerable degree of relatedness among some of the plasmids from Proteus spp.

Nineteen plasmid-containing Enterobacter spp. were isolated in addition to 8 Klebsiella spp., two Citrobacter spp., and two Hafnia alvei. With the exception of epidemiologically related isolates, no general similarities were found in plasmids within or between these different species of enteric genera. These results indicate a high degree of diversity in the plasmids found from the above enteric genera as was found with E.coli plasmids.

The comparison of fingerprints between E.coli and OEG showed that there were very few common plasmids. The only example where a plasmid was found to be common to an E.coli isolate and an OEG isolate was in one patient, as previously described, where the same TcTp R-plasmid was harboured by an

E.coli and an Ent.cloacae from the patient's rectal swab. This plasmid was also found in an Ent.agglomerans isolate from the wound of a different patient.

DISCUSSION

Patients and wounds studied:

The decision to study wounds from patients in the PVD Unit and geriatric patients was based on the fact that non-invasive sampling techniques could be used, although the diversity of coliforms isolated was likely to be high and multiple species common. This approach avoided the complications inherent in the study of faecal specimens. However, to understand better the role of coliforms in these wounds, an investigation of all species of potential pathogens present was first undertaken. In conjunction with this an investigation of the influence of antimicrobial therapy on the wound flora and a suggestion as to whether or not the organisms isolated were acquired from an environmental source or were part of the patients' own flora, was necessary.

No attempt was made to classify the wounds studied into those that were actively infected and wounds that were colonised with potential pathogens because many factors contribute to the distinction between colonisation and infection. Factors such as underlying pathology, type and site of wound and number and type of organisms isolated influence the host parasite relationship. Using the

information available about the patient's clinical history, treatment in the unit, description of the wound and organisms isolated, an attempt was however, made to indicate whether treatment had influenced wound healing and the elimination of potential pathogens.

Correlation of organisms isolated with wound duration and grade:

The 48 vascular ulcers studied were sampled for an average of 1.77 weeks; the mean wound grade was 1.88. Potential pathogens were isolated from 79 per cent of vascular ulcers. Forty-eight per cent of vascular ulcers were colonised/infected with coliforms, 29 per cent with Pseudomonas spp. and 35 per cent with Staph.aureus. Acinetobacter spp., Strep.faecalis, beta-haemolytic streptococci and anaerobes were each isolated from less than 10 per cent of all vascular ulcers studied. The short duration of these wounds after admission to the PVD Unit and the low wound grade suggests that the wounds were relatively easy to treat. In many instances this treatment was in the form of improving circulation to the wound by repairing major blood vessels to the limbs and by good wound management. Coliforms and Pseudomonas spp. were isolated from a large proportion of specimens suggesting that the wounds were

colonised/infected with both environmental organisms (Pseudomonas spp.) and organisms from the patient's own gut flora (coliforms). Staph.aureus was isolated from 35 per cent of vascular ulcers and is usually endogenous from the patient's skin, although environmental contamination may play a role.

The longer duration (2.33 weeks) and higher wound grade (2.26) of amputation sites in comparison to vascular ulcers may be due in part to the severity of trauma following amputation. This is supported by the observation that potential pathogens were isolated from fewer specimens (59 percent) in comparison to vascular ulcers. Although amputations were performed through healthy tissue the blood supply at the site of amputation was often reduced and predisposed to colonisation. The proximity to an amputation site of a wound, for example a vascular ulcer, would increase the probability of contamination during surgery. This may explain the isolation of potential pathogens from more than half the specimens. Coliforms were isolated from 44 per cent of amputation sites, a number similar to that from vascular ulcers. However, the number of specimens yielding Pseudomonas spp. (18%) was lower than from vascular ulcers and the proportion of specimens yielding Strep.faecalis was higher. This probably reflects a lower incidence of contamination with

environmental organisms and a higher incidence of contamination by faecal flora compared to vascular ulcers. The numbers of specimens from which Staph.aureus were isolated was also lower possibly reflecting a lower incidence of contamination from endogenous sources compared to vascular ulcers.

Surgical vascular repair sites had a mean healing time (1.70 weeks) similar to that of vascular ulcers. The mean grade (2.24) however, was high and similar to that of amputation sites. Surgical trauma probably played an important part in the clinical appearance of the wounds although they were generally quick to heal and less than half yielded potential pathogens. As these wounds were the result of surgery to repair blood vessels one would expect a low incidence of environmental contamination and a high level of endogenous organisms in the case of abdominal wounds, e.g. aortic bifurcation graft wounds, where the probability of contamination by gastro-intestinal commensals was higher. This appears to be the case as only 6 per cent of surgical vascular repair sites yielded Pseudomonas spp., whereas 30 per cent possessed coliforms and 18 per cent contained Strep.faecalis.

The 26 decubitus ulcers studied, as expected, differed in many respects from other groups of wounds studied. The longer duration (3.19 weeks), higher wound grade (2.77) and larger proportion of wounds from which potential pathogens were isolated (96%) compared to wounds in PVD patients, reflects both the type of wound studied and the type of patient. Patients with decubitus ulcers were geriatric most of whom were in a very poor general state of health and unable to properly care for themselves. Most of these patients were admitted to hospital after the decubitus ulcers had developed and despite general care and attention to hygiene by the nursing staff these wounds were difficult to heal. The types of organisms isolated from these wounds were similar to those isolated from patients in the PVD Unit. The main differences lay in the numbers and multiplicity of species isolated from individual wounds.

At least one species of coliform was isolated from each decubitus ulcer on at least one occasion. Sixty five per cent of decubitus ulcers also contained Pseudomonas spp. Although the numbers of specimens from which Staph.aureus were isolated (19%) was lower than in patients from the PVD Unit, the numbers of beta-haemolytic streptococci (23%) and anaerobes (31%) was much higher.

Wounds in patients in the PVD Unit that were sampled on only one occasion had either resolved completely or markedly improved by the week following specimen collection. These wounds would therefore not have been sampled again. Conversely, wounds that were sampled on two or more occasions had failed to resolve completely and although an improvement was seen in the clinical appearance of many of them, further specimens were obtained. As expected, the results indicated that the mean wound grade of wounds which were sampled on only one occasion was lower than the mean wound grade of the initial wound from wounds that were sampled on more than one occasion.

The mean duration of decubitus ulcers tended to be underestimated as few of the wounds were actually seen to resolve except in the small number of cases that it was possible to study over an 8-10 week period. Many of the patients seen over short time periods either died or were transferred, thus leading to underestimation of length of "healing times" for these wounds. Therefore, in many of the cases, where wounds were only sampled on one occasion, the patient died without resolution of the wound. This explains why the mean wound grade from geriatric patients was higher from wounds sampled on only one occasion compared to the initial mean grade of wounds sampled on multiple occasions.

Decubitus ulcers tended to be colonised/infected with more species of potential pathogen per wound compared to other wound groups. Due to the poor blood supply in these patients and problems such as incontinance which facilitated contamination with faecal flora, the high rate of isolation of potential pathogens was as expected. Beta-haemolytic streptococci and anaerobes were isolated from a much higher percentage of these wounds than from patients in the PVD Unit. Again, the isolation of anaerobes probably reflects faecal contamination of the wounds and may partly explain the higher wound grade as large numbers of potential pathogens present in many decubitus ulcers could contribute to local tissue damage and necrosis, further accentuating the problems of wound hygiene and improving circulation.

Although the grade of wound provided an indication of the presence of potential pathogens in the wound grade III wounds did not always contain potential pathogens. Wound grade did however, indicate that the higher the grade the greater the probability that potential pathogens would be isolated, although the type of wound and trauma associated with the wound also played an important part. The rate of healing

of the wound also correlated with the presence of potential pathogens but because a wound was slow to heal did not mean that potential pathogens were necessarily present.

The results suggested that the isolation of Staph.aureus, coliforms or any potential pathogen, correlated with a significant increase in the healing time of wounds containing these bacteria. The presence of Pseudomonas spp. did not appear to have a similar effect. These results must be interpreted with caution as only a small proportion of wounds possessed a single potential pathogenic species on all occasions sampled; therefore, the calculation of healing time in the presence of Staph.aureus, coliforms and Pseudomonas spp. did not take into account the isolation of other potential pathogens from the same wound either before, at the same time, or after the isolation of the potential pathogen in question. Similarly, calculation of healing time in the presence of any potential pathogen did not take into account isolation of more than one species or changes in the species isolated from subsequent specimens. In addition, the number of specimens from which the potential pathogen in question was isolated, was not considered. Despite the limitations of the method used

for the calculation of healing time, these results provide the basis for further investigation of the role of individual species in slowing the process of wound healing.

Thus, although there appears to be an association between the isolation of some potential pathogens and a reduced rate of wound healing, the presence of potential pathogens may not be a direct cause of the increased healing time. It may be that the physiology of wounds with an intrinsically poor healing potential are more prone to colonisation than wounds with a high healing potential.

Milton as a topical antimicrobial agent:

Milton (sodium hypochlorite, NaOCl) is a solution commonly used both for the disinfection of inanimate objects and as an antiseptic agent after surgery. The antimicrobial activity of Milton is due to its disruptive action on protein which results in protein hydrolysis and the conversion of hypochlorite to chloride. Milton is therefore active against both Gram-positive and Gram-negative bacteria (Smith et al, 1974; Bloomfield and Miles, 1979). Due to its action on all protein, Milton has also been used as an agent to clear necrotic debris from wounds (Bunyan, 1967). This combined antimicrobial and desloughing action of Milton may therefore

have influenced the numbers of potential pathogens isolated from the wounds to which it was applied. However, there was no significant reduction in the numbers of specimens from which either Pseudomonas spp. or Staph.aureus were isolated when Milton was applied topically to the wound. There was a significant increase in the number of wounds from which coliforms were isolated when Milton was applied topically. This increase could be due to a number of factors, the two main reasons being either contamination of stock Milton solutions with coliforms or bias in determining which wounds were treated topically with Milton.

The possibility of contamination of Milton stock solutions was not investigated as no particular coliform(s) was common to the wounds studied and therefore contamination of the wounds from a central source was unlikely.

It appears more likely therefore, that this apparent increase in the isolation of coliforms from wounds treated with Milton was due to the selection of wounds of poor clinical appearance for treatment. Furthermore, these wounds had a greater chance of being colonised/infected with coliforms than wounds that, according to appearance, did not require topical

therapy. Maintenance of a wet wound environment by treating topically with Milton may also have increased the numbers of coliforms isolated.

Systemic therapy:

A wide range of systemic antimicrobial agents were prescribed during the study. Many were administered for problems unrelated to colonisation/infection of the wounds studied e.g. for respiratory or urinary tract infections. Antimicrobial agents prescribed to large numbers of patients and which might be expected to have a direct effect on the wound flora included cephalosporins, flucloxacillin, penicillin V and metronidazole.

First, second and third generation cephalosporins were prescribed in varying numbers of patients during the study. Cephalosporins are generally active against Gram-negative bacteria; some have activity against Staph.aureus and a small number have limited activity against Pseudomonas spp. (Garrod et al, 1981). There was a small but significant reduction in the numbers of specimens from which coliforms were isolated when cephalosporins were prescribed. There was also a significant reduction in the numbers of specimens from which Pseudomonas spp. were isolated despite the fact that few

patients were treated with cephalosporins which had activity against Pseudomonas spp. Cephalosporins had no effect on the incidence of Staph.aureus isolation in the wounds studied.

Flucloxacillin is active against Gram-positive bacteria and is commonly prescribed as treatment for Staph.aureus infections. The results from this study however, indicated that there was no reduction in the numbers of specimens from which Staph.aureus was isolated when flucloxacillin was administered systemically. Significant reductions were however found in the numbers of specimens from which coliforms and Pseudomonas spp. were isolated when flucloxacillin was prescribed. This suggests that there are factors influencing the isolation of potential pathogens other than systemic therapy. This suggestion is supported by the observations on other individual agents.

Penicillin V is commonly prescribed for infections with Streptococcus spp., Clostridium spp. and sensitive Staph.aureus. There was a significant reduction in the numbers of specimens from which Staph.aureus was isolated when penicillin V was prescribed, despite the fact that most of the Staph.aureus isolated were resistant to penicillin. A

significant reduction was also found in the incidence of isolation of coliforms but not Pseudomonas spp. when penicillin V was prescribed.

Metronidazole is only active against anaerobic bacteria. However, there appeared to be a significant reduction in the numbers of specimens which yielded Pseudomonas spp. but not coliforms or Staph.aureus, when metronidazole therapy was administered.

Combined topical and systemic therapy:

The statistical analysis of results from a consideration of all systemic therapy and combined systemic and topical therapy suggests the following; the numbers of coliforms isolated was reduced in the presence of systemic therapy alone, but not when topical therapy was also administered. Pseudomonas spp. were isolated from fewer specimens when systemic therapy was prescribed, irrespective of whether or not topical therapy was also administered. Neither systemic nor topical therapy had an effect on the isolation of Staph.aureus from these wounds.

Many of the patients studied were prescribed systemic antimicrobial agents for infections other than at the wound site studied or as prophylaxis for surgery. In many cases therefore, the presence of antimicrobial agents was not directed against the organisms present in the wound. In contrast, Milton would only have been applied topically if the wound appeared to be colonised/infected with bacteria. Correlation of these two methods of treatment suggests the following conclusions; coliforms were isolated from a large proportion of all types of wounds studied. Their presence was not affected by systemic therapy (considered collectively) and they were present in significantly greater numbers of wounds treated with Milton. This suggests that wounds which appeared clinically to require topical treatment were predisposed to colonisation with coliforms, i.e. the wound was wet, necrotic etc., but this topical treatment did not appear to reduce the numbers of coliforms isolated. Similarly treatment with systemic antimicrobial agents, for whatever reason, did not eradicate coliforms from wounds.

In contrast, the numbers of Pseudomonas spp. were not affected by Milton topical therapy, but they did appear to be reduced by systemic therapy. Many of the patients studied

were being treated systemically with antimicrobial agents prophylactically during surgery to repair blood vessels to the limbs. It may have been the results of surgery, i.e. increased blood supply coupled with restoration of local host defences and healing of the wound, rather than systemic therapy, that had an indirect effect in lowering the numbers of Pseudomonas spp. isolated from a number of the wounds studied.

Staph.aureus was not affected either by the application of Milton topical therapy or the collective use of systemic antmicrobials. These organisms were isolated from similar numbers of all types of wounds studied thus suggesting little direct effect of any type of therapy on Staph.aureus in the wounds studied.

The lack of correlation between treatment with antimicrobial agents prescribed in the patients and isolation of potential pathogens in wounds was unexpected; in particular, the demonstration of positive correlation between events for which there appears to be no rational explanation. This illustrates some of the problems arising from performing statistical analysis on biological results. For the results of statistical analysis to be valid, random unbiased samples

are required. The results from Milton topical therapy and systemic therapy suggests that random samples were not obtained. It is also apparent that factors other than antimicrobial therapy introduced bias which influenced the isolation of potential pathogens from the wounds studied.

Investigation of individual wounds:

One of the features of some of the vascular ulcers studied was that in cases where amputation was performed after the ulcer was first sampled, the same species of potential pathogens were later isolated from the amputation site. The decision to amputate was based on the previous failure of the site to respond to alternative measures and/or the improbability of affecting resolution by other means. Despite the isolation of potential pathogens from the amputation sites the wounds healed, which demonstrated that in these cases the presence of potential pathogens was secondary to underlying pathology.

In general, if a patient possessed two or more wounds of a similar type, then the probability of isolating the same species of potential pathogen from these multiple sites was high. In cases where a patient had two or more wounds of

different types, there was a tendency for overlap of different species colonising/infecting different sites but with additional organisms present in some wounds.

Of 88 wounds sampled on two or more occasions, only 12 yielded the same species of potential pathogens on all occasions sampled. This demonstrates that within a short time period the bacterial flora of a wound can change substantially, although the change usually involves acquisition or loss of one species of potential pathogen rather than complete replacement of the original wound flora. Sampling variation may account for a proportion of the differences in results obtained from sequential specimens from the same wound and different wounds from the same patient. However, it is unlikely that these differences affected the overall patterns of results found.

The overall picture suggests that neither systemic nor Milton topical therapy have greatly influenced the presence of potential pathogens in the wounds studied. However, in a small number of patients changes in the wound flora appeared to be directly due to antimicrobial therapy.

As previously described the application of gentamicin both topically and systemically, resulted in the eradication of both Pseudomonas spp. and Staph.aureus from the wound of patient 89. Although therapy was successful in this instance,

the use of gentamicin topically is not normally recommended due to the relative ease of development of resistance to aminoglycosides (Mawer and Greenwood, 1977).

The emergence of resistance during therapy:

The isolation of E.coli with low level resistance to various aminoglycosides from patient No.100 was probably the result of gentamicin therapy. This however, cannot be proven in this particular patient as only one specimen was obtained after the start of therapy and no E.coli isolates were available from the patient prior to the commencement of gentamicin therapy.

The development of resistance to naladixic acid in E.coli and Prot.mirabilis, isolated following systemic therapy with naladixic acid in patient No.139, can be positively attributed to therapy. This demonstrates the danger of using naladixic acid as a single antimicrobial agent since it is known to select resistant mutants in a number of bacterial species (Atlas et al, 1969; Cederberg et al, 1974)).

Open wounds as a model for the study of plasmid behaviour:

Although innately a simpler system for study than the gut, these results demonstrate that different types of wound are both complex and variable and cannot be represented by a single model system. Differences in the host physiology between, for example, decubitus ulcers and amputation sites, may considerably influence the behaviour as well as numbers and diversity of colonising/infecting organisms.

It is possible that some plasmids may influence the ability of coliforms to colonise these sites. Although no such correlations were discerned in this study, they cannot be excluded since they might well have been concealed in the variation described above. However, future studies could be designed to examine these possibilities.

Coliforms isolated:

As previously discussed, the colonisation/infection of wounds with coliforms could be due to contamination by the patients own faecal flora. Rectal swabs were not obtained from all patients from whom wound swabs were collected. However, in those patients from whom rectal swabs were collected, not all wounds yielded coliforms. The following features were nevertheless apparent, 103 *Proteus* spp. were

isolated from the 168 specimens from wounds which contained coliforms. Only 76 E.coli were isolated from the same 168 specimens. The ratio of E.coli to Proteus spp. isolation was therefore 1:1.36. 174 E.coli isolates were found in the 90 coliform-containing rectal swabs compared to 35 Proteus spp. giving a ratio of 1:0.2. If all coliforms present in the wounds studied were faecal contaminants and if each coliform isolated from a rectal swab had the same probability of colonising/infecting a wound, then one would expect to find a similar ratio of E.coli:Proteus spp. in rectal isolates as that apparent in wounds. The numbers of Proteus spp. found in wounds were much larger than expected. Two possibilities may account for this. Firstly, although E.coli were more commonly isolated from faecal material, Proteus spp. were better able to colonise/infect surface wounds than E.coli. Alternatively, contamination from sources other than the patient's faecal flora may have contributed to the colonisation/infection of wounds with Proteus spp.

The ratios of E.coli:Klebsiella spp., E.coli:Enterobacter spp. and E.coli:Citrobacter spp. were 1:0.18, 1:0.11 and 1:0.07 respectively from rectal swabs. The corresponding values from wound swabs were 1:0.33, 1:0.43 and 1:0.25, all of which were higher than in rectal material. As

with Proteus spp., these results suggest that the isolation of Klebsiella spp., Enterobacter spp. and Citrobacter spp. from wounds was not due solely to the same probability of colonisation/infection from faecal material as E.coli. The remaining coliform species isolated from wound and/or rectal swabs were isolated only in small numbers and could not be assessed in this way.

The role of hands of both patients and staff is now well recognised as an important route for the transmission of a number of coliform species. The isolation of Klebsiella spp., Enterobacter spp. and Serratia spp. from the hands of staff, more often than the isolation of other coliforms, suggests that this may be an important source for contamination by these bacteria (Knittle, Eitzman and Baer, 1975). In addition, during outbreaks of infection with Kleb.aerogenes (Hable et al, 1972), Ent.cloacae (Mayhall et al, 1979) and Ser.marcescens (Maki et al, 1973; Schaberg et al, 1976) the epidemic strains were isolated from the hands of staff in the clinical units concerned. However, during an outbreak of infection with Prot.mirabilis, the epidemic strain was not isolated from the hands of any of the staff in the unit (Chow et al, 1979). These observations suggest that certain species of coliforms can be more easily transmitted by this route than others.

One of the questions this work was designed to answer was "Does plasmid transfer occur *in vivo* among the coliforms isolated from the sites studied?" The potential for plasmid transfer is dependent on a number of criteria. One that will be considered here was that a minimum of two coliforms was present in the site studied and a second criterion was that at least one of the coliforms present possessed a Tra^+ plasmid.

From the wounds studied, two or more coliforms were isolated from 75 of the 168 coliform-containing specimens and from 78 of the 91 rectal swabs. These results indicate that the first criterion for plasmid transfer *in vivo* was fulfilled by the 153 specimens which yielded multiple coliforms.

Also of importance in determining whether or not plasmids could transfer is the species of coliforms present in the site studied. It is apparent (Platt and Sommerville, 1981; Platt *et al*, 1984) that plasmid transfer occurs more readily between some species of coliforms than others. Therefore, the distribution of different species in any individual site will contribute to the probability of plasmid transfer *in vivo*.

Plasmid distribution - E.coli/OEG:

The results reported here confirm previous work on R-plasmids (Platt et al, 1986) and extend these investigations by including a detailed consideration of Tra^+ R^- plasmids. The most striking difference between E.coli and OEG was the large proportion of OEG which did not contain plasmids.

Not only were fewer E.coli isolates plasmid-free compared to OEG but the mean number of plasmids present in E.coli was higher and after the exclusion of plasmid-free isolates, the mean number of plasmids remained higher than in OEG.

The proportion of E.coli that were resistant was significantly higher than the resistant sub-population of OEG. Differences were also found in the ability of the plasmids in the two resistant sub-populations to transfer resistance determinants. Thirty-nine per cent of all E.coli possessed at least one Tra^+ R^+ plasmid whereas 24 per cent harboured Tra^+ R^- plasmids. In contrast, 4 per cent of all OEG contained Tra^+ R^+ plasmids and 15 per cent harboured Tra^+ R^- plasmids.

Sensitive E.coli/resistant E.coli:

Sensitive E.coli possessed a mean of 1.55 plasmids compared to 2.63 in resistant isolates. Thus, 100 sensitive E.coli isolates would harbour a total of 155 plasmids and 100 resistant E.coli isolates, 263 plasmids, a difference of 108 plasmids. A further difference in the plasmid population of the two E.coli groups was that 73 per cent of resistant E.coli contained at least one Tra^+ R^+ plasmid.

The stable acquisition of a single Tra^+ R^+ plasmid by 73 per cent of sensitive E.coli would have led to an expected increase of 73 plasmids per 100 isolates. The observed increase in resistant E.coli was 108 plasmids. This suggests that in many instances more than one plasmid had been acquired either by sequential transfer, co-transfer of more than one Tra^+ plasmid or by mobilisation of a Tra^- plasmid by a Tra^+ plasmid.

Forty-five per cent of sensitive E.coli contained Tra^+ R^- plasmids compared to only 7 per cent of resistant E.coli. This could be due to a number of factors, the most important being that if a Tra^+ R^+ plasmid was acquired by an isolate already possessing a Tra^+ R^- plasmid then its detection would be obscured by the Tra^+ R^+ plasmid using the methods described. Incompatibility may have led to the loss of Tra^+ R^- plasmids

if any were incompatible with incoming Tra^+ R^+ plasmids. Thirdly, acquisition of a resistance transposon by a Tra^+ R^- plasmid would have converted it into a Tra^+ R^+ plasmid; selection pressure in the form of antimicrobial therapy or incompatability would result in loss of the Tra^+ R^- plasmid.

Sensitive OEG/resistant OEG:

When the above argument was applied to OEG the same conclusions were reached with regard to the acquisition of multiple plasmids. Thus acquisition of single plasmids by many OEG isolates appears to be less common than the acquisition of two or more plasmids by a small number of coliform isolates. This may reflect the different recipient ability of individual isolates where some isolates can easily acquire plasmids but other isolates are very poor plasmid recipients (Platt et al, 1986).

Sensitive E.coli/sensitive OEG:

The major difference between these two coliform sub-populations was that a significantly higher proportion of sensitive OEG did not contain any plasmids compared to sensitive E.coli. After exclusion of plasmid-free isolates differences between the plasmid content of the two sensitive

coliform sub-populations were still apparent. However, these were not as marked as when comparing total or sensitive isolates.

The mean number of plasmids in sensitive E.coli was higher (mean 2.00) than in sensitive OEG (mean 1.31). The proportion of isolates containing Tra^- and Tra^+ R^- plasmids were very similar with no significant difference between sensitive E.coli and OEG. Moreover, as significantly more sensitive OEG isolates (76%) were plasmid-free compared to sensitive E.coli (14%), this suggests that a higher proportion of OEG were poor recipients and did not easily acquire plasmids compared to E.coli.

Resistant E.coli/resistant OEG:

The most striking differences between these groups lay in the characteristics of the plasmids in both resistant coliform sub-populations.

Whereas no significant difference was found in the total number of resistant E.coli possessing Tra^+ plasmids compared to resistant OEG, Tra^+ R^+ plasmids were significantly more common in resistant E.coli compared to resistant OEG.

In addition to 73 per cent of the resistant E.coli that possessed resistance markers encoded by Tra^+ R^+ plasmids, 55 per cent of all resistant E.coli isolates (these comprised 27% which did not possess Tra^+ R^+ plasmids and 28% which did) also possessed resistance properties which were not present on Tra^+ plasmids. In E.coli isolates with plasmids, these resistance determinants could have been present on Tra^- R^+ plasmids. However, this is unlikely as Tra^- R^+ plasmids could only have entered the cell if mobilised by Tra^+ plasmids. As no Tra^+ plasmids were present in the isolates and the plasmids present were not mobilised by RP4 it would appear unlikely that these resistances were plasmid-encoded and were more likely to be chromosomally-mediated.

Ninety-two per cent of resistant OEG possessed resistance markers which were not specified by Tra^+ R^+ plasmids. As with E.coli it would appear that they were chromosomally-mediated.

One explanation for the higher percentage of resistant OEG which possessed chromosomal-resistance properties compared to resistant E.coli is as follows: plasmids were more common in E.coli; therefore, in the presence of antimicrobial therapy

there was a high probability of a Tra^+ R^+ plasmid being present in the E.coli population encoding resistance to the relevant antibiotic.

The presence of this plasmid and any isolates with relevant chromosomal mutations would result in the survival of these isolates during therapy. With removal of antibiotic selection pressure, recolonisation of the site and re-establishment of a stable bacterial population, there would be the potential for the dissemination of the plasmid throughout the coliform population able to acquire the plasmid by conjugation. This may in turn have led to a greater proportion and wider variety of the coliform population containing the Tra^+ R^+ plasmid.

As E.coli possess more plasmids than OEG the potential for plasmid dissemination between E.coli isolates appears to have been greater than between E.coli and OEG or between different OEG isolates. In turn this may have reduced the tendency for selection of E.coli with chromosomal mutations and increased the selection of plasmid-containing isolates.

The high proportion of chromosomal resistance in OEG and small proportion of resistant OEG which possessed Tra^+ R^+ plasmids compared to E.coli may have been due to the selection of OEG isolates with chromosomally-encoded resistance. As

plasmids were less common in OEG than E.coli it is likely that OEG were less able to acquire and stably maintain plasmids compared to E.coli as a result of the recipient ability of the individual coliforms and the host range of the plasmids present. During antimicrobial therapy therefore, fewer OEG would possess Tra^+ R^+ plasmids. Survival of OEG during therapy would therefore be more dependent on the selection of isolates with chromosomal mutations. In time, this may have led to the selection and survival of OEG isolates with chromosomal resistance determinants.

Resistance determinants specified by Tra^+ R^+ plasmids:

From the 58 coliforms containing Tra^+ R^+ plasmids 16 different patterns of resistance were found. The most common pattern was resistance to ampicillin alone which was present as a single resistance marker in 18 isolates. In addition, ampicillin resistance was present in 8 of the other patterns with a total of 47 (81%) isolates containing Tra^+ R^+ plasmids that specified ampicillin resistance. The large percentage of plasmids carrying ampicillin resistance could be due to two main factors. Ampicillin-resistant plasmids detected in this collection could have come to dominate by clonal selection of a small number of plasmids which were originally resistant to

ampicillin. Alternatively, the spread of ampicillin-resistance transposons to a wide variety of existing plasmids could have led to the large proportion of diverse plasmids encoding ampicillin resistance.

Matthew (1979) showed that of 363 Gram-negative bacteria which harboured plasmids specifying beta-lactamase activity, 77.4 per cent coded for either TEM-1 or TEM-2 enzymes. The genes for both TEM-1 and TEM-2 are known to be encoded by transposons (Matthew and Hedges, 1976). It has also been demonstrated that the nucleotide sequence specifying TEM beta-lactamase activity was similar in a variety of compatibility groups of naturally-occurring plasmids (Heffron et al., 1975). It would appear likely therefore, that the widespread presence of ampicillin-resistance on Tra^+ plasmids has been caused by acquisition of transposons by a large number and variety of plasmids rather than the dissemination of a few plasmids originally coding for these beta-lactamase enzymes. This will be further considered later.

The second most common pattern of resistance determined by Tra^+ R^+ plasmids was ampicillin and tetracycline resistance which was detected in 11 isolates. A further three isolates possessed ampicillin/tetracycline resistances with at least one other resistance determinant.

Twenty of the Tra^+ R^+ plasmids studied conferred tetracycline resistance. Seven of these also determined sulphamethoxazole resistance.

Ampicillin, streptomycin and sulphamethoxazole resistances were found in ten isolates and in combination with at least one other marker, in a further 5 isolates. Twenty-four isolates possessed streptomycin resistance either as a single marker or in combination with at least one additional resistance.

Sulphamethoxazole resistance was transferred from 23 isolates. Of these 16 also possessed streptomycin resistance, three possessed tetracycline resistance and 4 possessed both streptomycin and tetracycline resistance, with additional resistance determinants also being transferred in some instances.

Trimethoprim, kanamycin and chloramphenicol resistances were each transferred from a small number of isolates. In all cases at least one additional resistance determinant was also transferred from these isolates.

All of the above resistance determinants have previously been demonstrated on transposons from naturally-occurring plasmids, e.g. ampicillin-resistance by Tn1 (Hedges and Jacob, 1974) and Tn2 (Heffron et al, 1975), tetracycline

resistance by Tn10 (Foster, Howe and Richmond, 1975), and sulphamethoxazole resistance by Tn4 (Kopecko and Cohen, 1975). Streptomycin resistance was also encoded by Tn4 and by Tn7 (Barth et al, 1976), trimethoprim resistance by Tn7, kanamycin resistance by Tn5 and Tn6 (Berg et al, 1975) and chloramphenicol resistance by Tn9 (Gottesman and Rosner, 1975). Thus, transposon-mediated resistance was probably the basis of much of the resistance detected in the plasmids studied.

The number of resistance patterns among the Tra^+ R^+ plasmids studied was limited. Additionally, many of the resistance markers appeared to be linked, e.g. ampicillin, streptomycin and sulphamethoxazole resistance was transferable from 15 isolates (together and sometimes with additional resistance determinants) which suggests that Tn4 might have been present in these plasmids (Cohen, 1976). Moreover, the sequential acquisition of transposons may have been the principal mechanism by which the plasmids accumulated multiple antibiotic resistance determinants. However, further investigation into the genetic structure of these plasmids (e.g. by fingerprinting) is required before we can determine whether common plasmids are present or whether any particular segments of DNA are common to a number of the plasmids studied.

Tra^+ R^- plasmids:

Plasmid R300B cannot be mobilised by plasmids of incompatibility groups C, F, HI, J, H or T (Hughes and Datta, 1983). This suggests that the 6 plasmids found in this study capable of mobilising plasmid pHH1310a, but not plasmid R300B, belong to one of the above incompatibility groups. Plasmids of the above incompatibility groups and from groups B, I, M, P, W, X, N, HII and D, can all mobilise plasmid pHH1310a (Hughes and Datta, 1983). This indicates that the Tra^+ R^- plasmids in this study which mobilised pHH1310a and R300B belonged to the latter incompatibility groups.

Ten isolates of Proteus spp. were found which mobilised both plasmids pHH1310a and R300B although no Tra^+ R^- plasmid DNA was subsequently detected in the transconjugants on agarose gels. This phenomenon has been described by other workers (Hughes and Datta, 1983) who found that from 104 coliforms with plasmids able to mobilise the Tra^- R^+ plasmids, pHH1310a and/or R300B, the mobilising Tra^+ R^- plasmid was either only transiently present or not detected at all in 20 of the resulting transconjugants. Three of these plasmids were from Proteus spp. and none were found from Proteus spp. where plasmid DNA was stably inherited by E.coli K12 recipient. These results suggest that plasmid host range

may play an important role in plasmid dissemination and in particular, that plasmids from Proteus spp. may be of a narrow host range only being found within this coliform genera.

Twenty-four per cent of all E.coli in this study possessed Tra^+ R^- plasmids. This figure is similar to the 17 per cent found among 300 faecal E.coli isolates (Lewis, 1968) from people outside hospitals and 33 per cent of 60 E.coli isolates from faeces of healthy pigs, cattle and humans (Smith and Linggood, 1970).

Inhibition of E.coli K12 growth:

The observation that a number of isolates of E.coli inhibited the transfer of RP4 during experiments to mobilise Tra^- plasmids led to the recognition that these E.coli isolates produced putative colicins which inhibited the growth of the E.coli K12 recipients used. For this reason, experiments were repeated using Ent.cloacae as a recipient and Sal.typhimurium as an intermediate. Both Ent.cloacae and Sal.typhimurium were resistant to the factors produced by the clinical E.coli isolates and could be used to detect Tra^+ plasmids and for mobilisation by RP4.

Results from these experiments showed that a further 4 isolates could be detected which harboured Tra^+ R^+ plasmids and 5 which possessed Tra^+ R^- plasmids. Three of the 9 resultant transconjugants also produced factors which inhibited E.coli K12 growth, suggesting that in these three cases the inhibitory factor was encoded by the Tra^+ plasmid or by a plasmid mobilised by the Tra^+ plasmid.

These results indicate that the frequency of plasmid transfer may have been underestimated in many studies if the recipient strains were inhibited by factors produced by the wild type isolates.

Pseudomonas spp.:

It was found that none of the 113 Pseudomonas spp. isolated from 30 patients encoded resistances other than intrinsic and that none possessed plasmids. It would therefore appear that despite the high rate of carriage of plasmids by the coliforms isolated from some sites, these plasmids were not transferred to any of the Pseudomonas spp. in vivo. Plasmid-mediated antimicrobial resistance was therefore not a problem in the Pseudomonas spp. population isolated from the patients studied.

Mobilisation of cryptic plasmids:

No plasmids which possessed resistance determinants appeared to be mobilised by plasmid RP4. However, if a plasmid had specified ampicillin, tetracycline or kanamycin resistance, mobilisation of these determinants would not be detected as RP4 possesses these resistance markers. Furthermore, low frequency mobilisation of a cryptic plasmid would also not have been detected as the only available method of determining successful mobilisation of Tra^- R^- plasmids was gel electrophoresis. Thus, if any of the 12 single colonies tested had acquired RP4 and had also acquired additional plasmid DNA, would mobilisation have been recorded as positive. Although it is not known how efficiently RP4 mobilises other plasmids this plasmid was chosen because it has a broad host range and therefore would transfer into all of the coliform genera studied. However, incompatibility with other Inc P plasmids would also result in failure to detect mobilisation of plasmids of this Inc group.

Three coliform isolates, which did not possess Tra^+ plasmids, contained plasmids which were mobilised by RP4. Two of the three coliforms containing mobilisable cryptic plasmids were isolated from the same wound. One was harboured by an Ent.cloacae isolate and the other by a Cit.freundii isolate.

Both plasmids were of the same molecular weight ($\leq$10Md). It seems likely therefore, that these plasmids had been mobilised into the two coliform isolates *in vivo* by a Tra^+ plasmid which was not transferred itself or was subsequently lost from both isolates. The third isolate contained a mobilisable cryptic plasmid which was isolated from the same patient but from an *Ent.cloacae* isolate from a rectal swab. In this instance the plasmid was much larger, approximately 62 Md.

To determine whether the plasmids from the *Ent.cloacae* and *Cit.freundii* are the same and if they were related to the cryptic plasmid from the rectal *Ent.cloacae* isolate requires further investigation of the plasmid DNA.

Restriction endonuclease digests of plasmid DNA generates characteristic fingerprints for individual plasmids and was applied to all plasmid-containing coliforms and transconjugants containing Tra^+ plasmids to assess the relatedness of plasmids present in the coliforms studied.

The application of plasmid fingerprinting to clinical situations:

Many previous plasmid investigations have included the determination of phenotypic characteristics specified by plasmids and studies of their distribution in different

bacterial populations. Considerable emphasis has been placed on the study of plasmids in bacteria resistant to antimicrobial agents which had been implicated in epidemics of infection. However, until recent years the investigation of plasmids in epidemic bacteria was limited by the methods available to distinguish between different plasmids. The comparison of resistance determinants carried by plasmids and estimates of their size, although useful in suggesting the presence of common plasmids in two or more isolates, does not confirm the molecular relatedness of plasmids from different isolates. Similarly, incompatibility grouping has been used on a number of occasions to determine whether R-plasmids from bacteria associated with outbreaks of infection were related. As the methods used to determine the incompatibility group of plasmids are labour-intensive, they are not practical when considering large groups of plasmids which do not have phenotypic markers in common.

Recent advances in methods to investigate the molecular structure of plasmids, in particular plasmid fingerprinting by restriction endonuclease digestion has greatly aided the investigation of for example, the epidemiology of antibiotic-resistant bacteria.

Plasmid fingerprinting has been used to assess the relatedness of plasmids within and between different genera of both Gram-positive (McGowan et al, 1979 and Hershfield, 1979) and Gram-negative (O'Brien et al, 1982 and Markowitz et al, 1980) bacteria. Plasmid fingerprints have enabled comparison of isolates in the same outbreak of infection (Sadowski et al, 1979) and of isolates from different epidemics or geographical locations (Schaberg et al, 1976). These studies were carried out to determine whether or not plasmids present in different isolates with similar phenotypic characters were distinguishable with regard to fingerprints. In addition plasmid fingerprint analysis can be used to monitor changes in epidemic plasmids during outbreaks of infection (Rubens et al, 1981). These authors showed that a transposon was responsible for the dissemination of gentamicin resistance among plasmids in E.coli, Ser.marcescens, Kleb.pneumoniae, Ent.cloacae and Ps.aeruginosa.

Thus plasmid fingerprint analysis can be used not only to assess the relatedness of different plasmids but also to monitor changes in plasmids which have acquired additional phenotypic characters.

There are however, a number of limitations in the use of restriction endonucleases to fingerprint plasmids which influence the conclusions that can be drawn from the results obtained. For example, the fingerprints of two plasmids with small differences in base sequence would be identical unless the differences were within the specific cleavage site of the restriction endonuclease used. In the latter instance alterations, even of a few bases, could lead to major changes in the plasmid fingerprint. Thus, plasmids with considerable sequence homology, as recognised by electron microscope hetroduplex analysis which can detect changes in DNA sequences of greater than 50 to 75 base pairs (Cohen et al, 1978), may have minimal similarity at the fingerprint level. However, the use of two or more enzymes which have different recognition sites should enable the distinction between minor base changes at specific sites and major differences in sequence homology.

Plasmid fingerprinting in the analysis of faecal flora contaminating wounds:

It was suggested earlier that many of the coliforms isolated from wounds were contaminants from the patients' own faecal flora. Twenty-one patients, from whom rectal swabs were obtained also yielded coliforms from their wound swabs.

Twelve coliforms isolated from wound and rectal swabs from the same patient and which were identical with regard to colonial morphology, API 20E code and resistance determinants, did not harbour plasmids. As plasmid-free isolates formed a minority (39%) of all coliforms studied, the lack of plasmids in these 12 pairs of coliforms could not be taken as evidence that the same organism was present in the patient's wound and rectal swab. Nineteen pairs of isolates, in addition to being homogeneous with regard to these characteristics, also possessed plasmids which were indistinguishable with regard to molecular weight and restriction fingerprints. Nine pairs of isolates from wound and rectal swabs, which were homogeneous with regard to colonial morphology and API 20E code, differed with regard to resistance determinants (6 isolates) and/or plasmid profile (9 isolates). We can therefore conclude that of 28 pairs of isolates, 19 were common to both wound and rectal swabs from the same patient and were therefore probably acquired by contamination from the patient's own faecal flora. Many more wound isolates, including the 12 plasmid-free and 9 which showed different characteristics which distinguished them from rectal strains carried by the same patient, may also have

been faecal contaminants but as we could not confirm isolation of the corresponding isolate from the rectal swab, their origin cannot be confirmed.

Six patients each had two coliforms common to both sites and 7 had one coliform common to both wound and rectal swabs. Therefore, of the 21 patients from whom wound and rectal coliforms were compared, 13 had identical coliforms in both sites. Thus, faecal contamination plays an important role in the colonisation/infection of wounds and confirms what in the past has been generally believed, but was largely unproven.

Of the coliform species represented in the 19 examples of paired clinical isolates from wound and rectal swabs, 12 were E.coli, 6 Proteus spp. and one Kleb.pneumoniae. Previous results (Table 17, Table 25) suggested that E.coli was more commonly isolated from rectal swabs and OEG from wound swabs. Plasmid profiles and fingerprints confirmed that many of the OEG isolates from wounds were also present in the same patient's faecal flora and conversely that a small proportion of faecal E.coli colonised/infected these wounds. This further supports the proposal that, in particular, Proteus spp. are better able to colonise/infect wounds than are E.coli.

The ability of Proteus spp. to colonise/infect wounds more efficiently than E.coli may reflect the different natural habitats of these organisms. Whereas the natural and most common habitat of E.coli is the gastro-intestinal tract of man and animals, Proteus spp. are often isolated from environments such as soil in addition to faecal material. Constant colonisation of the gut by E.coli may result in a degree of immunity against that species which reduces its ability to colonise/infect wounds, whereas Proteus spp. present in the gut transiently and in smaller numbers, may not provoke the same degree of immunity. Alternatively, physical characteristics of wounds such as lower temperature and availability of nutrients may also favour colonisation/infection with Proteus spp. rather than E.coli.

Plasmid transfer in vivo:

Although previous studies of the incidence of R-plasmid transfer in vivo have concluded that such events are rare (Anderson et al, 1973; Smith, 1969; Jarolmen and Kemp, 1969; Smith, 1971) no attempt was made to quantitatively relate the results to a baseline. The results are dependent on a number of factors such as donor and recipient ability of coliforms in a common site and antibiotic therapy. However,

more recent studies involving in vivo transfer in individual patients (Datta et al, 1981; Platt et al, 1984) and a comparison of the in vivo R-transfer rate and in vivo R-transfer potential (Platt et al, 1986) suggest that in vivo transfer may be more common than was previously suggested. The present investigation was designed to extend earlier work and to determine whether conjugative plasmids not associated with resistance transferred at a detectable level.

Forty-eight different wound and rectal sites were studied where two or more coliforms were isolated at any one time, at least one of which harboured a conjugative plasmid. A transfer potential of 280 events was possible from 96 isolates which possessed conjugative plasmids. Four instances of plasmid transfer in vivo were observed which gives an in vivo transfer rate of 1:70. This figure suggests fewer transfer events had taken place in vivo than in an earlier study in this department involving a different patient group (Platt et al, 1986). However, the previous study involved only conjugative R-plasmids where an R-plasmid transfer rate of 1:21.5 was found. The value found in the present study for R-plasmid transfer, 1:50, was lower than previously found. A number of factors may have influenced these results.

It has been suggested that the majority of plasmids from OEG had a narrow bacterial host range and that many were unstable in E.coli K12. In addition, very few plasmids were found in OEG which had similar fingerprints to plasmids from E.coli isolates. This suggests that many E.coli plasmids may not be transferable to or stably maintained in OEG isolates. Thus, the generic composition of coliforms involved in the calculation of R-transfer indices will influence the values obtained and may contribute to the differences between this and our previous study.

Out of a total of 129 possible transfer events among 26 patients, only one was observed in vivo which involved conjugative plasmids not associated with resistance. Although this suggests that plasmid transfer involving these plasmids may be less common in vivo than transfer of conjugative R-plasmids, the number of positive transfer events detected in vivo was too small for accurate comparison of the in vivo transfer rate of conjugative R-plasmids with conjugative plasmids not associated with resistance.

In this study plasmid transfer occurred *in vivo* independently of antimicrobial therapy although under many circumstances such treatment might influence the rate of transfer or its detection (Anderson *et al*, 1973; Guinee, 1970; Smith, 1970).

Other clinical implications of plasmid fingerprint results:

A number of instances were found where the analysis of plasmids and their fingerprints has led to the identification of apparently identical coliforms from two or more patients, suggesting that cross-infection has occurred.

Three *E.coli* strains (96RAA1, 97RAA1b and 98RAA1a) isolated from different patients' rectal swabs, harboured identical plasmids. All three were in-patients of the PVD unit at the same time, suggesting that cross-infection had led to the spread of the *E.coli* isolate. A number of possible sources and directions of cross-infection could be identified; for example, one patient may have cross-infected the other two patients. Alternatively, the coliform may have been spread sequentially from one patient to the second and from this patient to the third. Moreover, the source may have been a

fourth patient who was not studied or a common environmental source which led to the spread of this plasmid containing E.coli to at least three patients.

A second instance of cross-infection was found when Ent.cloacae isolates harbouring the same plasmid were isolated from a wound swab from one patient (141JC1) and a rectal swab from a second patient (143RJC1). These two patients were present in the same geriatric unit at the same time.

The isolation of a Kleb.pneumoniae (101RCD1) and a Kleb.oxytoca (105CH1) which harboured an identical plasmid from different patients in the same geriatric unit, suggested that not only had cross-infection occurred but that plasmid transfer had occurred either in one of these patients from whom only one species of the Klebsiella was isolated or in a third patient who was not studied. Alternatively, an environmental source of contamination and site for plasmid transfer cannot be discounted.

Three E.coli isolates (56AA1a, 56AA2b and 56AA1b) from one patient harboured a plasmid identical to the plasmid present in E.coli isolates from wound (107AA1) and rectal (107RAA2a) swabs from a second patient. However, these two patients were present in different hospital units two years apart. This suggests that plasmids can be maintained in an

E.coli population and transferred from one E.coli strain to another with possible transfer of strains from one patient to another with no apparent change in the fingerprint of the plasmid over a long period of time.

Thus, the use of plasmid fingerprinting can confirm instances of cross-infection and also yield indirect evidence for both plasmid transfer in vivo and plasmid stability.

Plasmids from E.coli:

The fact that few plasmids common to two or more clinical E.coli isolates were detected within the three categories of plasmids found (i.e. conjugative R-plasmids, conjugative plasmids not associated with resistance and non-conjugative plasmids) may have been partly due to the fingerprinting methods used.

E.coli often harbours multiple plasmids and when these were separated into transconjugants the restriction strategy might not have permitted optimal comparison of both donor and transconjugant plasmid fingerprints. Although the enzyme PstI was used to generate fingerprints from all clinical isolates and transconjugants neither it nor any other single enzyme was suitable to compare all plasmid-containing strains as the range of fragmentation patterns generated was too great. Twenty-six

per cent of the clinical isolates studied gave fewer than 6 fragments and 38 per cent produced more than 20 fragments when digested with PstI. Moreover, 70 per cent of the clinical E.coli isolates harboured more than one plasmid and as would be expected the more plasmids harboured by a clinical isolate, the higher the number of fragments seen with fingerprint analysis (Table 24). Therefore, E.coli isolates which harboured multiple plasmids were more likely to have been digested with EcoRI and HindIII than other enzyme combinations. In the analysis of individual plasmids within a multi-plasmid-containing strain, digestion with EcoRI and HindIII should give rise to fewer fragments per plasmid than with PstI and SmaI. Although more than 6 fragments may have been obtained from a clinical isolate with multiple plasmids on digestion with Eco RI or HindIII, the number generated from each individual plasmid may have been too small to see similarities between a number of isolates.

It has been demonstrated that plasmids within an incompatibility group have a "core" of DNA common to that group (Falkow, 1975; Chabbert et al, 1979). Therefore as over 25 different incompatibility groups have been identified (Jacob et al, 1977) some restriction fragments common within each group might have been anticipated. However, this depends on the

number of restriction sites within the core recognised by the different enzymes used and whether there was transposition of other DNA into the core of DNA associated with individual incompatibility groups.

Despite the problems in comparing fingerprints from isolates containing different numbers of plasmids, it was reasonable to assume that a number of isolates with characteristics in common, such as resistance determinants, would also have common fingerprints. A small proportion of the E.coli plasmids had restriction fragments in common but in general the fingerprints showed that most plasmids were not closely related. Analysis of some of the categories of plasmids, for example even those that carried common resistance determinants, showed very few isolates to have similar fingerprints. This may have been due to the dissemination of resistant transposons among diverse plasmids. Comparison of fingerprints of plasmids grouped on the basis of other characteristics, e.g. plasmid size, may reveal similarities. However, this may again reflect the common core of different incompatibility groups.

By comparing plasmids with common resistance determinants it was hoped to identify either endemic plasmids carrying the same resistance markers or the dissemination of a

common resistance transposon throughout a number of different plasmids. The recognition of endemic strains was however, rare, suggesting that cross-infection was not common among the patients studied.

Common restriction fragments in different plasmids which could be attributed to the presence of resistance transposons were not detected. This may have been partly due to the method of fingerprinting used and the fact that PstI was the only enzyme used to digest plasmid DNA from all isolates. Ampicillin-resistance was the most often detected resistance determinant found among the plasmids studied. The most common mechanism of resistance to ampicillin is production of TEM enzymes (Matthew, 1979) which is indicative of the presence of Tn 1/3 (Medeiros, 1984). This transposon yields characteristic fragments of 2,800 and 700 base pairs when digested with PstI (Heffron, 1983) therefore one might have expected to find these fragments in some of the fingerprints resulting from PstI digestion of ampicillin-resistant plasmids. Several plasmids produced fragments in this size range but without the presence of a known control this transposon could not be unequivocally recognised.

A further feature which may have confused attempts at plasmid recognition was that, in many instances, more than one plasmid was detected in the transconjugant. This may have been due to the presence of common resistance determinants on more than one conjugative plasmid or the mobilisation of a Tra^- R^+ plasmid by a Tra^+ R^- plasmid. Alternatively co-transfer of more than one plasmid only one of which carried the resistance determinant, mobilisation of a Tra^- R^+ plasmid by a Tra^+ R^+ plasmid or mobilisation of a Tra^- R^- plasmid could have accounted for the presence of multiple plasmids in the transconjugants.

It is possible that some conjugative R-plasmids have arisen by the transposition of resistance determinants to a second plasmid with conjugative ability but no resistance determinants. Had this happened, then it should have been possible to isolate both the newly formed conjugative R-plasmid and the original Tra^+ R^- plasmid from the bacterial population concerned. However, no such plasmids were found in the present study. The transposition of a resistance transposon into a conjugative plasmid would have led to a small increase in the molecular weight of the plasmid and some alteration to the plasmid fingerprint. No examples of this were found either from isolates from the same patient or from

E.coli isolates in general. However, we cannot assume that there was no transposition of resistance determinants since failure to detect the newly formed conjugative R-plasmid and the original conjugative plasmid not associated with resistance, might have been due to instability of the plasmids. The time elapsed since transposition should have led to selection of stable plasmids but since the two plasmids would now be incompatible in a single host, it is perhaps unlikely that both plasmids would be maintained in the population at detectable levels. An alternative approach would be to compare the fingerprint of each given plasmid with all Tra^+ R^- plasmids of similar size. The comparison of any Tra^+ R^+ plasmid with all Tra^+ R^- plasmids and the converse comparison is both time-consuming and complex, and requires a more efficient system of comparing fingerprints than visual comparison.

The most striking conclusion from the results found from E.coli isolates was that there were very few plasmids which had similar fingerprints, either within or between the three categories of plasmids found i.e. Tra^+ R^+, Tra^+ R^- and Tra^-. More strains possessed multiple plasmids than a single plasmid. Both plasmids from an isolate which harboured two plasmids must have belonged to different incompatibility

groups, similarly plasmids from 7 different incompatibility groups must have been represented in those isolates which harboured 7 plasmids. Thus 6 isolates, each harbouring 5 or more plasmids, must harbour a minimum of 30 plasmids. Since only 27 incompatibility groups are currently recognised and some are more prevalent among E.coli than others, duplicates of a number of incompatibility groups must be present. If, as previously discussed, different incompatibility groups have common cores then this should be evident in fingerprint analysis. As no large groups of similar plasmids were seen among the isolates, despite the necessary presence of multiple plasmids of the same Inc group, this lack of similarity suggests that the pool of plasmids present in the E.coli isolate studied was large and diverse.

Resistance determinants, plasmid profiles and plasmid fingerprints from each of two clinical E.coli isolates from which more than one different transconjugant was isolated, were studied in an attempt to determine which plasmid(s) were conjugative and which encoded resistance determinants. Both clinical isolates studied harboured more than one plasmid and transferred resistance determinants.

The results from clinical isolate 90RAA3 (Page 233, Table 28) and three transconjugants isolated from it suggested that only one plasmid of the 5 detected was conjugative. The 75 Md plasmid and one <10 Md plasmid harboured by the clinical isolate were both non-conjugative and were not mobilised by the 80 Md plasmid which was conjugative. All three plasmids of <10 Md found in the transconjugants were too small to encode transfer genes and must therefore have been mobilised by the conjugative 80 Md plasmid. As TCT-1, which harboured only the 80 Md plasmid, specified resistance to AppSmSu, these resistance determinants must have been specified by the 80 Md conjugative plasmid. All fingerprint fragments of this conjugative R-plasmid, when digested with both EcoRI and HindIII, were also present in the fingerprints of both additional transconjugants and the clinical isolate. Additional fingerprint fragments in the clinical isolate, TCT-2 and TCT-3 should correspond to the presence of both mobilised and non-mobilisable plasmids from the clinical isolate. The increased level of Sm resistance in TCT-2 could be attributed to either high or low level resistance specified by one of the small non-conjugative plasmids which results in the transconjugant exhibiting full Sm resistance since low level Sm resistance was specified by the 80 Md plasmid. This small

plasmid could also have encoded resistance to Ap and/or Su since the 80 Md plasmid already encoded these resistance determinants.

Failure to express SmSu resistance by TCT-3 in which one of the < 10 Md plasmids present in TCT-2 had not been mobilised may have influenced the expression of these resistance determinants resulting in apparent sensitivity to SmSu although resistance to Ap was shown.

The results from clinical isolate 107RAA1 (Page 236, Table 28) suggested that the 50 Md plasmid was conjugative and encoded resistance to Ap. In addition, the 75 Md plasmid was also conjugative and could mobilise the < 10 Md plasmid. One of the latter two (or perhaps both) plasmids encoded resistance to ApKmSm although from these results we cannot determine which plasmid carried the resistance determinants or if both plasmids carried different or overlapping resistance determinants. The difference in levels of resistance to KmSm shown by TCT-2 and TCT-3 may have been due to difference in gene expression. Resistance to Su was not transferable and can therefore be attributed to chromosomal resistance.

Plasmids from enteric genera other than E.coli

Fingerprint analysis of plasmids from OEG were similar to those obtained from E.coli in that there was very little overall similarity within and between the three categories of conjugative R-plasmids, conjugative plasmids not associated with resistance and non-conjugative plasmids. One of the major differences between plasmids from E.coli and those from OEG was that very few OEG possessed resistance determinants as described earlier. However, a number of additional differences were found when the fingerprints were analysed.

Twenty-one (78%) conjugative plasmids from 27 OEG isolates were unstable in E.coli K12 recipients. In contrast to this, only 8 (9%) conjugative plasmids from 86 E.coli isolates were unstable, one in the clinical isolate, 6 in an E.coli K12 recipient and one in an Ent.aerogenes recipient.

Eleven unstable OEG plasmids were transferred from Proteus spp.: in fact no Proteus spp. plasmids were found which were stable in E.coli K12. No plasmids from Proteus spp. were found to have similar fingerprints to plasmids from any other coliforms. This supports the previous findings that plasmids from Proteus spp. are of a narrow host range and that they belong to a limited number of incompatibility groups (Coetzee et al, 1972).

Only 6 OEG isolates harboured conjugative plasmids which were stable in E.coli K12. The conjugative plasmid from two of these isolates was also found in a clinical E.coli isolate. None of the remaining 4 conjugative plasmids from Enterobacter spp. and Klebsiella spp. which were stable in E.coli K12 were found in other enteric genera. The remaining 10 conjugative plasmids from Enterobacter spp., Klebsiella spp. and Citrobacter spp. were unstable in E.coli K12. Two isolates possessed an indistinguishable plasmid, the remaining 8 had different fingerprints. These results suggest that the majority of conjugative plasmids can be transferred and stably maintained within different Klebsiella spp. and Enterobacter spp. but that few of them are stable in E.coli.

Although it appears that plasmids from Proteus spp. showed a considerable degree of similarity the other enteric genera appeared to possess different plasmid pools. Plasmid within these pools may have different host ranges with most stable within a limited number of coliform species and only a small number having a broad host range.

The host range of plasmids is rarely studied in a systematic way possibly because the methods for doing so are time-consuming. The results presented here suggest that host range studies may be of value in the assessment of taxonomic coherence between different coliform species.

The host range of plasmids also has implications for the evolution of R-plasmids. Broad host range plasmids possessing resistance transposons may be able to disseminate into different hosts that harbour indigenous Tra^+ R^- plasmids of narrow host range. Transpositional conversion of such Tra^+ R^- plasmids would lead to the development of Tra^+ R^+ plasmids with a greater ability to disseminate (especially under conditions of antibiotic selection pressure) throughout the species to which that plasmid can easily transfer. Thus, there would be not only spread of a broad host range plasmid but also dissemination of narrow host range plasmids carrying resistance determinants.

The strategy used to determine which enzymes should be used to digest plasmid DNA from both the clinical isolates and transconjugants, had the disadvantage that it proved impossible to analyse all features initially intended. A contributing factor to many of the problems was the fact that many of the clinical isolates and transconjugants harboured multiple

plasmids and clearly analysis of fingerprints of multi-plasmid strains posed problems not encountered with single plasmid strains. However, the analysis of plasmid fingerprints in conjunction with plasmid profiles offers a solution to many of the problems.

Incomplete cleavage of an enzyme recognition site resulting in the generation of a weak, partial digest, is relatively simple to recognise in single plasmid strains whereas a weak band in multi-plasmid strain could be attributed to the fragment of a constituent plasmid present in a low copy number.

Excessively bright bands in single plasmid strains can usually be attributed to the presence of doublets or triplets whereas in multi-plasmid strains they are often due to single fragments from small multi-copy plasmids. The summation of molecular weights of such bands and correlation with the presence of small plasmids in the plasmid profile, could presumptively resolve these differences.

Thus the interpretation of plasmid fingerprint data from strains harbouring different numbers of plasmids requires considerable experience and remains subjective. However, a high degree of correlation can usually be achieved especially when plasmid profiles are also considered.

FUTURE DEVELOPMENTS

The emphasis in this study has been to characterise plasmids among coliforms and to use in vitro results to suggest how these findings may be reflected in the in vivo behaviour of plasmids in coliforms in the human host. The results presented demonstrate that this approach can provide clarification of many aspects of clinical infection and epidemiology. However, the abundance and diversity of plasmids among coliforms that colonise man will require the development of more refined techniques, especially for the characterisation of clinical isolates that harbour multiple plasmids, before unequivocal interpretation of their clinical significance is possible.

The extension of conjugation experiments to further segregate individual plasmids from multi-plasmid transconjugants will answer a number of questions about the characteristics of individual plasmids in these transconjugants. Previous conjugation experiments were carried out using a 24 hr incubation period. Therefore, the demonstration of two or more large plasmids in a transconjugant indicates that a minimum of one was conjugative. Thus, transconjugants that harbour multiple plasmids may have arisen

by several mechanisms including the transfer of two or more conjugative plasmids or mobilisation of a non-conjugative plasmid(s) by a conjugative plasmid. Conjugation experiments performed using transconjugants that possess multiple plasmids as donors, shorter conjugation periods to facilitate plasmid segregation, e.g. 0.5, 1, 2, 4 hr, and subsequent characterisation of transconjugants may resolve the following questions:-

(1) Which individual plasmids had conjugative ability?

(2) Which plasmids were mobilised by conjugative plasmids?

(3) Were any Tra^+ R^- plasmids present in transconjugants which also harboured Tra^+ R^+ plasmids?

(4) In transconjugants which acquired resistance determinants, which plasmids specified these determinants and were they conjugative or mobilised by conjugative plasmids?

Tra^- plasmids, especially those with molecular weight of > 20 Md, have received little attention. Although RP4 was chosen to investigate the mobilisation of Tra^- plasmids as previously discussed, it would be interesting to carry out a more systematic study. Using this approach, various plasmids belonging to different incompatibility groups in different bacterial hosts would provide valuable information with regard to the mobility and host range of Tra^- plasmids and their ability to participate in the genetic flexibility of the bacterial host.

Throughout the present study the computing facilities (both hardware and software) available were limited and although a standardised output of plasmid fingerprints was achieved, analysis of the fingerprints other than by visual comparison was not possible. However, development of appropriate software to compare and cluster fingerprint data may lead to the recognition of a number of features not apparent on visual inspection. These include the detection of transposons in R-plasmids, common DNA cores in plasmids of the same incompatibility group, similarities in fingerprints of non-conjugative plasmids and identification of groups of plasmids specific to particular coliform genera or species.

The present coliform collection does not contain sufficient numbers of any single genus, other than Escherichia, for comparative analysis of plasmid distribution, mobility and fingerprinting to be statistically valid. The collection and analysis of larger numbers of one or more specific coliform genera may therefore enable better interpretation of the significance of plasmids in enteric genera other than E.coli.

The numbers of individual genera were also too small to determine whether plasmids directly influence the ability of coliforms to colonise wounds. However, an investigation of factors encoded by plasmids other than antibiotic resistance for example, enhanced ability to adhere to mamalian cells, resistance to the bacteriocidal action of serum and resistance to phagocytosis may contribute to defining the function of plasmids in clinical infection with coliforms. In this respect the further development of computer-aided analysis of plasmid fingerprints may prove especially useful in the recognition of clusters of related plasmids.

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APPENDIX 1

Questionnaire used for recording patient details when wound swabs were obtained.

Specimen No: D.of B: / / Hospital No.:
Patient No: Surname: Christian names:
Date of specimen: Address:

Date admitted: Ward No.
Underlying Pathology/General condition:

Surgery: Yes / No Date:

LESION:
Hospital acquired: Yes / No
Aetiology
Site
Duration
Description
Wet/Damp/Dry

THERAPY:
Systemic

Topical

Other infected sites: Yes / No Specimen No: Date:

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1. **Platt, D.J. and Sommerville, J.S. (1981)** A simple method for the detection of resistance plasmids in Serratia species by transfer to members of the genus Enterobacter. Journal of Antimicrobial Chemotherapy, 8, 145-152.

2. **Platt, D.J. and Sommerville, J.S. (1981)** Serratia species isolated from patients in a general hospital. Journal of Hospital Infection, 2, 341-348.

3. **Platt, D.J., Sommerville, J.S. and McGroarty, J.A. (1983)** Characterization of spontaneous resistant variants of Serratia marcescens selected in the presence of carbenicillin. Journal of Antimicrobial Chemotherapy, 12, 329-335.

4. **Platt, D.J., Sommerville, J.S. and Gribben, J. (1984)** Sequential acquisition of R-plasmids in vivo by Salmonella typhimurium. Journal of Antimicrobial Chemotherapy, 13, 65-69.

5. **Platt, D.J., Sommerville, J.S., Kraft, C.A. and Timbury, M.C. (1984)** Antimicrobial resistance and the ecology of Escherichia coli plasmids. Journal of Hygiene, 93, 181-188.

6. **Platt, D.J. and Sommerville, J.S. (1984)** Effect of RP4 on growth and pigmentation in Serratia marcescens. Microbios Letters, 27, 7-13.

7. **Platt, D.J., Chesham, J.S. and Kristinsson, K.G. (1986)** R-plasmid transfer in vivo: a prospective study. Journal of Medical Microbiology, 21, 325-330.

Journal of Antimicrobial Chemotherapy (1981) **8**, 145–152

A simple method for the detection of resistance plasmids in *Serratia* species by transfer to members of the genus *Enterobacter*

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A simple conjugation system is described for the transfer of resistance plasmids from *Serratia* species to members of the genus *Enterobacter*. A surface-mating technique was employed using a supplemented minimal agar medium. Inhibition of the recipient was mediated by the antimicrobial agent under investigation and counter-selection against the donor was achieved by the incorporation of rhamnose as the carbon source. Plasmid transfer and expression occurred readily in the presence of the antimicrobial agent. The plasmids transferred were RP4, R446b and four naturally occurring serratia plasmids designated pDJP1, 2, 3, and 4. The incidence and frequency of plasmid transfer to *Enterobacter* species were markedly higher than to *Escherichia coli* K12.

Introduction

The role of *Serratia* species in nosocomical infection is now well established (Yu, 1979). The increasing incidence of such infection in Great Britain (Brookes, Chambers & Tabaqchali, 1979; Platt & Sommerville, in preparation*) and the frequency of multiple drug resistance in these organisms make it desirable to monitor the resistance mechanisms and to discriminate between plasmid-mediated resistance and chromosomal mutation.

The physical detection of plasmids in drug resistant strains does not show that the resistance is plasmid mediated since chromosomal mutation is common in *Serratia* (Traub & Kleber, 1977). Acquisition by mutation, of multiple resistance to unrelated drugs in *Ser. marcescens* has also been reported (Traub & Fukushima, 1978). Transfer of resistance (R+) plasmids from *Serratia* spp. has been described, employing *Escherchia coli* K12 as the recipient (Cooksey, Thorne & Farrar, 1976; Hedges, 1979). However plasmid transfer from *Serratia* spp. to *E. coli* is often inefficient (Hedges, 1979).

Bradley (1980) has shown that in transferring plasmids between strains of *E. coli*, surface mating was always as good as, and frequently superior to, broth mating.

*Data presented at the British Society for the Study of Infection, Scottish/Scandinavian Conference, St Andrews, Scotland, September 1980.

0305–7453/81/080145 + 06 $01.00/0

This communication describes a simple (one step) plate mating system for R plasmid transfer from *Serratia* species to members of the genus *Enterobacter* in which donor counterselection is achieved by having, as a carbon source in a supplemented minimal medium, a carbohydrate (rhamnose), utilizable by the recipient but not by the donor.

Materials and methods

Organisms and plasmids

Clinical isolates of *Serratia* and *Enterobacter* species were obtained from the routine diagnostic laboratory in Glasgow Royal Infirmary. They were identified by the API system (20E), determined on two occasions (Rubin, 1979), and confirmed by deoxyribonuclease production and susceptibility to colistin and cefazolin. *E. coli* K12 (strains J53 and J62) were kindly provided by N. Datta (Royal Postgraduate Medical School, Hammersmith Hospital). The characteristics of the plasmids and their bacterial host strains are shown in Table I.

Media

Cystine-lactose electrolyte deficient (CLED) agar (Oxoid CM301) was used for growth and maintenance of donor and recipient cultures, isosensitest agar (Oxoid CM471) for sensitivity testing and nutrient agar (Oxoid CM3) for the growth of organisms for DNA extraction and gel electrophoresis. Minimal agar (MA) (Clowes & Hayes, 1968) provides nitrogen as ammonium salts and carbon as glucose. It was modified as described below. Sterile supplements were added to molten, cooled basal medium to give the final concentrations shown:

MA1—glucose replaced by rhamnose (0·2%).
MA2—MA1 supplemented with glucose (0·01%) and proteose peptone 3 (Difco) (0·01%).
MA3—MA2 supplemented with proline and methionine.
MA4—MA2 supplemented with proline, histidine and tryptophan.
MA5—MA supplemented with proline, histidine and tryptophan.
All amino acids were used at a final concentration of 20 mg/l.

Antimicrobial agents

The final concentration in MA plates for the selection of transcipients were: carbenicillin (Beecham) 100 mg/l, tetracycline (Sigma) 15 mg/l, kanamycin (Sigma) 25 mg/l, trimethoprim (Sigma) 10 mg/l, streptomycin (Sigma) 15 mg/l, gentamicin (Roussel) 10 mg/l, and sulphamethoxazole (Wellcome) 50 mg/l.

Disc diffusion sensitivity tests were carried out as described by Ericsson & Sherris (1971), with discs containing nalidixic acid 30 μg, ampicillin 10 μg, carbenicillin 100 μg, sulphamethoxazole 25 μg, trimethoprim 1·25 μg, tetracycline 10μg, kanamycin 30 μg, chloramphenicol 30 μg, streptomycin 10 μg, gentamicin 10 μg, tobramycin 10 μg, amikacin 10 μg, cefazolin 30 μg, cefamandole 30 μg, colistin sulphate 25 μg, (Oxoid); rifampicin 50 μg (Mast).

Surface mating technique

About 30 colonies of each recipient strain were harvested from overnight cultures

Table I. Characteristics of plasmids

Plasmid	Approximate molecular weight	Resistance determinants	Bacterial host	Source
RP4	36	Ap Tc Km	(1) *E. coli* K12 J53 (2) *Ser. marcescens* (GRI 2677)	N. Datta RP4 transfer from J53 into clinical isolate.
R446b	47	Sm Tc	(1) *E. coli* K12 J53 (2) *Ser. marcescens* (GRI 2677)	N. Datta R446b transfer from J53 into clinical isolate.
pDJP1	45	Su Tc Km Ap Gm Tm	(1) *Ser. marcescens* (GRI 7679) (2) *E. coli* K12 J53	Clinical isolate Transferred from *Ser. marcescens* via *Enterobacter* (intermediate host).
pDJP2	70	Su Tc Km Ap Gm[a] Tm Sm Cm	(1) *Ser. marcescens* (GRI 14680) (2) *E. coli* K12 J53	Clinical isolate Transferred from *Ser. marcescens* via *Enterobacter* (intermediate host).
pDJP3 pDJP4	70 36 [b]	Su Km Ap Gm Tm Sm	(1) *Ser. liquifaciens* (GRI 4180) (2) *E. coli* K12 J53	Clinical isolate Transferred from *Ser. liquifaciens* via *Enterobacter* (intermediate host).

Ap, Ampicillin; Tc, tetracycline; Km, Kanamycin; Sm, streptomycin; Su, sulphonamide; Gm, gentamicin; Cm, chloramphenicol; Tm, tobramycin.
[a]Gentamicin resistance encoded by this plasmid was low level (MIC = 8 mg/l).
[b]All transcipients examined contained both plasmids.

on CLED agar and suspended in 5 ml of sterile distilled water. Some of each suspension was inoculated, with a sterile swab, onto one-half of a plate of the appropriate selective MA and allowed to dry. Donor suspensions were prepared in the same way and up to five per plate were inoculated at right angles across the recipient, as streaks 7–10 mm wide. The plates were incubated at 37°C for 48 h. Transcipient clones appeared as colonies at the interface of donor and recipient and the results were recorded as follows: −= 0 colonies, ±= 1 to 10 colonies, += 11 to 50 colonies, 2+ = semiconfluent growth at the interface of donor and recipient and 3 + = confluent growth over the complete donor streak. Plasmid transfer was confirmed after purification of transcipient clones, by disc sensitivity testing and by comparison of the electrophoretic patterns of plasmid DNA in agarose gels. This method provides internal controls since if mutants of donor or recipient emerged, they would appear on areas of the plate other than the cross-streaks.

DNA extraction and electrophoretic separation of plasmids

The equivalent of one or two loopfuls of an overnight culture on nutrient agar at 37°C were suspended in 300 µl Tris-borate buffer (pH 8·2) in polyallomer tubes. Two hundred microlitres of sodium dodecyl sulphate (10%) were added and the tubes were vortex-mixed immediately for 10 s. Strains of serratia which failed to lyse completely by this procedure were placed in a water bath at 50°C for about 5 min. The crude lysates were centrifuged at 40,000 **g** for 15 min at room temperature. One-hundred microlitres of the supernate was loaded onto the gels (0·7–1% agarose) together with 5 µl of bromophenol blue (0·05%) and the gels sealed with molten agarose. Electrophoresis, plasmid visualization and molecular weight determinations were as described by Datta *et al.* (1979).

Selection of Enterobacter *recipients*

Forty-five clinical isolates of *Enterobacter* spp. were screened for their ability to acquire the plasmids RP4 and pDJP1 from *Ser. marcescens*. Selection was for transfer of kanamycin resistance on medium MA1.

E. coli K12, J53 and J62 were tested in parallel as recipients on MA3 and MA4 respectively. In addition, transfer of the same plasmids from *E. coli* K12, J53 to *E. coli* K12, J62 was examined on MA4 and MA5.

Results

In the system described, transcipient colonies appeared at the interface of the cross-streaks (Figure 1). They had acquired resistance determinants compatible with those shown in Table I and exhibited characteristic bands in agarose gels (Figure 2).

Of 45 clinical isolates of enterobacter screened as recipients for RP4 and pDJPI, 12 strains (7 *Ent. cloacae*, 3 *Ent. aerogenes*, 1 *Ent. sakazakii*, and 1 *Enterobacter* sp.) acquired both plasmids. Two further *Ent. cloacae* acquired pDJP1 only and another *Ent. cloacae* acquired RP4 only.

Ent. cloacae GRI 8380, *Ent. sakazakii* GRI 9280, and *Ent. aerogenes* GRI 9880 were chosen as the best recipients representing each of the *Enterobacter* spp. These were used as recipients for the transfer of plasmids RP4, R446b, pDJP1, 2, 3, and 4. The medium employed was MA2 which contained glucose (0·01%) and peptone

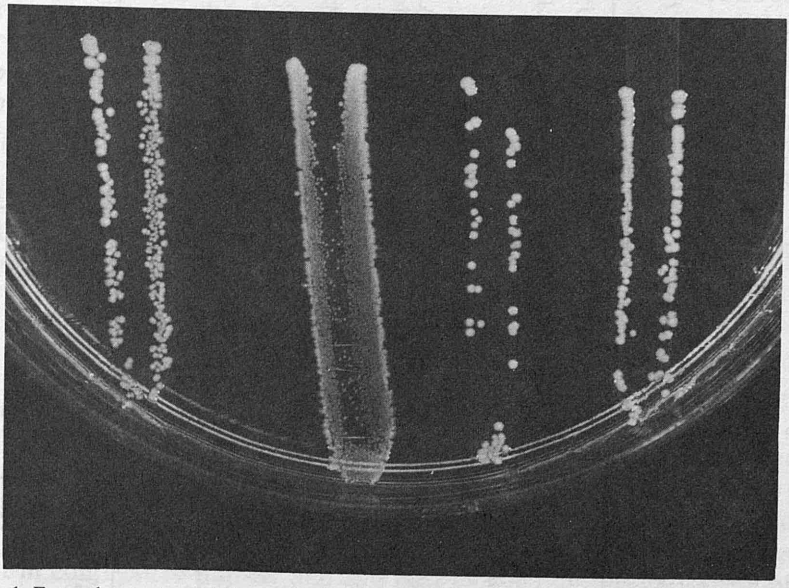

Figure 1. Enterobacter transcipients obtained on medium MA2 containing 25 mg/l kanamycin.

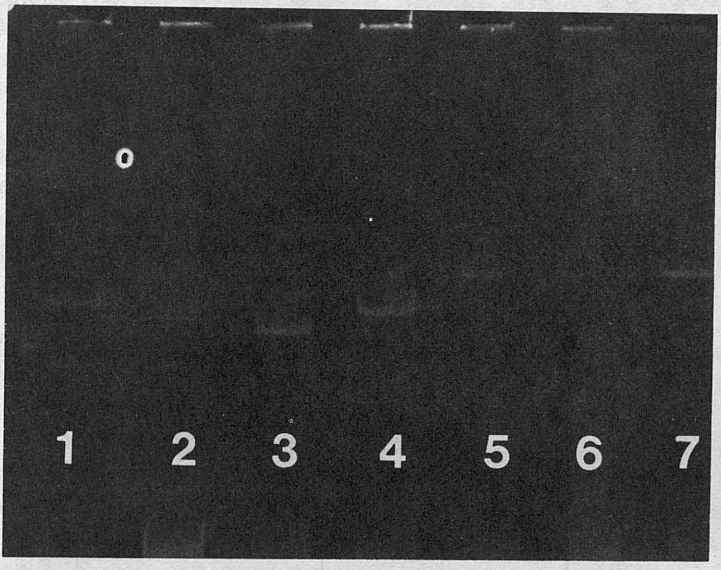

Figure 2. Agarose gel (0·7%) of plasmids transferred from *Serratia* spp. to *Ent. aerogenes*. Tracks 1 and 7 contain molecular weight standards R702 (MW46) and R1 (MW62), respectively, and R446b (track 2) RP4 (track 3) pDJP1 (track 4) pDJP2 (track 5) pDJP3 & 4 (track 6).

Table II. Transcipients obtained from surface mating of *Serratia* spp./*Enterobacter* spp., *Serratia* spp./*E. coli* K12 and *E. coli* K12 J53/*E. coli* K12 J62

Plasmid	Donor: *Serratia* spp. Recipient: *Enterobacter* spp.				Recipient: *E. coli* K12		Donor: *E. coli* K12 J53 Recipient: *E. coli* K12	
	Ent. aerogenes GRI 9880		*Ent. cloacae* GRI 8380	*Ent. sakazakii* GRI 9280	J53	J62	J62	
	MA1 Medium	MA2 Medium	MA2 Medium	MA2 Medium	MA3 Medium	MA4 Medium	MA4 Medium	MA5 Medium
RP4	+	3+	2+	2+	−	−	3+	2+
R446b	+	3+	+	2+	−	−	+	+
pDJP1	+	3+	+	2+	−(±)	−	3+	2+
pDJP2	+	3+	2+	+	−(±)	−	2+	+
pDJP3 pDJP4	±	2+	±	±	−(±)	−	2+	+

−, 0 transcipients; ±, 1–10 transcipients; +, 11–50 transcipients; 2+, semi-confluent growth at interface; 3+, confluent growth throughout streak.
( ), Result obtained on one occasion of three tested.

(0·01%). These were incorporated to minimize metabolic stress. The transfer of plasmids to the three selected *Enterobacter* strains and to the *E. coli* controls, on the appropriate media are shown in Table II. *Ent. aerogenes* exhibited the highest transfer frequency of the three enterobacter recipients examined and the number of transcipients obtained was consistently higher on MA2 than MA1. *Ent. cloacae* and *Ent. sakazakii* were both slightly less efficient recipients, particularly for pDJP3/pDJP4, which always co-transferred. Transfer from serratia to *E. coli* K12 J53 was rare and variable. No transfer was observed from serratia to *E. coli* K12 J62. When *E. coli* K12 J53 was the donor of the same plasmids to strain J62, transfer occurred at high frequency irrespective of the carbon source provided (glucose or rhamnose).

Discussion

The results demonstrate that plasmids are transferred more readily from *Serratia* spp. to members of the genus *Enterobacter* than to *E. coli* K12. The inefficient transfer from *Serratia* to *E. coli* K12 confirms the findings of Hedges (1979) and our own unpublished observations using broth systems. The same plasmids were efficiently transferred between strains of *E. coli* K12. Thus *E. coli* can act as a good recipient in conjugative transfer and as a host for the replication of these plasmids. The barrier must therefore be an intergeneric one.

Cooksey *et al.* (1976) showed that 3 of 39 multiresistant *Ser. marcescens* transferred resistance to *E. coli* K12 whereas 19 of these strains transferred R factors to a strain of *Klebsiella pneumoniae*. These findings, together with our own, indicate that the intergeneric barrier is less marked between *Serratia* or *Klebsiella* and *Enterobacter* than between *Serratia* and *E. coli*, perhaps a reflection of their phylogenetic relationship.

Many surface mating systems employ double drug selection, often with nalidixic acid to prevent donor growth, if the donor is sensitive to that drug. However, this requires that conjugation and selection are carried out as separate steps since nalidixic acid inhibits plasmid transfer (Bradley *et al.*, 1980). Selection against the donor by providing it with no carbon source eliminates both of these disadvantages and enables the most multiply resistant serratia isolates to be tested for R transfer. Rhamnose was chosen because strains of *Ser. marcescens* are uniformly unable to utilize it as a sole carbon source (Grimont, Grimont & de Rosnay, 1977). The small amounts of glucose and peptone are insufficient to function as carbon sources and their precise role remains unclear but their presence consistently increased the transfer frequency.

The use of MA2 provides a simple method for screening clinical isolates of *Serratia* spp. for the presence of conjugative plasmids which encode drug resistance. This will facilitate epidemiological studies of serratia and may have application to the study of plasmid transfer from other organisms unable to utilize rhamnose.

Acknowledgements

We are indebted to Naomi Datta for providing the *E. coli* strains and plasmids RP4 and R446b.

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Serratia species isolated from patients in a general hospital

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Summary: A survey carried out between 1 May 1979 and 30 April 1980 yielded 119 isolates of *Serratia* spp. from 58 patients. Ninty-one per cent of the patients were compromised, 86 per cent had undergone recent surgery and 79 per cent had received antimicrobial therapy within 14 days prior to serratia isolation.

The principal sources of the isolates were the intensive care and peripheral vascular units. The combined use of antibiogram, serological and phage typing revealed both epidemiological episodes and sporadic isolation of these organisms suggesting that the population of *Serratia* spp. in this hospital is heterogeneous.

Fifty-seven per cent of the isolates were resistant to carbenicillin and 30 per cent resistant to gentamicin. Gentamicin resistance was exclusively plasmid-mediated whereas resistance to carbenicillin was equally divided between plasmid-mediated and chromosomal mechanisms.

Introduction

The role of *Serratia marcescens* and *Ser. liquifaciens* as pathogens is now well established and has been extensively reviewed (Grimont and Grimont, 1978; von Graevenitz and Rubin, 1979; Yu, 1979). A high incidence of isolation of these organisms, and their frequent association with morbidity is common in the United States but they are rarely encountered in routine specimens in Great Britain (Brooks, Chambers and Tabaqchali, 1979).

Few reports have described changes in the frequency of isolation of these organisms over a significant time span. Black and Hodgson (1971), reported serratia isolation during a four month survey in Glasgow Royal Infirmary. This communication presents the results of a similar survey carried out 10 years later in the same hospital, compares the isolation incidence and discusses the epidemiology and antimicrobial resistance of the isolates obtained from the current survey.

Materials and methods

Patient related factors

Recent surgery was defined as surgery within 14 days prior to the first isolation of a *Serratia* spp. and recent antimicrobial therapy as systemic treatment within 14 days prior to the first serratia isolation. Patients predisposed to infection with low grade pathogens as a result of underlying physiological processes or mechanical intervention were termed 'compromised'.

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Organisms

In the routine diagnostic laboratory at Glasgow Royal Infirmary coliform isolates considered to warrant sensitivities are identified by the API (20E) system. Isolates with API profiles that corresponded to *Ser. marcescens*, *Ser. liquifaciens* or *Serratia* spp. were collected. Since API profiles do not discriminate well between genera within the tribe *Klebsiellae* (Rubin, 1979) they were determined on two occasions and apparent serratia isolates confirmed by deoxyribonuclease production (Black, Hodgson and McKechnie, 1971) and resistance to colistin and cefazolin (20 μg/ and 30 μg/disc, respectively). The ability to produce prodigiosin was tested by growth at 30°C on CLED agar (Oxoid CM301). Eighty-two isolates were characterized in detail by the methods described below.

Screening tests for β-lactamase production

The production of β-lactamase was detected using nitrocefin (cell suspension method; O'Callaghan *et al.*, 1972) and 'betatest' strips (Medical Wire Co. Ltd.). The latter employ penicillin as substrate and acid production denotes hydrolysis of substrate.

Sensitivity testing, transfer and physical detection of R-plasmids

Antimicrobial sensitivity was determined by disc diffusion. R-plasmid transfer to *Enterobacter aerogenes* was carried out using a surface mating technique and plasmids detected in agarose gels as previously described (Platt and Sommerville, 1981).

Gentamicin MICs were determined using a broth dilution method (Phillips, Warren and Waterworth, 1976) in sensititre plates (Seward Laboratories, UAC House, Blackfriars Road, London); these were carried out on serratia isolates resistant to gentamicin by the disc test and on *E. coli* K12 transconjugants after acquisition of the plasmids coding for gentamicin resistance in serratia.

Typing methods

Antibiograms were constructed in the following way: the inhibition zone radii of the test organisms, obtained with carbenicillin, gentamicin, chloramphenicol, kanamycin and streptomycin, were compared to the zones obtained with *Ser. marcescens* NCTC 1377. The latter were determined on 12 separate occasions. A test organism was considered to be resistant if the inhibition zone radius of a given antimicrobial was less than $(\bar{x} - 3\sigma)$ where $\bar{x}$ and σ were the mean and standard deviations, respectively, of 12 determinations of the standard *Ser. marcescens*. Serological and phage typing was carried out in the Division of Hospital Infection, Central Public Health Laboratory, Colindale using the methods described by Pitt, Erdman and Bucher (1980).

Results

Isolation incidence

During the year from 1 May 1979 to 30 April 1980, 119 isolates of *Serratia* spp. were obtained from 58 patients. None of the isolates was pigmented. Wound swabs

provided 67 isolates (56 per cent), urine specimens 21 (18 per cent), sputum or tracheal aspirates 17 (14 per cent), unspecified specimens 11 (9 per cent) and blood cultures three (3 per cent). The distribution of 86 isolates from 43 patients in wards where *Serratia* spp. were isolated from more than one patient is shown in Table I: with the exception of one medical ward all episodes of serratia isolation were associated with surgical units.

Table I. *Source of serratia isolates excluding wards containing only one serratia-positive patient*

Ward	Number of	
	Isolates	Patients
Vascular surgery	28	14
Respiratory intensive care	26	8
ENT surgery	12	4
General surgery	8	6
Cardiothoracic surgery	6	5
Orthopaedic surgery	4	4
Medical wards	2	2

Patient factors associated with colonization

Ninety-one per cent of patients were compromised of whom 33 per cent were diabetic and 21 per cent had underlying malignant disease. Eighty-six per cent of the patients had undergone recent surgery and 79 per cent had had recent antimicrobial therapy with drugs to which *Serratia* spp. are intrinsically resistant (i.e. ampicillin, tetracycline, colistin or cephalosporins). Cephalosporins were the most commonly prescribed antimicrobial in this group and had been given to 53 per cent of the total patients (67 per cent of the patients that had received antimicrobial therapy).

Antimicrobial susceptibility

All isolates showed the primary resistance characteristic of the genus *Serratia*. Fifty-seven per cent of the isolates were resistant to carbenicillin, 32 per cent to kanamycin and gentamicin, 30 per cent to tobramycin and 11 per cent to streptomycin. No amikacin resistance was observed. The results of characterization of 82 of the 119 isolates are shown in Table II. The most resistant isolates (antibiograms A, B and C) contained R-plasmids demonstrated by agarose gel electrophoresis and transfer to *Ent. aerogenes*.

The plasmid designations, approximate molecular weight and resistance determinants transferred are shown in Table III. pDJP1 mediated high level gentamicin resistance and low level tobramycin resistance, pDJP2 mediated low level gentamicin resistance. Plasmids pDJP3 and pDJP4 always co-transferred and together mediated low level resistance to both drugs. The MICs of gentamicin and tobramycin are shown in Table IV. The level of resistance determined by these plasmids was consistently higher in the *Serratia* spp. than in *E. coli* K12.

Table II. *Characterization of 82 clinical isolates of* Serratia *spp.*

	Antibiogram					Number of			Physical plasmid detection and resistance transfer	β-Lactamase screening test		Serotype	Phage lysis pattern
	Cb	Gm	Cm	Km	Sm	Patients	Isolates	Wards					
A	■	■	■	■	■	1	1	1	+	+*	−†	0:2/4 (1 isolate)	1, 2, 4, 7
B	■	■	■	■	□	8	18	2	+	+	+	0:2/4 (1 isolate)	1, 2, 4, 7
												0:14 (17 isolates)	1, 6, 7, 11, 12
C	■	■	□	■	■	1	7	1	+	+	+	NT (7 isolates)	NT
D	■	□	■	□	□	7	7	5	−	±	−	0:14 (4 isolates)	1, 6, 7, 11, 12
												0:12 (1 isolate)	NT
												0:4 (1 isolate)	2
												0:3 (1 isolate)	1, 4
E	■	□	□	□	□	3	14	1	−	±	±	0:14 (14 isolates)	1, 6, 7, 11, 12
F	□	□	■	□	■	1	1	1	−	+	−	0:5 (1 isolate)	2
G	□	□	■	□	□	3	3	3	−	±	−	0:14 (1 isolate)	4
												0:4 (1 isolate)	1, 2.
												NT (1 isolate)	NT
H	□	□	□	□	□	19	31‡	12	−	±	−	0:5 (8 isolates)	2

■, Resistant; □, Sensitive; *nitrocefin method; †acidimetric method; ‡23 isolates of mixed serotype with none predominant including 4 of 0:14; NT, non-typable.
Cb, carbenicillin; Gm, gentamicin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin.

Table III. *Characteristics of gentamicin resistant serratia isolates*

Plasmid			
Designation	Molecular weight (approximate)	Antibiogram	Resistance determinants transferred to *Enterobacter*
pDJP1	70 Md	A	Ap Gm Km Sm Cm Su Te
pDJP2	45 Md	B	Ap Gm Km — — Su Te
pDJP3 }	70 Md		
pDJP4 }	36 Md	C	Ap Gm Km Sm — Su —

Ap, ampicillin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Cm, chloramphenicol; Su, sulphamethoxazole; Te, tetracycline.

β-Lactamase activity was detected in all isolates using nitrocefin but only in certain isolates (antibiograms A, B, C and E) when the acidimetric method (penicillin substrate) was used.

Epidemiology

The combination of phage and serotyping shows the serratia population in the Royal Infirmary to be heterogenous. Eleven distinct strains were identified. The most common serotype was 0:14 and it comprised 49 per cent of the 82 isolates characterized. Phage typing of the 0:14 strains subdivided the isolates into two groups, containing 35 and five isolates respectively. Eight isolates of identical phage type, belonged to serotype 0:5. Other serotypes were observed infrequently. Fourteen isolates were non-typable by either method. The typing results were combined with the antibiogram to show the relationship between the different strains and the units from which they were isolated (Figure 1). The three isolates from the renal dialysis unit were obtained from the same patient. This patient had previously been in the intensive care unit. All isolates with antibiogram B contained plasmids indistinguishable from pDJP1; 17 were serotype 0:14 and one reacted with 0:2 and 0:4 antisera. The only other serotype 0:2/4 isolate in this study originated from a general surgical ward and contained pDJP2. Other serotype 0:14 isolates of the same phage type but with different antibiograms were associated with the peripheral vascular and surgical ENT units. Although some of these isolates were

Table IV. *The levels of plasmid coded gentamicin and tobramycin resistance determined by broth dilution MIC in* Serratia *spp. (wild type) and* Escherichia coli *K12 (transcipients)*

	MIC (mg/l) in *Serratia*		MIC (mg/l) in *Escherichia coli*	
Plasmid	Gentamicin	Tobramycin	Gentamicin	Tobramycin
pDJP1	>64	32	>64	8
pDJP2	8	2	4	1
pDJP3 }	8	8	4	4
pDJP4 }				

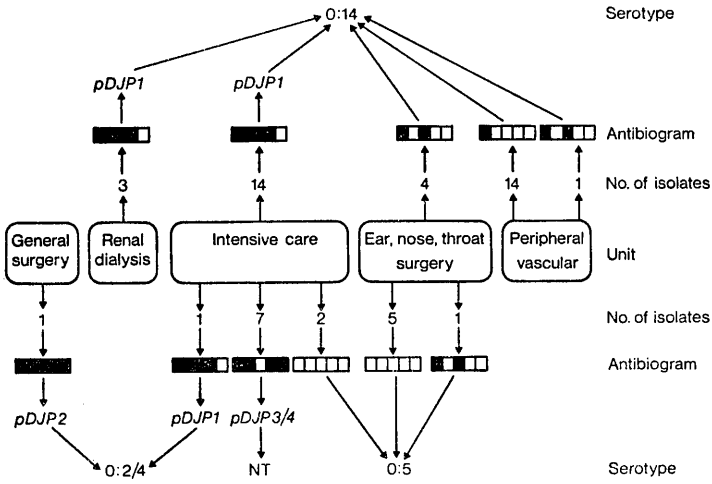

Figure 1. The distribution of multiply antibiotic resistant strains of *Serratia* spp. and strains belonging to prevalent serotypes within Glasgow Royal Infirmary. All isolates within serotypes were of identical phage type. Antibiograms are shown as in Table II and plasmid designations as in Table III.

resistant to carbenicillin and chloramphenicol neither plasmids nor resistance transfer could be demonstrated.

Discussion

In 1970 Black and Hodgson (1971) obtained 10 isolates of *Ser. marcescens* from eight patients in this hospital. All of their isolates were sensitive to carbenicillin and gentamicin. Comparison with the results reported in this paper indicates a marked increase in both the isolation incidence and antimicrobial resistance. A further difference between the two studies is the predominant site of isolation of these organisms. In the earlier study isolates were obtained exclusively from urine or sputum specimens whereas most of our isolates (56 per cent) were from wound swabs.

The association between serratia isolation and compromised hosts, recent surgery and previous antimicrobial therapy (especially cephalosporins) closely parallels the findings of Yu *et al.* (1979), and suggests that the situation in Great Britain may be approaching that observed in the United States.

Reports of gentamicin resistant serratia in Great Britain have been few and brief (Severn, 1977; Meers, Foster and Churcher, 1978; Stephens, Potten and Bint, 1979). Datta *et al.* (1980), found no *Serratia* spp. in an extensive survey of gentamicin resistant enterobacteria. Plasmid-mediated resistance is common among American isolates of *Serratia* spp. (Hedges, 1979) but does not appear to have been reported in Great Britain. The most surprising finding was that there appeared to be three distinct plasmids associated with gentamicin resistance. The high level of resistance conferred by pDJP1 suggests that this plasmid probably determines an

aminoglycoside modifying enzyme (Davies and Smith, 1978). The lower level of resistance associated with pDJP2 appears similar to that described by Alvarez and Regueiro (1980).

Each of the plasmids determined a TEM-type β-lactamase (J. Roud, pers. comm.). However, the plasmid containing isolates accounted for only half of the carbenicillin resistance observed. The carbenicillin-resistant isolates in which neither plasmids nor resistance transfer could be demonstrated, produced only weak β-lactamase reactions (Table II) suggesting that chromosomal mutation is a common mechanism of carbenicillin resistance among these isolates of serratia.

The association of gentamicin resistance with the intensive care unit is consistent with the role of gentamicin usage in the selection of resistant organisms (Yu *et al.*, 1979).

The prevalence of serotype 0:14 was not unexpected since this appears to be the most commonly observed serotype (Pitt *et al.*, 1980). However, the finding that 35 isolates belonged to the same phage subtype demonstrates that this strain is endemic and since 17 of these contained the plasmid pDJP1 suggests that this plasmid was acquired within the hospital. The range of antibiograms associated with this strain emphasizes the role of antibiotic usage as a selection pressure leading to the emergence of resistance (Figure 1). It is also of interest that the only two isolates of serotype 02:/4 both contained apparently different resistance factors that determined gentamicin resistance. One was obtained from a patient in a general surgical unit (pDJP2) and the other from a patient in intensive care (pDJP1). The chronology of isolation suggests that this may have been acquired from one of the serotype 0:14 isolates. Further characterization of these plasmids is in progress.

We would like to thank Dr T. L. Pitt for phage and serotyping these isolates.

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Characterization of spontaneous resistant variants of *Serratia marcescens* selected in the presence of carbenicillin

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Spontaneous carbenicillin-resistant variants were isolated from pigmented *Serratia marcescens* GRI 2677 with frequencies as high as 1 in $7{\cdot}4 \times 10^4$. One-hundred and thirteen such variants were characterized with respect to pigmentation, antimicrobial susceptibility and β-lactamase production.

Sixty-eight were less pigmented than the parent culture, 84 showed low level resistance to five aninoglycosides and 17 showed increased β-lactamase activity. Thirty-three variants were unstable and reverted to the parental phenotype at high frequency. One designated GRI 2677-8, was additionally auxotrophic and was further characterized.

On the basis of these criteria the variants were assigned to one of 21 sub-groups. These results are discussed in relation to resistant clinical isolates and a possible mechanism that explains the diversity of variants.

Introduction

Serratia marcescens is intrinsically resistant to ampicillin but most 'wild' strains including the type culture *Ser. marcescens* NCTC 1377 are sensitive to carbenicillin. However, in a recent epidemiological survey we showed that carbenicillin resistance could be attributed to R-plasmids in only half of the isolates (Platt & Sommerville, 1981*a*) and suggested that chromosomal mutation might account for resistance in the remaining isolates.

Spontaneous mutation is common in *Ser. marcescens.* It affects pigmentation (see Williams & Quadri, 1980) and gives rise to cross resistance among both the aminoglycosides (Traub & Fukushima, 1979) and also the unrelated antimicrobial agents trimethoprim, chloramphenicol and nalidixic acid (Traub & Kleber, 1977).

Clinical isolates of *Ser. marcescens* are rarely pigmented (Clayton & von Graevenitz, 1966) and often resistant to antimicrobials whereas those obtained from soil, water and insects are usually pigmented and drug-sensitive (Grimont & Grimont, 1978).

The aims of this study were to determine, firstly, the proportion of carbenicillin-resistant variants present in cultures of a sensitive strain; and, secondly, whether spontaneous acquisition of the carbenicillin-resistant phenotype was associated with a decrease in pigmentation, cross-resistance with other antimicrobials and increased β-lactamase activity.

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Materials and methods

Organisms

Ser. marcescens GRI 2677 is a pigmented carbenicillin-sensitive clinical isolate originally obtained from the sputum of a patient in Glasgow Royal Infirmary. It was stored as a clinical isolate by harvesting between 10 and 50 colonies from a pure culture (Meynell & Meynell, 1965). On revival, ten single colonies were tested individually for homogeneity of resistance pattern and on the basis of the results obtained two sub-populations (denoted A and B) were defined.

Media

Cysteine-lactose-electrolyte-deficient (CLED) agar (Oxoid CM301) was used throughout for growth.

Isosensitest agar (Oxoid CM471) was used for disc diffusion sensitivity testing and as the source of the inoculum for β-lactamase determination. Minimal agar was prepared as described by Clowes & Hayes (1968).

Incubation

All cultures were incubated at 30°C.

Sensitivity testing

Disc diffusion sensitivity tests were carried out as previously described (Platt & Sommerville, 1981*a*, *b*). The radii of inhibition zones were measured and the following criteria used to grade resistance:

++ = resistant = no inhibition zone,
+ = partial resistance = an inhibition zone radius more than 3 mm smaller than the mean of 12 determinations of the parent culture where 3 mm is greater than 3 standard deviations calculated from the same 12 determinations.

Selection of carbenicillin-resistant variants

CLED agar containing 400 mg/l of carbenicillin was inoculated with 0·1 ml of a bacterial suspension containing between 3 and 8×10^7 organisms/ml and incubated for 36 h at 30°C. Colonies were counted and 113 of these were subcultured on drug-free CLED agar for subsequent characterization. Sub-population A was additionally tested in a similar way on plates containing 100 mg/l of carbenicillin.

Pigment phenotype

Pigmentation variants were assessed subjectively on CLED agar by comparison with the parent organism. They were classified into four categories of decreasing colour p^+ (parent strain), $p^{\pm}$, $p^{\pm}$ and p^- (non pigmented).

Detection of β-lactamase

Tests for the production of β-lactamase were carried out with 'betatest' strips (Medical Wire Co. Ltd.) in accordance with the manufacturer's instructions, and with nitrocefin. Induced and uninduced cultures were tested. Subinhibitory concentrations of carbenicillin were used for induction. The cell suspension method was

used for nitrocefin (O'Callaghan *et al.*, 1972) in microtitre trays. The same strain of *Ser. marcescens* carrying the plasmid RP4 (TEM 2) was used as a positive control throughout.

GRI 2677-8

The reversion of non-growing cells of GRI 2677-8 was examined in distilled water. The organism was harvested from a young (8 h) culture, washed three times and resuspended in distilled water at 30°C. The total count, number of variants and number of revertants was determined by dilution plate counts on CLED agar at two to three day intervals for a period of 21 days.

Results

The pigmentation of *Ser. marcescens* is enhanced on CLED agar and this greatly facilitated the reproducible classification of pigmentation variants.

When disc-diffusion sensitivity tests were carried out on ten identically pigmented colonies of the revived culture of GRI 2677 two resistance patterns were found. The first (sub-population A) was identical to that of the type strain of *Ser. marcescens* (NCTC 1377). Inhibition zone radii to carbenicillin and cefamandole were 16 mm and 7 mm respectively. In the second resistance pattern (sub-population B) a smaller carbenicillin inhibition zone was seen (radius 12·5 mm) and there was no zone with cefamandole. Of the ten colonies tested, four belonged to sub-population A and six to sub-population B.

The number of resistant variants obtained after selection in the presence of 400 mg/l of carbenicillin differed for sub-populations A and B in that, for A the frequency was 1 in $1{\cdot}9 \times 10^7$ and for B, 1 in 10^5.

When resistant variants were selected from sub-population A in the presence of a lower concentration (100 mg/l) of carbenicillin, the frequency was 1 in $7{\cdot}4 \times 10^6$. Most of these variants were indistinguishable from sub-population B and were not characterized further. However, one non-pigmented variant, designated GRI 2677-8, produced smaller colonies on conventional media and unlike the parent organism, it failed to grow on minimal agar. It produced no zone around discs containing carbenicillin and cefamandole and was partially resistant to streptomycin, kanamycin, gentamicin, tobramycin and amikacin. Overnight broth cultures contained a high proportion (between 10 and 20%) of fully sensitive, pigmented prototrophic organisms, indistinguishable from the parent. Furthermore within individual colonies of this non-pigmented variant, clearly defined regions of pigmentation were seen after three to five days incubation (Figure 1). When GRI 2677-8 was suspended in distilled water, the total count decreased by one log unit, the number of variants decreased by more than three log units and the number of revertants increased by more than one log unit over 21 days (Figure 2).

Characteristics of 113 variants selected in the presence of 400 mg/l of carbenicillin are shown in Table I. Considerable differences are seen both between individual variants and between the variants derived from the two sub-populations. Cross-resistance with the aminoglycosides was found in 81% of the variants from sub-population B but was absent in those from A. All parent colonies and variants produced a weak inducible beta-lactamase detectable with nitrocefin. However, 15% of the variants produced an inducible enzyme which gave a positive reaction in the

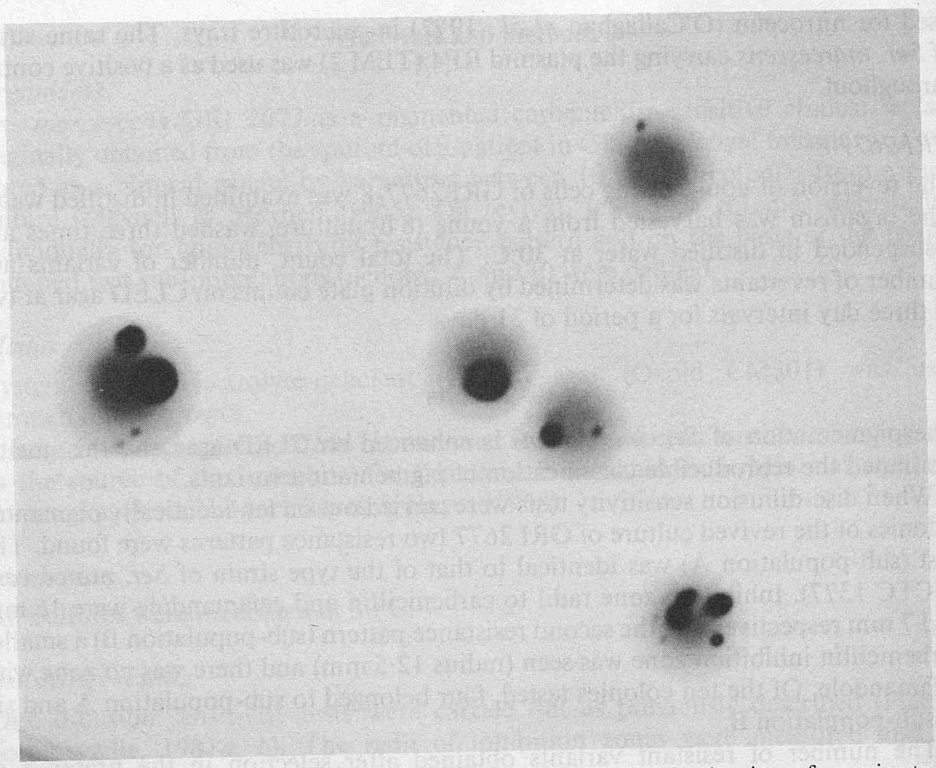

Figure 1. Colonies of *Ser. marcescens* GRI 2677-8 showing clearly defined regions of reversion to the pigmented parental phenotype.

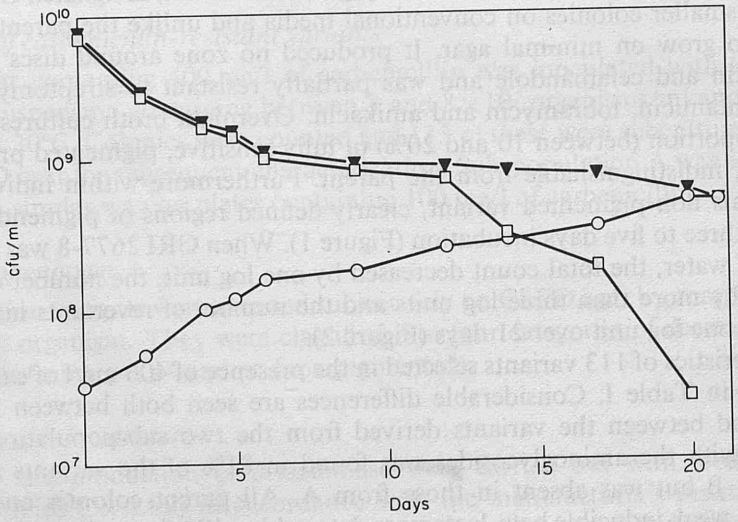

Figure 2. Viability measurements of GRI 2677-8 at 30°C in distilled water. ▼, Total count; □, non-pigmented resistant variant; ○, pigmented sensitive revertant (parental phenotype).

Table I. Characterization of carbenicillin-resistant variants of *Ser. marcescens* selected in the presence of 400 mg/l of carbenicillin

Sub-population	Number	Pigmentation phenotype	Carbenicillin resistance*	Aminoglycoside resistance†	β-lactamase #
A	1	p^+	+	−	+
A	4	p^+	+	−	−
A	1	$p^\pm$	+	−	−
A	3	p^-	+	−	−
B	3	p^+	++	+	−
B	2	p^+	++	−	−
B	1	p^+	+	+	+
B	19	p^+	+	+	−
B	15	p^+	+	−	−
B	3	$p^\pm$	+	+	+
B	13	$p^\pm$	+	+	−
B	1	$Up^\pm$	++	+	+
B	1	$Up^\pm$	++	+	−
B	9	$Up^\pm$	+	+	+
B	21	$Up^\pm$	+	+	−
B	6	$p^\pm$	+	+	−
B	1	p^-	++	+	+
B	1	p^-	++	+	−
B	1	p^-	+	+	+
B	4	p^-	+	+	−
B	3	p^-	+	−	−

*++, no zone of inhibition; +, significantly reduced inhibition zone.

†+, significantly reduced zone of inhibition with streptomycin, kanamycin, gentamicin, tobramycin and amikacin.

#, detected by acidimetric test with penicillin as substrate.

U, indicates that these variants were unstable, and gave rise to mixtures containing fully pigmented, sensitive organisms indistinguishable from the parent clone.

acidimetric test with penicillin as substrate. Typically these variants produced a stronger and/or faster reaction with nitrocefin. In no instance were these reactions comparable to the RP4 control. Sixty per cent of the variants were less pigmented than the parent organism.

Discussion

The presence of two sub-populations in the revived culture may be an artifact of storage or reflect heterogeneity in the original clinical isolate. However, the quantitative difference in the response of the two sub-populations to the selective pressure imposed by carbenicillin is interesting and suggests that a two step process may be involved. This is further supported by the finding that selection of sub-population A with a lower concentration of carbenicillin produced variants with the characteristics of sub-population B.

The aminoglycoside cross-resistance seen in many of the variants is similar to that described by Traub & Fukushima (1979) except that they selected their resistant variants with an aminoglycoside. However, their data also shows that three variants so selected also acquired resistance to carbenicillin. These authors suggested that

their results might be explained by modification of drug uptake brought about by changes in the cell envelope. Our results accord with this hypothesis since both pigment and β-lactamase are associated with the cell envelope (Williams & Quadri, 1980; Sykes & Matthew, 1976). The production of pigmentation variants by strains of *Ser. marcescens* is well recognized (Bunting, 1946). The antimicrobial sensitivity of $p^{\pm}$, $p^{\pm}$ and p^{-} variants of GRI 2677, isolated in the absence of selection pressure, were identical to the parent strain (unpublished observations). This excludes the possibility that selection of weakly pigmenting variants co-selects for resistance and suggests the converse, that selection for resistant variants *per se* predisposes to lesser pigmentation.

Sherratt (1981) has suggested that most spontaneous mutations in *Escherichia coli* are probably the result of illegitimate recombinations. The reversion of GRI 2677-8 to the parental phenotype during growth is a random event consistent with back-mutation as determined by fluctuation tests (Platt & Sommerville, 1981*c*). The results shown in Figure 2 demonstrate population reversion consistent with phenotypic adaptation. However, these apparently disparate findings, in conjunction with the diversity of spontaneous variants described and their high frequency of isolation strongly suggest the involvement DNA rearrangement by illegitimate recombination (Cullum & Saedler, 1981).

The results presented substantiate the suggestion (Platt & Sommerville, 1981*a*) that a significant proportion of carbenicillin resistance in clinical isolates of *Serratia* is mediated by chromosomal mechanisms and further suggests that selection of such variants may result in cross resistance to the aminoglycosides. Although increased β-lactamase activity was detected in some of the variants there was no correlation with the level of increased carbenicillin resistance (Table I). We would therefore consider modification of the permeability barrier to be the principle mechanism of resistance in the variants described.

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Sequential acquisition of R-plasmids *in vivo* by *Salmonella typhimurium*

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Salmonella typhimurium, resistant only to trimethoprim and sulphamethoxazole, was isolated from the faeces and blood of a chronic alcoholic patient in acute renal failure. The isolates harboured an 18 Md non-conjugative plasmid. He was dialysed peritoneally and treated with ampicillin; four days later there was no clinical improvement and his peritoneal dialysis fluid (PDF) had become infected. *Salm. typhimurium* was isolated from faeces and PDF. Both isolates were additionally resistant to ampicillin and contained two plasmids (55 Md and 18 Md). Therapy was changed to chloramphenicol and gentamicin was added to the PDF. Two weeks later *Salm. typhimurium* was again isolated from PDF and faeces. The PDF isolate was unchanged but 4% of the colonies isolated from this faecal specimen were resistant to chloramphenicol and had acquired an additional 62 Md plasmid. From all PDF and faecal specimens two different strains of *Escherichia coli* and one strain of *Klebsiella pneumoniae* were isolated which contained plasmids indistinguishable, on the basis of molecular weight and transferable resistance markers, from those acquired by *Salm. typhimurium*. The transferability of these plasmids *in vitro* to *E. coli* K12 and to the patient's initial *Salm. typhimurium* was studied and the results discussed.

Introduction

The transfer of R-plasmids among the enterobacteria and the clinical problems associated with resistant coliforms are well known (see Stuart-Harris & Harris, 1982). Although epidemiological studies provide ample evidence of the spread of plasmids among bacterial populations and of cross-infection from patient to patient (Knight & Casewell, 1981; Platt & Sommerville 1981*a*), most of this evidence implies rather than demonstrates *in-vivo* transfer. There have been few reports of R-plasmid transfer *in vivo* that have allowed the event to be assessed in relation to the patients commensal flora. Anderson, Gillespie & and Richmond (1973) described R-plasmid transfer in the gut of human volunteers with a laboratory strain of *Escherichia coli* as the donor and more recently Datta, Richards & Datta (1981) reported the acquisition by a strain of *Salmonella typhi*, of two plasmids that conferred resistance to chloramphenicol and co-trimoxazole.

We report here the sequential acquisition of two R-plasmids *in vivo* by a strain of *Salm. typhimurium* which caused septicaemia in a patient who had a long history of chronic alcohol abuse.

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Materials and methods

Clinical specimens were processed by standard methods (Cruickshank *et al.*, 1975). Coliforms were identified by API (20E) and the identity of *Salm. typhimurium* confirmed serologically. Plasmid DNA was demonstrated as previously described (Platt & Sommerville, 1981*b*). Resistance transfer to a nalidixic acid-resistant *E. coli* K12 J53-1 (Bachmann, 1972) was carried out by standard methods described in detail by Kraft, Platt & Timbury (in press). The method of resistance transfer to the patient's initial *Salm. typhimurium* strain differed only in the use of trimethoprim (100 mg/l) to select against donor organisms. Each of the organisms listed in Table I were used as donors in resistance transfer to *E. coli* K12. The two *E. coli* and one *Klebsiella pneumoniae* isolates were used singly and together in conjugation experiments with the patient's initial *Salm. typhimurium* isolate as the recipient. These experiments were repeated in selenite broth base (Oxoid CM 395) containing 4 g/l sodium biselenite.

Case history

A 54-year-old man was admitted to Glasgow Royal Infirmary on 30 April 1982 with profuse diarrhoea of three to four weeks' duration. He was confused in time and place, very dehydrated, apyrexial and had a long history of alcohol abuse. He became anuric and pyrexial on 1 May at which time he was transferred to the Renal Unit and a peritoneal catheter inserted for dialysis. Blood and faecal cultures were taken. *Salm. typhimurium* was isolated from both. The organism was resistant to trimethoprim and sulphamethoxazole but sensitive to all other antimicrobial agents tested including ampicillin and chloramphenicol. Antimicrobial therapy was initially with intravenous ampicillin but later changed to oral chloramphenicol and intraperitoneal gentamicin.

Results

The bacterial isolates from clinical specimens are shown in Table I together with their resistance patterns, plasmids and resistance determinants transferred to *E. coli* K12. The initial *Salm. typhimurium* isolated from venous blood and faeces, was resistant to trimethoprim and sulphamethoxazole and harboured an 18 Md plasmid. Four days later faecal and PDF isolates of *Salm. typhimurium* were resistant to ampicillin and contained an additional plasmid (mol. wt, 55 Md) identical, on the basis of molecular weight and transferable resistance markers, to one carried by a non-lactose-fermenting *E. coli* present in both sites. The patient's therapy was changed from ampicillin to chloramphenicol and gentamicin was added to PDF. Two weeks later *Salm. typhimurium* was again isolated from these sites. The PDF isolate was unchanged but 4% of the colonies isolated from the faecal specimen were resistant to chloramphenicol and had further acquired a plasmid of molecular weight 62 Md that corresponded to a plasmid in the lactose fermenting *E. coli* and the *K. pneumoniae.* Intraperitoneal gentamicin had been discontinued three days before this specimen was collected and was recommenced one day after. One further faecal specimen was examined ten days later. No *Salm. typhimurium* was isolated from primary plates and of 254 colonies obtained after selenite enrichment none was resistant to chloramphenicol though all retained ampicillin resistance.

Table I. Characterization of isolates

Clinical specimen	Date of collection	Isolates	Resistance pattern*	Plasmids (approx. mol. wt $\times 10^6$)	Transferable resistance
Venous Blood	1.5.82	*Salmonella*	Tp Su	18†	None
	5.5.82	*typhimurium*			
	9.5.82				
Peritoneal	5.5.82	*Salm. typhimurium*	Ap Tp Su	18†, 55	Ap
dialysis	7.5.82	*E. coli* (lac^-)	Ap	55, 70†	Ap
fluid	13.5.82	*E. coli* (lac^+)	Ap Su Tc Cm pSm	18†, 62, 80†	Ap Su Tc Cm pSm
	19.5.82	*K. pneumoniae*	Ap Su Tc Cm pSm	62	Ap Su Tc Cm pSm
Faeces‡	1.5.82	*Salm. typhimurium*	Tp Su	18†	None
	5.5.82	*Salm. typhimurium*	Tp Su Ap	18†, 55	Ap
	11.5.82	*Salm. typhimurium*	Tp Su Ap	18†, 55	Ap
	18.5.82	*Salm. typhimurium*	Tp Su Ap	18†, 55	Ap
			Tp Su Ap Tc Cm pSm	18†, 55, 62	Ap Su Tc Cm pSm
	28.5.82	*Salm. typhimurium*	Tp Su Ap	18†, 55	Ap

No *Salm. typhimurium* was isolated from blood cultures taken on 11.5.82 and 15.5.82.
*Tp, trimethoprim; Su, sulphonamide; Ap, ampicillin; Tc, tetracycline; Cm, Chloramphenicol; pSm, low level streptomycin.
†Non-conjugative plasmids.
‡All faeces specimens contained each of the coliforms isolated from peritoneal dialysis fluid in addition to the *Salm. typhimurium* shown.

The 18 Md plasmid detected in the initial *Salm. typhimurium* was non-conjugative and was not mobilised to *E. coli* K12 by either the 55 or 62 Md conjugative plasmids. A similar plasmid (i.e. 18 Md) was detected in the lactose-fermenting *E. coli* which also harboured an 80 Md non-conjugative plasmid. The non-lactose-fermenting *E. coli* carried a 70 Md non-conjugative plasmid.

In conjugation experiments to *E. coli* K12 and to the initial *Salm. typhimurium* isolate the 55 Md plasmid specifying ampicillin resistance transferred readily in all crosses. The 62 Md plasmid which carried multiple resistance was transferred at low frequency from the patient's *E. coli* and *K. pneumoniae* to *E. coli* K12 but no transconjugants were obtained in *in-vitro* crosses with the patient's initial *Salm. typhimurium* isolate as the recipient. This plasmid transferred readily to *E. coli* K12 from the faecal isolate of *Salm. typhimurium* that carried it. From conjugation mixtures containing the patient's two *E. coli* and *K. pneumoniae* strains as donors and his initial *Salm. typhimurium* as the recipient, all of the transconjugants isolated contained a single plasmid of 55 Md which conferred ampicillin resistance. No transfer of any plasmids was seen when conjugation was carried out in selenite broth.

Discussion

Septicaemia caused by *Salm. typhimurium* is uncommon in otherwise healthy individuals and this case may be related to the patient's chronic alcohol abuse. The relationship between antimicrobial therapy and the acquisition of both plasmids is interesting but not unexpected (Anderson *et al.*, 1973; Guinée, 1965). Although his infection had been established for three to four weeks before he was admitted to hospital, the first *Salm. typhimurium* isolates were sensitive to the antimicrobial agents to which his commensal gut flora mediated resistance. This suggests that the selection pressure imposed by ampicillin treatment was required for the ampicillin resistant strain to emerge. The small number and transient isolation of chloramphenicol-resistant *Salm. typhimurium* may have been affected by his concommitant therapy with gentamicin but since procedures for the isolation of salmonellae involve the incubation of faeces in selenite broth we considered the possibility that the chloramphenicol-resistant organisms might be a laboratory artifact. However, no plasmid transfer was demonstrated in selenite broth and the fact that we could not transfer the 62 Md plasmid that specified chloramphenicol resistance to this strain of *Salm. typhimurium in vitro,* weighs against this hypothesis and further suggests that this strain of *Salm. typhimurium* was a poor plasmid recipient. Variable recipient ability has been shown in many organisms including *Salm. typhimurium* (Sanderson, Janzer & Head, 1981) *Enterobacter* species (Platt & Sommerville, 1981*b*) and *Haemophilus parainfluenzae* (Platt & Guthrie, in preparation) and suggests the need to consider recipient ability as a specific parameter in epidemiological studies.

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Antimicrobial resistance and the ecology of *Escherichia coli* plasmids

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SUMMARY

Four hundred and seven clinical isolates of *Escherichia coli* were examined for the presence of plasmids. These isolates comprised 189 which were collected irrespective of antimicrobial resistance (VP) and 218 which were collected on the basis of high-level trimethoprim resistance (TPR). The VP isolates were divided into drug sensitive (VPS) and drug-resistant (VPR) subpopulations.

Plasmids were detected in 88% of VP isolates (81% of VPS and 94% of VPR) and 98% of TPR isolates. The distribution of plasmids in both groups and subpopulations was very similar. However, there were small but statistically significant differences between the plasmid distributions. These showed that more isolates in the resistant groups harboured plasmids than in the sensitive subpopulation (VPS) and that the number of plasmids carried by resistant isolates was greater. Multiple drug resistance was significantly more common among TPR isolates than the VPR subpopulation and this was paralleled by increased numbers of plasmids.

Fifty-eight per cent of VPR and 57% of TPR isolates transferred antimicrobial resistance and plasmids to *E. coli* K12. Of the R^+ isolates, 60% carried small plasmids (MW < 20Md) and 52% of these co-transferred with R-plasmids. These results are discussed.

INTRODUCTION

Since the first reports of transferable resistance to antimicrobials in Japan (Watanabe, 1963), the importance of plasmids both to their bacterial hosts and indirectly to man has been progressively appreciated (see Harwood, 1980). Our understanding of the involvement of plasmids in infection has expanded beyond their role in drug resistance with the realization that more and more clinically important characteristics are or can be plasmid-mediated. These include a variety of virulence attributes – colonization ability (Gaastra & de Graaf, 1982), invasiveness (Harris *et al.* 1982), resistance to host defences (Moll, Manning & Timmis, 1980) and toxin production (Wilshaw *et al.* 1980, 1982). Furthermore, plasmid modification of biochemical characters used in the identification of pathogens can result in failure to recognize and treat infections (Smith & Parsell, 1975).

The development of simple and rapid techniques for the physical detection of plasmids which extend to a wide range of bacterial genera has been a major recent advance in this field (Eckhart, 1978; Birnboim & Doly, 1979; Platt & Sommerville,

1981*a*). Apart from the advantage these techniques confer for the correlation of the presence of particular plasmids with bacterial characteristics such as invasiveness (Sansonetti, Kopecko & Formal, 1982), their simplicity also allows the investigation of large numbers of organisms on a prospective basis to study aspects of their ecology. Because of its clinical relevance (see Stuart-Harris & Harris, 1982) and the importance of resistance markers to recombinant DNA technology, plasmid-mediated drug resistance has received most attention. Many studies have demonstrated the clinical importance of plasmids and transposition (Datta, Nugent & Richards, 1980; Platt, Sommerville & Gribben, 1984) but the quantitative aspects remain ill-defined. Early studies relied exclusively on the transfer of resistance markers which, without physical characterization of the plasmids, can lead to misinterpretation of results (Kraft, Platt & Timbury, 1983, 1984).

Transferable resistance in the enterobacteria often accounts for as little as 20% of the resistance detected leaving the greater part unexplained. However, published estimates show that the incidence of transferable resistance varies and depends upon many factors such as the range of genera and the choice of resistance marker studied, and also whether the collection of organisms investigated includes epidemiologically related isolates. Datta *et al.* (1980), in a collection of enterobacteria selected for trimethoprim and gentamicin resistance found 23% and 78% respectively to be transferable. In a survey of *Serratia* spp. isolated in one year, Platt & Sommerville (1981*b*) demonstrated 100% transferability of gentamicin resistance compared to only 50% in the case of carbenicillin resistance, the remaining 50% being due to mutation (Platt, Sommerville & McGroarty, 1983). Furthermore, the choice of recipient strains in conjugation experiments can markedly influence transferability (Sanderson, Janzer & Head, 1981). Although *Escherichia coli* K12 is almost invariably used it may give a falsely low estimate of R-transfer when different enteric genera are used as plasmid donors (Cooksey, Thorne & Farrar, 1976; Platt & Sommerville, 1981*a*).

Previous studies in this department (Kraft, Platt & Timbury, 1983; Sommerville & Platt, unpublished) suggested that R-plasmids represented only a small proportion of the extrachromosomal DNA pool in enterobacteria. However, such a pool could be expected to contribute significantly to the fluidity of resistance genes (Sherratt, 1982). We were therefore prompted to investigate plasmids in isolates of *E. coli* collected irrespective of antimicrobial resistance with the aim of studying the contribution of R-plasmids relative to the overall extrachromosomal gene pool. Here we report the distribution, transfer and mobilization of plasmids in these isolates compared with those obtained from *E. coli* isolates collected on the basis of trimethoprim resistance.

MATERIALS AND METHODS

Bacteria

A total of 407 clinical isolates of *E. coli* were collected between 1979 and 1983, obtained from the routine diagnostic laboratories of Glasgow Royal Infirmary and other hospitals in the Eastern District of Glasgow; of these, 189 were collected from patients with vascular disease (147 from rectal swabs and 42 from wound swabs). The isolates from vascular patients (VP) were divided into 'antibiotic sensitive'

(VPS) and 'antibiotic resistant' (VPR) subpopulations by comparison with *E. coli* K12 in a disk-diffusion sensitivity test as previously described (Platt & Sommerville, 1981*b*). The remaining 218 isolates were *E. coli* from urinary-tract infections collected on the basis of high-level trimethoprim resistance (MIC > 1024 mg/l) (TPR).

E. coli K12 J53-1, J62-1 (Nal^r) and J53-2, J62-2 (Rif^r) (Bachmann, 1972) were used as recipients in conjugation experiments.

Isolation and identification

Standard procedures were used for the isolation of organisms from clinical material (Cruickshank *et al.* 1975). In addition, rectal swabs were plated directly onto CLED agar (Mast DM110) and isosensitest agar (Oxoid CM471) with the following antibiotic disks: carbenicillin 100 μg, cefazolin 30 μg, tetracycline 10 μg, kanamycin 30 μg, streptomycin 10 μg, chloramphenicol 30 μg, sulphamethoxazole 25 μg and trimethoprim 1·25 μg. This facilitated the isolation of resistant strains present in the specimen in small numbers. Any coliforms growing within the inhibition zones were purified before identification by the API 20E system.

Detection and characterization of plasmids

Plasmid DNA was obtained in crude SDS lysates from cultures of both donors and transconjugants grown on nutrient agar (Oxoid CM3). It was subjected to electrophoresis in 0·7 % agarose gels and plasmid molecular weight was estimated as previously described (Platt & Sommerville, 1981*a*).

Resistance transfer to *E. coli* K12 was carried out using a standard broth mating technique and the antimicrobial susceptibility of transconjugants determined by disk sensitivity testing.

Statistical analysis

The chi-squared test was used to compare the plasmid distributions and also the degree of multiple drug resistance. Other results were compared using a chi-squared 2×2 contingency test incorporating Yates correction for continuity (Siegel, 1956).

RESULTS

Four hundred and seven isolates of *E. coli* were studied. Of the 189 VP isolates from vascular patients, 90 were fully sensitive (VPS) to all of the antimicrobial agents tested (ampicillin, tetracycline, chloramphenicol, trimethoprim, sulphamethoxazole, streptomycin, and kanamycin) and 99 were resistant (VPR) to at least one drug. There was no evidence of cross-infection during the collection of these isolates. The 218 isolates collected on the basis of high-level trimethoprim resistance (TPR) were all resistant to at least one other agent. The numbers of isolates with different degrees of multiple resistance are shown in Table 1. There was significantly more multiple drug resistance among the TPR isolates than the VPR subpopulation ($P < 0{\cdot}001$), which shows that the collection of trimethoprim-resistant organisms selects for multiple drug resistance. This is further supported by the observation that the small number of trimethoprim-resistant strains among the VPR isolates were also multiply-resistant.

Table 1. *The distribution of multiple antibiotic resistance among TPR isolates, VPR isolates and the trimethoprim resistant subgroup of VPR isolates*

Number of antimicrobial agents to which isolates were resistant	TPR: isolates collected on the basis of trimethoprim resistance		VPR: resistant subpopulation of vascular patient isolates		Trimethoprim-resistant subgroup of VPR isolates
	Number	(%)	Number	(%)	Number
1	0		18	(18)	0
2	8	(4)	24	(24)	0
3	17	(8)	27	(27)	1
4	52	(24)	12	(12)	1
5	77	(35)	12	(12)	8
6	39	(18)	1	(1)	0
7	25	(11)	5	(5)	5
Total	218	(100)	99	(100)	15

Plasmids were detected in most of the 407 isolates; 98 % of TPR isolates, 94 % of VPR isolates and 81 % of VPS isolates. Some isolates harboured up to seven plasmids. The distribution of plasmids in TPR and VP isolates and VPR and VPS subpopulations is shown in Fig. 1 (*a–d*) respectively. The principle feature of these distributions is their similarity which shows that the acquisition of antimicrobial resistance by *E. coli* has not dramatically affected the number of plasmids harboured. However, there were small but significant differences when the individual distributions were compared. The VPS subpopulation (*d*) contained more plasmid-free isolates than the VPR subpopulation (*c*) ($P < 0{\cdot}01$) and the VPR isolates contained more plasmid-free organisms than the TPR collection (*a*) ($P < 0{\cdot}001$). Comparison of the overall plasmid distributions showed that a significantly greater proportion of VPR isolates contained more plasmids than did the VPS isolates ($P < 0{\cdot}05$) and similarly TPR isolates carried more plasmids than VPR isolates ($P < 0{\cdot}01$).

To exclude the possibility that small plasmids (<20 Md) contributed disproportionately to these data, the distribution of potentially self-transmissible plasmids (>20 Md) (Broda, 1979) was calculated (Fig. 2*a–d*). A comparable similarity in the distributions is apparent which indicates that small plasmids are equally distributed among the different populations. Statistical comparison of the distributions also produced similar results which indicates that the resistance-associated differences are the result of changes in the numbers of large, potentially self-transmissible plasmids.

Resistance transfer was demonstrated from 57 % and 58 % of TPR and VPR isolates respectively but from only 31 % of the VP isolates overall. Comparison of the plasmid distribution in TPR and VPR isolates that transferred resistance to *E. coli* K12, showed no significant difference in the numbers of plasmids present. However, there were considerably more large plasmids (>20 Md molecular weight) in the TPR isolates than in VPR subpopulation; this difference was significant ($P < 0{\cdot}01$). The mobilization of small plasmids from TPR and VPR isolates that transferred resistance is summarized in Table 2. There were no significant differences between the two collections as regards the number of small plasmids

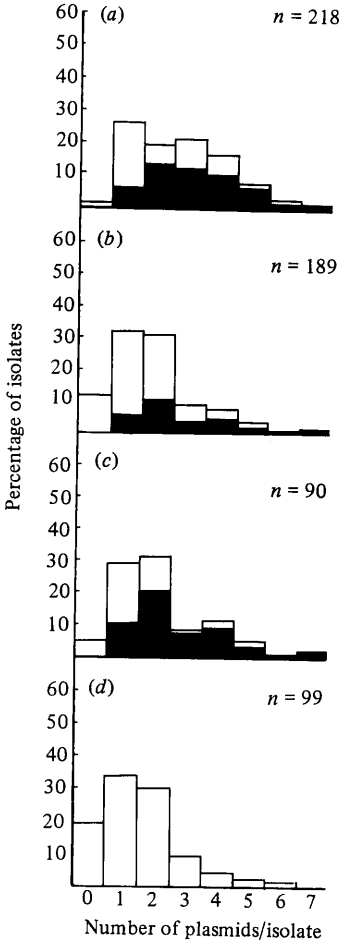

Fig. 1.

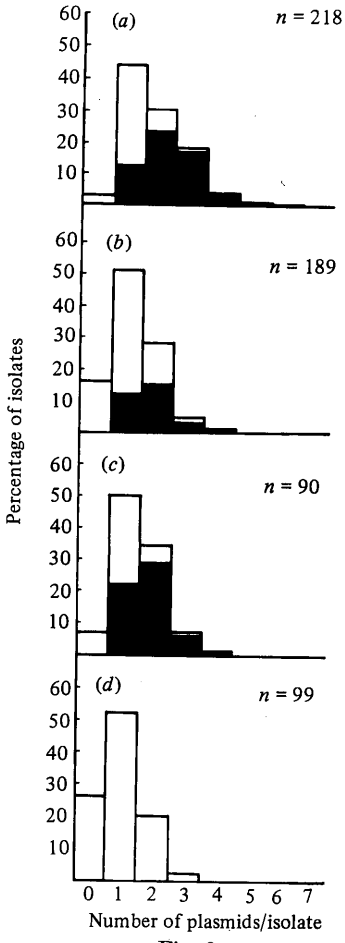

Fig. 2.

Fig. 1. The distribution of plasmids in *E. coli* collected on the basis of high-level trimethoprim resistance (TPR) (*a*) and isolates collected from patients with vascular disease (VP) (*b*). (*c*) and (*d*) are resistant and sensitive subpopulations of VP isolates respectively. ■, Proportion of isolates harbouring self-transmissible R-plasmids.

Fig. 2. The distribution of plasmids > 20 Md in *E. coli* collected on the basis of high level trimethoprim resistance (TPR) (*a*) and isolates collected from patients with vascular disease (VP) (*b*). (*c*) and (*d*) are resistant and sensitive subpopulations of VP isolates respectively. ■, Proportion of isolates harbouring self-transmissible R-plasmids.

present or the number mobilized ($P > 0{\cdot}05$). Over half the isolates contained small plasmids and 52 % of these were mobilized by the R-plasmid(s) present.

DISCUSSION

Our most notable findings were the large number of plasmids in the fully sensitive isolates and the broad similarity of the distribution of plasmids in sensitive and resistant isolates. This suggests that R-plasmids comprise only a

Table 2. *Mobilization of small plasmids (< 20 Md) by transferable R-plasmids from VPR isolates and isolates collected on the basis of trimethoprim resistance (TPR)*

	VPR		TPR	
	Isolates	Plasmids	Isolates	Plasmids
No. of isolates transferring resistance	58	—	124	—
Small plasmids present	33 (57 %)	62	79 (64 %)	124
Small plasmids mobilized	17 (29 %) (52 %)*	32 (52 %)	41 (33 %) (52 %)*	69 (56 %)
Small plasmids not mobilized	16 (28 %)	30 (48 %)	38 (31 %)	55 (44 %)

* Small plasmids mobilized as a proportion of small plasmids detected.

Table 3. *The increased plasmid content of resistant populations (VPR and TPR) of* E. coli *relative to the sensitive population (VPS)*

	E. coli population		
	VPS	VPR	TPR
Total no. of plasmids detected	141	226	590
No. of isolates	90	99	218
No. of isolates harbouring plasmids	73	93	214
No. of plasmids per isolate calculated after exclusion of plasmid free isolates from each population	1·93	2·43	2·76
Plasmids per isolate as a percentage of VPS	100	126	143

minor part of the extrachromosomal gene pool of *E. coli*. Our results also indicate that the acquisition of resistance has resulted in a small but significant change in the distribution of plasmids.

Plasmids that were too small to be self-transmissible, appear to play a minimal role in antibiotic resistance. However, they were widely distributed among our isolates and the frequency with which they were mobilized was comparable to the transfer frequency of R-plasmids (Table 2). This suggests that the lack of transfer genes does not materially diminish their mobility and that their contribution to evolution within the accessory gene pool of bacteria may have been underestimated.

Early work with collections of resistant enterobacteria led to the belief that the use of antibiotics results in the formation and dissemination of conjugative R-plasmids (Anderson, 1965). Our results support this suggestion. In particular, when each of the resistant groups was compared with the sensitive VPS group there was a 26 % increase in the total number of plasmids carried by the VPR subpopulation whereas the corresponding increase in the TPR collection was 43 % (Table 3). Thus, it appears that the acquisition of drug resistance was associated with an increase in the number of plasmids harboured; an increase in the incidence of multiple drug resistance between the two resistant collections was paralleled by a further increase in plasmid numbers. It is interesting that despite these differences the proportion of transferable resistance remained unchanged.

Hughes & Datta (1983) and Datta & Hughes (1983) demonstrated that many bacteria isolated in the pre-antibiotic era contain conjugative plasmids. They

concluded that conjugative plasmids were as common then as they are in drug-sensitive strains today and suggested that the R-factors prevalent today are derived from them. Furthermore, their interpretation of these results was in cotrast to Anderson's conclusions (1965). However, our finding of large numbers of plasmids in sensitive isolates together with the increased number of plasmids in resistant isolates reconciles both views. Thus, a small number of transfer events, which occur under the selection pressure imposed by antibiotic usage, can provide a large potential for macro- and micro-evolution of plasmids by the mechanisms described by Cohen *et al.* (1977).

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Effect of RP4 on growth and pigmentation in *Serratia marcescens*

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Abstract

The plasmid RP4 was studied in two pigmented strains of *Serratia marcescens*. The acquisition of RP4 resulted in the production of paler colonies with a lower growth rate than the plasmid-free parent organisms. Elimination of the plasmid either spontaneously or by treatment with ethidium bromide restored both pigmentation phenotype and growth rate to former levels.

Comparison of the spectra of prodigiosin extracted from parent strains and those carrying RP4 showed no qualitative differences in absorption peaks. The electrophoretic mobility of pigment extracted from RP4$^+$ and RP4$^-$ strains was identical. When pigment was extracted quantitatively from parent strains and transconjugants, and standardised to a constant cell density the absorbance at 540 nm was the same. This indicated that the decrease in pigmentation associated with RP4 carriage results from plasmid supression of growth rate.

Introduction

Strains of *Serratia marcescens* isolated from the terrestrial environment are usually pigmented (Grimont and Grimont, 1978) whereas clinical isolates are almost always non-pigmented (Wilkowske *et al.*, 1970; Platt and Sommerville, 1981a). The latter are often multiply resistant to antimicrobial agents and such resistance may be either plasmid mediated (see Hedges, 1980; Platt and Sommerville, 1981b) or the result of mutation (Traub and Fukushima, 1979; Platt *et al.*, 1983). Non-pigmented variants arise spontaneously when pigmented strains of *S. marcescens* are subcultured (Bunting, 1946).

Preliminary studies (Platt and Sommerville, 1981c) showed that when the plasmid RP4 was transferred to a pigmented strain of *S. marcescens* the transconjugants were less pigmented than the recipient and, furthermore, that pigmentation was restored to the former level when the plasmid was eliminated.

Plasmid carriage imposes a biosynthetic burden on the host strain (Sherratt, 1982), and Zund and Lebek (1980) showed that many R-plasmids prolonged the generation time of *E. coli*. They also found a positive correlation between plasmid molecular size and increase in doubling time. Since the pigment prodigiosin is a secondary metabolite (Williams and Quadri, 1980) its production is likely to be affected by growth rate. The aims of the study reported here were to determine whether there were qualitative differences in the pigment produced by *S. marcescens* carrying RP4 and whether the effect was related to growth rate.

Materials and methods

Media

Cysteine-lactose-electrolyte-deficient (CLED) agar (Oxoid CM301) was used for growth rate determination. A medium of identical formulation but lacking the indicator bromo-thymol blue was prepared in this laboratory from Oxoid ingredients. Broth media corresponding to the two CLED agars were similarly prepared but without agar.

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Isosensitest agar (Oxoid CM 471) was used for sensitivity testing and Brain Heart Infusion Broth (Oxoid CM 225) for conjugation. Nutrient agar (Oxoid CM3) was used for the growth of organisms for DNA extraction and electrophoresis.

Organisms and plasmids

Two pigmented strains of *S. marcescens* were studied, GRI 2677 and GRI 179. Both were isolated from clinical material obtained from epidemiologically unrelated patients, several years apart. Neither strain contained plasmids. RP4 was kindly provided by Professor N. Datta (Royal Postgraduate Medical School, Hammersmith Hospital, England) and was transferred to *S. marcescens* from strains of *Escherichia coli* K12.

Detection of Plasmids

Agarose gel electrophoresis was used for the confirmation of plasmid transfer and the determination of molecular weight as previously described (Platt and Sommerville, 1981b). Plasmids were eliminated using ethidium bromide by the method of Bouanchaud *et al.* (1969).

Conjugation to *S. marcescens*

Plasmid transfer from *E. coli* K12 was carried out by standard overnight broth mating. Cefazolin (100 μg/ml) was used to select against the donor, and an appropriate plasmid specified resistance determinant to select against the recipient.

Determination of colony radial growth rate (K_r)

Colony radial growth rate was determined as described by Pirt (1967). Bioassay plates 243 mm square (Nunc) containing 250 ml CLED agar were inoculated with dilutions of each culture to give between thirty and sixty colonies each separated by at least 10 mm. This prevented competition for nutrients from influencing colony radial expansion. Plates were incubated at 30°C.

At intervals after the appearance of colonies (about 15 h) the diameter of a minimum of twenty colonies was measured using a Leebrook Microbiological Zone Reader (Leebrook Scientific Instrument Company Ltd, 22 Goddington Road, Stroud, Kent, England) giving a linear magnification of 7.5:1. The colony radial growth rate (K_r) was calculated in μm/h from plots of the mean colony radius against time.

Pigment extraction and spectroscopy

For the qualitative comparison of pigmentation in strains with and without plasmids, prodigiosin was extracted from early stationary cultures using acidic methanol by the method of Quadri and Williams (1972). Absorption spectra were determined between 300 and 700 nm using a Unicam SP800 spectrophotometer.

For the quantitative comparison of pigment in plasmid-carrying and plasmid-free *Serratia*, early stationary cultures were harvested by centrifugation, washed in distilled water and resuspended to constant absorbance at 680 nm (Meynell and Meynell, 1965) in tris-borate buffer (pH 8.2). Ten per cent sodium dodecyl sulphate (SDS) was added with gentle mixing to 1% final concentration. Lysis was completed by incubation at 50°C for 10 min.

Cell debris and chromosomal DNA were removed by centrifugation at 30,000 rpm (48,000 x *g*) for 15 min. The supernate was removed and spectral absorption determined using a Beckman DU-7 spectrophotometer.

Electrophoresis of prodigiosin

When agarose gel electrophoresis was used to confirm the acquisition of plasmids a single band of visible pigment was seen to migrate to the cathode just ahead of the bromophenol blue tracker dye.

This technique was used for the comparison of pigment from strains with and without plasmids and also to compare pigment extracted by organic solvents with pigments extracted by SDS lysis of cells.

Pigment extracted by organic solvents was diluted 1:1 with either tris-borate buffer or 10% SDS before electrophoresis.

After electrophoresis the pigmented bands were examined for fluorescence in ultra violet light (254 nm), and then stained for the presence of protein with Coomassie blue by the method of Weber and Osborn (1969).

Results

S. marcescens transconjugants that acquired RP4 produced colonies which were visibly less pigmented (Figure 1). The difference in pigmentation between RP4$^+$ and plasmid-free organisms was more pronounced for GRI 2677 than GRI 179.

Characterization of extracted pigment

When pigment was extracted qualitatively from the two strains of *Serratia*, with and without plasmids, the spectra obtained showed no differences in absorption peaks either between the two strains studied or between plasmid-free and plasmid-carrying organisms. The spectral characteristics of the extracted pigments were as previously reported by Quadri and Williams (1972), and Williams and Quadri (1980).

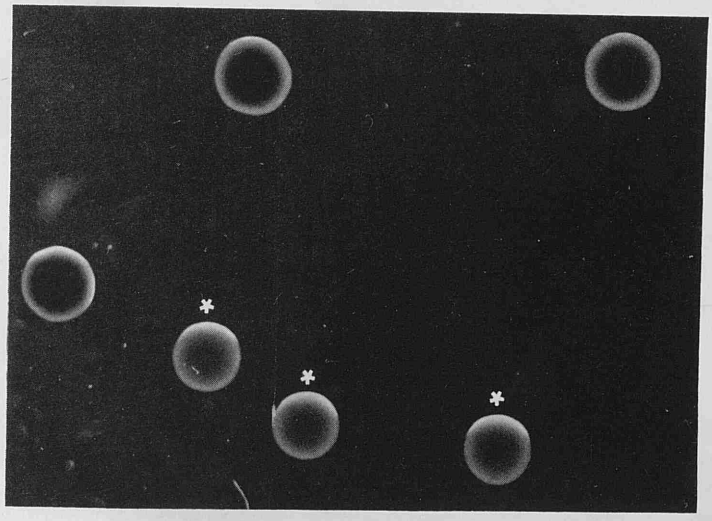

Figure 1 The effect of RP4 on pigmentation of *Serratia marcescens* GRI 2677; *indicates RP4 colonies.

The electrophoretic mobility of pigment extracted by SDS treatment from both strains of *S. marcescens* was identical and was unaffected by the presence of the plasmid studied. The pigment ran as a single band just ahead of the bromophenol blue tracker dye. Organically-extracted pigment in tris-borate buffer remained at the origin. However, organically extracted pigment diluted in SDS migrated to the same point in the gel as SDS extracted pigment. All bands fluoresced weakly in UV light and none stained with Coomassie blue.

Effect of plasmids on growth rate

Preliminary studies indicated that the absorbance (680 nm) of early stationary phase cultures of RP4$^+$ organisms was lower than the plasmid free strains. However, growth rate determination by viable count gave inconsistent results, and colony radial growth rate was determined because of its greater precision. The radial growth of colonies was linear between 15 and 60 h and is shown in Figure 2. The acquisition of RP4 lowered the radial growth rate of both strains of *S. marcescens* (Table 1). Elimination of plasmids either by ethidium bromide or spontaneous curing restored both the pigmentation phenotype and growth rate to former levels.

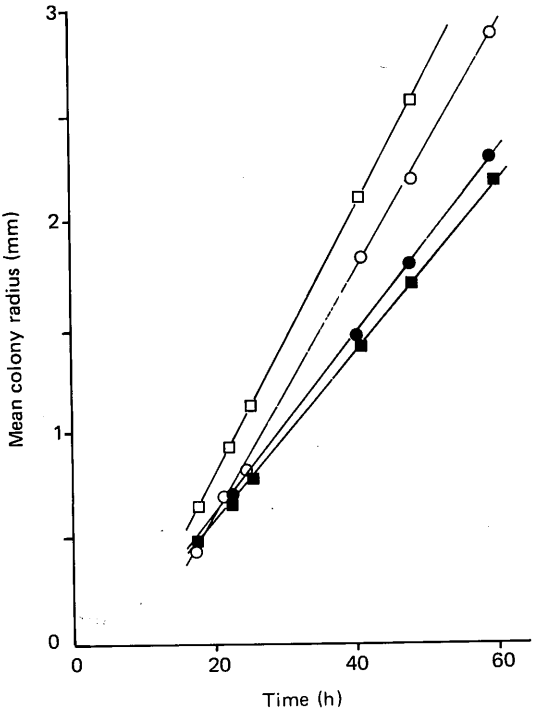

Figure 2 Radial growth* of colonies of *S. marcescens* GRI 179, ○; GRI 179 RP4, ●; GRI 2677, □; and GRI 2677 RP4, ■. *Mean colony radius was determined from measurements of a minimum of twenty colonies on three occasions; standard error on each determination was <1.5%.

Table 1 Colony radial growth rate (K_r) of strains of *S. marcescens*

Strain	Plasmid	K_r (μ/h)
GRI 179	none	59.4
	RP4	49.5
GRI 2677	none	64.1
	RP4	40.6

Spectroscopy of pigment extracted by lysis with SDS gave the principle absorption peak of prodigiosin hydrochloride at 539–540 nm with the characteristic shoulder at 510 nm (Figure 3). This shows the difference in peak height of pigment extracted from early stationary cultures of GRI 2677 and GRI 2677 RP4. When organisms containing RP4 were standardised to constant cell density and pigment extracted, no difference was found in the height of the 540 nm absorbance peak. This confirms that the plasmid associated differences in colonial pigmentation are a function of growth rate depression rather than direct interaction of plasmid with the pigmentation process.

Discussion

The production of pigmented variants of *S. marcescens* is well known (Bunting, 1946). The selection of spontaneous carbenicillin resistant variants is associated with a decrease in colonial pigmentation (Platt *et al.*, 1983), and similar results were obtained with kanamycin selection (unpublished). However, the correlation between decreased colonial pigmentation, the demonstrable acquisition of plasmid DNA, and the restoration of pigmentation to the level of the plasmid-free strain when the plasmid was eliminated suggested that the two events were causally related. The association of prodigiosin with the cell envelope (Williams and Quadri, 1980), and the influence of transferable plasmids on the cell envelope (Manning and Achtman, 1979), raised the possibility that the presence of plasmids might directly affect the pigment itself or its localisation within the cell envelope.

The electrophoretic behaviour of pigment, its fluorescence and failure to stain with Coomassie blue even after extraction by gentle methods (SDS) suggest that any association with cellular components, particularly protein, is weak. The identical electrophoretic migration of pigment extracted from R^+ and R^- organisms together with the absence of detectable differences in spectral absorption suggest that the phenotypic differences observed between R^+ and R^- organisms are not attributable to plasmid mediated alteration of the pigment structure.

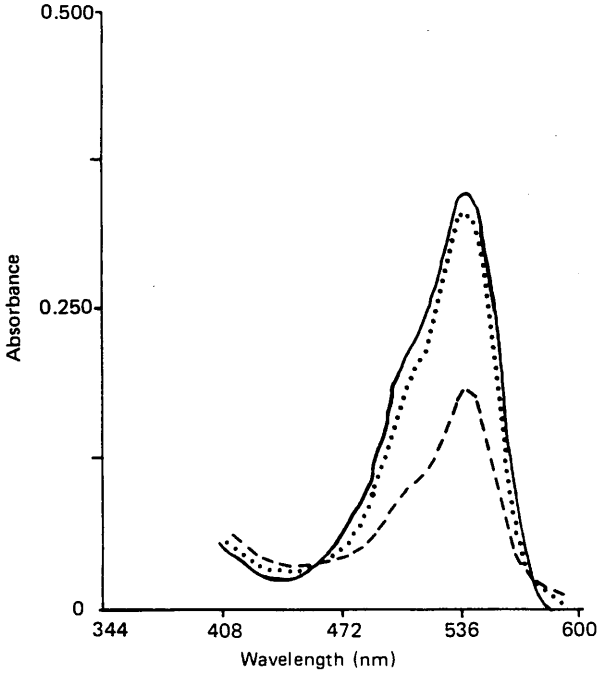

Figure 3 Absorption spectra of prodigiosin hydrochloride extracted from early stationary cultures of *S. marcescens* GRI 2677: plasmid free (———), RP4 (— — — —), RP4 standardised to the cell density of the plasmid free strain before extraction (· · · · · ·). Absorbance peak values (540 nm) respectively 0.351, 0.183 and 0.334.

The further possibility that the phenotypic change in pigmentation, associated with the acquisition of RP4, resulted from the role of prodigiosin as a secondary metabolite (Williams, 1973; Hopwood, 1981) is excluded by the demonstration that standardisation of early stationary cultures to constant cell density at 680 nm gave a constant absorbance maximum (Figure 3).

Holland and Dale (1980) showed that the plasmids R1 and R46 increased the number of pigmentation variants produced by *S. marcescens*. These observations, in conjunction with the results presented here suggest that the phenotype of pigmented *S. marcescens* may be a useful adjunct for the investigation of plasmid/host cell interactions.

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R-plasmid transfer *in vivo*: a prospective study

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Summary. Two hundred and eighty-seven coliform bacteria were isolated from 116 rectal swabs or faecal specimens obtained from 113 patients. By means of plasmid analysis and resistance transfer (R-transfer) *Escherichia coli* was found to differ from other enteric genera in plasmid distribution. Criteria were proposed that enabled in-vivo "R-transfer potential" and in-vivo "R-transfer rate" to be calculated. From each of 22 of the 113 patients numerous coliforms were isolated, of which at least one per patient contained one or more self-transmissible R-plasmids potentially transferable to 43 other coliforms. Evidence indicated that R-plasmid transfer had occurred *in vivo* on two of the 43 potential occasions. These results are discussed in the context of plasmid ecology in the human host.

Introduction

The clinical problems associated with infections caused by resistant coliform bacteria and the role of plasmids in resistance are well known (Casewell, 1982; Stuart-Harris and Harris, 1982). The importance of resistance transfer (R-transfer) *in vivo* is commonly inferred from epidemiological evidence (Knight and Casewell, 1981; Platt and Sommerville, 1981*a*). However, detailed studies of in-vivo transfer are few and have often been limited by the experimental system used. Jarolmen and Kemp (1969) and Smith (1971) concluded that in-vivo transfer in animals was a rare event influenced by the characteristics of the donor and recipient organisms, the plasmid, the host environment and antimicrobial therapy. Studies in man have also suggested the infrequency of in-vivo R-transfer (Hartley and Richmond, 1975; Petrocheilou *et al.*, 1976; Richmond and Petrocheilou, 1978). More recently, reports have described the acquisition of several R-plasmids by *Salmonella* species in individual patients (Datta *et al.*, 1981; Platt *et al.*, 1984*a*). The observation that most strains of *E. coli*, collected irrespective of drug resistance, harbour plasmids (Platt *et al.*, 1984*b*) prompted us to examine the distribution of plasmids among enteric organisms and to reconsider in-vivo R-transfer on a prospective basis.

Materials and methods

Specimen collection and processing

Sixty-two faecal specimens were obtained from the routine diagnostic laboratory and 54 rectal swabs from in-patients in the peripheral vascular disease unit, Glasgow Royal Infirmary. None of the latter were known to have any gastro-intestinal infection at the time of specimen collection, but patients with suspected mesenteric ischaemia were not excluded.

Faecal suspensions (*c.* 0·5 g/10 ml of sterile distilled water) and rectal swabs were plated on CLED (Mast DM110) and MacConkey (Oxoid, CM7B) Agars for isolation of single colonies. A direct sensitivity test was performed on Isosensitest Agar (Oxoid CM471) with antibiotic disks (Oxoid) containing carbenicillin 100 μg, cefazolin 30 μg, tetracycline 10 μg, kanamycin 30 μg, streptomycin 10 μg, chloramphenicol 10 μg, sulphamethoxazole 25 μg and trimethoprim 1·25 μg to select any resistant coliforms that were present in small numbers. The plates were examined after overnight incubation aerobically at 37°C. From the CLED and MacConkey plates six colonies of each distinct colony type were suspended in 2·5 ml of Isosensitest Broth (Oxoid, CM473) and plated separately for purity on CLED. They were tested for homogeneity in disk-diffusion sensitivity tests with disks containing: nalidixic acid 30 μg, rifampicin 50 μg, ampicillin 10 μg, carbenicillin 100 μg, cefazolin 30 μg, cefamandole 30 μg, colistin sulphate 25 μg, tetracycline 10 μg, kanamycin 30 μg, streptomycin 10 μg, gentamicin 10 μg, tobramycin 10 μg, amikacin 10 μg, sulphamethoxazole 25 μg, trimethoprim 1·25 μg, and chloramphenicol 10 μg.

Any coliform colonies growing within the inhibition zones on the direct sensitivity plates were similarly purified and their sensitivities were tested. Pure cultures were identified by means of the API 20E system and stored for further investigation.

Plasmid detection

Cell lysis, electrophoresis and plasmid visualisation were performed as described by Platt and Sommerville (1981*b*).

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DNA purification and digestion by restriction endonucleases

DNA was prepared from 5 ml of an overnight culture in Brain Heart Infusion Broth (Oxoid, CM225) by the alkaline SDS method of Birnboim and Doly (1979) as modified by Ish-Horowitz and Burke (1981). After extraction with a mixture of phenol and chloroform, DNA was precipitated with an equal volume of isopropanol at room temperature. The precipitate was dissolved in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA, *p*H 8·0); 50 μl of 7·5 M ammonium acetate were added with mixing followed by 300 μl of ice-cold ethanol. After at least 30 min at −18°C the precipitate was deposited by centrifugation and redissolved in 80 μl of TE buffer, treated with 9 μl of RNAase 1 mg/ml and incubated for 30 min at 37°C. A 10-μl volume of 2·5 M sodium chloride was added and DNA was obtained by isopropanol precipitation, ammonium acetate treatment, and ethanol reprecipitation as above. The final pellet was resuspended in 30 μl of TE buffer. The restriction enzymes *Hin*d III, *Pst* 1, *Sma* 1, *Alu* 1, and *Hae* III were obtained from BRL (Bethesda Research Laboratories) and 4 μl of each enzyme were used in a 50-μl reaction mixture in eppendorf tubes containing 10 μl of DNA with buffers, as recommended by the manufacturer. The tubes were incubated for 4 h at 37°C after which 5 μl of bromophenol blue tracker dye were added to each and the contents loaded on to 0·7–1·0% horizontal agarose gels. Electrophoresis was performed at 18 mA overnight. The running buffer was tris-borate, *p*H 8·2, containing ethidium bromide 0·3 μg/ml. The mol. wts of restriction fragments were calculated by comparison with *Hin*d III, *Pst* 1 and *Hae* III digests of phage lambda DNA.

Antibiotic resistance transfer

Organisms resistant to antimicrobial agents other than those to which the species was intrinsically resistant were tested for R-transfer after overnight broth mating by standard methods (Kraft *et al.*, 1983). Recipient organisms used were *E. coli* K12 J53-2 (lac^+, rif^r) and K12 J62-2 (lac^-, rif^r) (Bachmann, 1972) and *Enterobacter aerogenes* GRI 9880 (Platt and Sommerville, 1981*b*). Methods used for the confirmation of R-transfer were as described in detail by Kraft *et al.* (1983).

Antimicrobial therapy

The antimicrobial therapy prescribed during the 30 days preceding the collection of specimens was recorded.

Calculation of in-vivo "R-transfer potential" and in-vivo "R-transfer rate"

Two indices were calculated to describe the likelihood of R-transfer *in vivo*. The in-vivo R-transfer potential was the number of combinations of (i) a transferable plasmid of mol. wt $>20\times10^6$ in a donor with (ii) a potential recipient in the same specimen. The in-vivo R-transfer rate was the number of times transfer of a plasmid of mol. wt $>20\times10^6$ was detected/the R-transfer potential.

The following criteria were applied separately to each specimen for the purposes of the calculation. (a) A given specimen must have yielded at least two coliforms distinguishable biochemically or by resistance pattern. (b) At least one coliform must have harboured one or more R-plasmids transferrable to *E. coli* K12. (c) Each coliform that fulfilled criterion (b) was considered as a separate donor. (d) In the presence of a plasmid donor every other distinguishable coliform isolated from a given specimen was considered as a separate potential recipient. (e) When a single donor transferred more than one plasmid of mol. wt $>20\times10^6$ independently, the R-transfer potential was calculated separately for each plasmid; and conversely when co-transfer of two or more plasmids occurred without demonstrable segregation the R-transfer potential was calculated as for a single plasmid.

Statistical analysis

The chi-squared test was used to compare the plasmid distributions (Siegel, 1956).

Results

One hundred and sixteen specimens were obtained from 113 patients. Two hundred and eighty-seven coliforms were isolated, of which 161 were *E. coli*. The other 126 belonged to diverse enteric genera and are listed in table I, which also shows the numbers of plasmids harboured and the resistance of strains to antimicrobial agents. Drug resistance was more common among *E. coli* isolates than among those of other enteric genera. Although the numbers of isolates of enteric genera other than *E. coli* were too small for individual analyses, they appeared similar with respect to a low incidence of resistance and small numbers of plasmids. The latter is most clearly seen in fig. 1, which shows the distribution of plasmids among *E. coli* and other enteric genera. The distribution of plasmids was significantly different ($\chi^2_{5df}=54{\cdot}104$; $p<0{\cdot}001$) and, with the possible exception of *Enterobacter* spp. for which the sample size was very small, strains of other enteric genera contained fewer plasmids than *E. coli*. However, when the distribution of plasmids among strains that contained one or more plasmids was compared there was no significant difference between *E. coli* and other enteric genera ($\chi^2_{3df}=0{\cdot}696$; $p>0{\cdot}8$) (fig. 2). This indicates that the difference between *E. coli* and other enteric genera was due to differences in the number of plasmid-free strains rather than to an overall carriage of fewer plasmids by the other enteric genera.

The number of different coliforms isolated from each specimen varied between zero and seven and

Table I. The distribution of plasmids and antibiotic resistance among the genera studied

Organism	Total number of isolates	Number of isolates containing the stated number of plasmids per isolate							Total number of antibiotic-resistant isolates
		0	1	2	3	4	5	6	
E. coli	161	21(3)*	55(22)	47(20)	23(9)	7(4)	6(4)	2(1)	63
Klebsiella spp.	51	27(0)	12(3)	10(2)	2(1)	0	0	0	6
Proteus spp.	34	24(1)	6(0)	4(2)	0	0	0	0	3
Enterobacter spp.	22	5	3	4	5	3	1	1	0
Citrobacter sp.	16	9	4(1)	2	0	1	0	0	1
Salmonella sp.	1	1	0	0	0	0	0	0	0
Providencia sp.	1	1	0	0	0	0	0	0	0
Yersinia sp.	1	1	0	0	0	0	0	0	0
Total	287	89	80(26)	67(24)	30(10)	11(4)	7(4)	3(1)	73

* Number of isolates resistant to at least one antibiotic shown in parentheses.

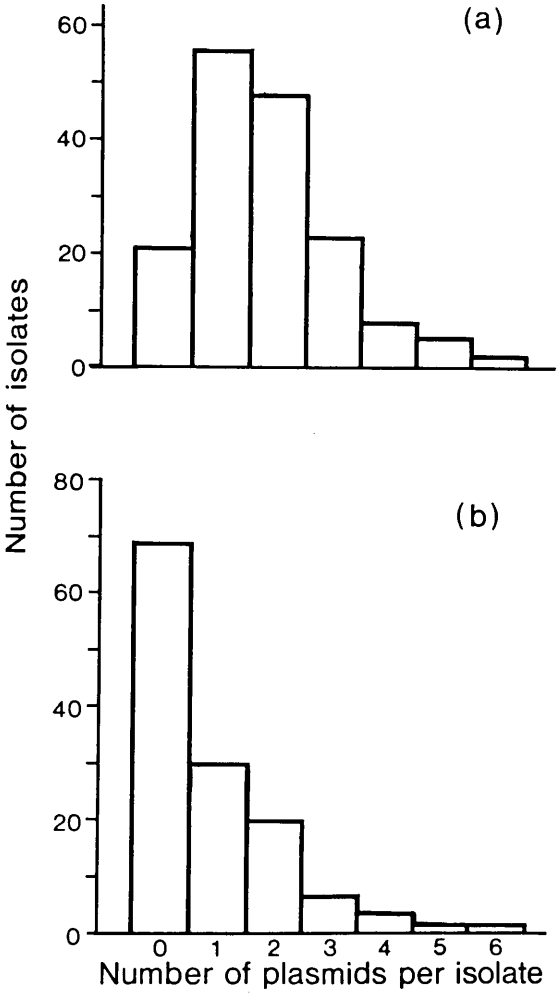

Fig. 1. The distribution of plasmids among isolates of (a) *E. coli*, (b) other enteric genera.

their distribution is shown in table II. The seven specimens that yielded no coliforms were all obtained from patients receiving at least two antimicrobial agents and included three patients undergoing gut "sterilisation' with Fracon (framycetin, colistin and neomycin). The modal value was one coliform per specimen.

From each of two patients, two coliforms were isolated (patient A, *E. coli* and *Klebsiella pneumoniae*; patient B, *E. coli* and *Citrobacter freundi*). The pairs of isolates each harboured transferrable R-plasmids that were indistinguishable on the basis of resistance markers transferred, mol. wt and restriction-enzyme fingerprints (table III). We interpret this finding as evidence of in-vivo R-transfer. The 36×10^6-mol. wt conjugative plasmid harboured by the strains of *E. coli* and *K. pneumoniae* isolated from patient A gave rise to transconjugants with two resistance patterns that differed with respect to sulphonamide resistance. Both Tc^r Tp^r pSm^r Su^r and Tc^r Tp^r pSm^r phenotypes were isolated with *E. coli* as the plasmid donor. However, only Tc^r Tp^r pSm^r transconjugants were obtained when *K. pneumoniae* was the donor and *E. coli* K12 the recipient. Transconjugants of both phenotypes were isolated from the *K. pneumoniae* strain when *Ent. aerogenes* replaced *E. coli* K12 as the recipient. Furthermore, when plasmids with the Su^s phenotype were retransferred to *E. coli* K12 from either of the above transconjugants both Su^s and Su^r phenotypes were isolated. This indicates that the difference in phenotype was the result of altered plasmid expression. Comparison of both *Hae* III and *Alu* 1 fingerprints of the Su^s and Su^r transconjugant plasmids showed that loss of the Su phenotype was associated with an increase in the size of the largest

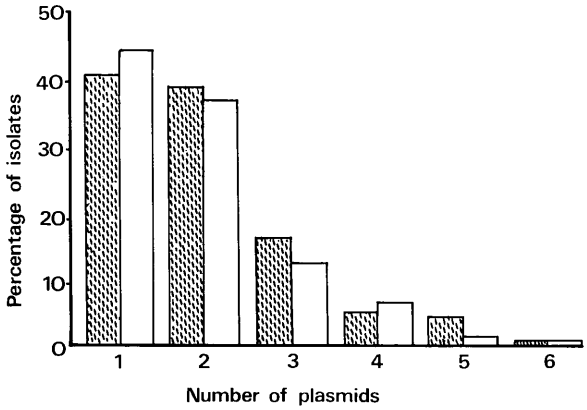

Fig. 2. The proportional distribution of plasmids among *E. coli* ▨ and other enteric genera □ after the exclusion of plasmid-free isolates.

Table II. The distribution of specimens from which distinguishable coliforms were isolated

Number of coliforms isolated	Number of specimens
0	7
1	30
2	26
3	22
4	22
5	5
6	2
7	2

restriction fragment (table III). Low level streptomycin resistance (pSm^r) was also plasmid-mediated and expressed in the transconjugants and in the *K. pneumoniae* donor but not in the *E. coli* donor from patient A.

Patient A had not received antibiotics within the previous 30 days. Patient B had been treated with amoxycillin for 5 days at the time the organisms were isolated and the plasmids described in the strains conferred resistance to amoxycillin.

R-plasmid transfer to *E. coli* K12 was demonstrated from 32 of the isolates studied and from at least one of the coliforms isolated from each of 30 patients. However, from eight of these patients no other coliform was isolated and therefore the in-vivo "R-transfer potential" was calculated on the basis of 22 patients and found to be 43. Combining these data with the two in-vivo transfer events described above gives an in-vivo R-transfer rate of 1/21·5.

Discussion

The development of rapid, simple methods for the physical detection, separation and fingerprinting of plasmid DNA from diverse genera (Platt, 1983) has provided the means to investigate plasmid ecology by enabling a large number of strains to be studied in detail. Thus, resistant organisms can be investigated with sensitive strains isolated in parallel, their plasmids compared and a better interpretation of the role of plasmids obtained. Hughes and Datta (1983) and Datta and Hughes (1983) studied

Table III. Summary of the characters of R-plasmids transferred *in vivo*

	Details of clinical isolate			Details of transconjugants			
Patient	Species	Resistance	Mol. wt (10^6) of plasmid	Resistance	Mol. wt (10^6) of plasmid	Restriction fragment sizes (kilobase pairs) with the given endonuclease	
A	*E. coli*	Tc Tp Su	62, 36	Tc Tp pSm Su*	36	*Hae* III 1·35, 1·30, 1·05, 0·74, 0·69 *Alu* 1 1·35, 1·25, 1·10, 0·98, 0·86	
				Tc Tp pSm*	36	*Hae* III 1·7, 1·30, 1·05, 0·74, 0·69 *Alu* 1 1·7, 1·25, 1·10, 0·98, 0·86	*Pst* 1 2·05, 1·85
A	*K. pneumoniae*	Tc Tp Su pSm	62, 36	Tc Tp pSm*	36	*Hae* III 1·7, 1·30, 1·05, 0·74, 0·69 *Alu* 1 1·7, 1·25, 1·10, 0·98, 0·86	
				Tc Tp pSm Su†	36	*Hae* III 1·35, 1·30, 1·05, 0·74, 0·69 *Alu* 1 1·35, 1·25, 1·10, 0·98, 0·86	
B	*E. coli*	Ap	62, 30, 10	Ap*	62	*Pst* 1 4·0, 3·3, 3·1, 2·8, 2·5, 2·3 1·8, 1·6, 1·3	
B	*C. freundi*	Ap	62	Ap*	62	*Sma* 1 10·0, 7·6, 6·9, 6·0, 5·1, 3·5, 2·6, 2·5, 2·25, 2·15, 2·0, 1·9	

TC = Tetracycline, Tp = trimethoprim, Su = sulphonamide, pSm = low level streptomycin, Ap = ampicillin.
* *E. coli* K12 transconjugants.
† *Enterobacter* transconjugant.

the transferability of various markers from Murray's collection of enteric bacteria isolated in the pre-antibiotic era and concluded that conjugative plasmids were as common then as they are in drug-sensitive strains today.

In a previous study of defined populations of *E. coli* we compared the frequency distributions of strains that harboured 0, 1, 2, 3 ... 7 plasmids per isolate (Platt *et al.*, 1984*b*). The population collected on the basis of antibiotic resistance possessed 26% more plasmids than the sensitive population. In this study the distribution of plasmids among *E. coli* isolates was similar. However, other enteric genera collected in parallel with *E. coli* harboured fewer plasmids (mean 0·89 plasmids/isolate compared with 1·78 plasmids/isolate among *E. coli*) (fig. 1). This suggests that *E. coli* is either a better plasmid recipient than other enteric genera or better able to maintain plasmids once acquired. Several possible explanations could account for this. The principal habitat of *E. coli* is the human and animal gut, where it is the predominant coliform; the other enteric genera tend to be minority residents and are also adapted to a free-living existence. Plasmid carriage constitutes a biosynthetic burden (Zund and Lebek, 1980; Sherratt, 1982). It seems likely that a well-adapted organism is readily able to accumulate plasmids. In the absence of selection pressure this may confer an energetic disadvantage on the host. Alternatively, the high spontaneous mutation rate in genera such as *Serratia* (Platt *et al.*, 1983) and *Klebsiella* (Smith, 1976) may confer sufficient genetic flexibility to reduce the benefits of plasmid carriage. It could also be argued that the differences in the distribution of plasmids might be attributable to the greater incidence of antibiotic resistance among *E. coli* isolates as compared with other enteric genera (table I). However, the effect of antibiotic resistance on plasmid distribution was minimal in our previous study (Platt *et al.* 1984*b*) and it seems unlikely that it would cause the striking differences observed here.

Earlier workers who found a low absolute incidence of R-transfer *in vivo* have emphasised such factors as donor ability, recipient ability (Jarolman and Kemp, 1969; Smith 1971) and possible inhibitory effects of other organisms (Anderson, 1975). These arguments reflect the systems studied and neglect the plasmid's contribution to its own transfer. In particular, we consider that specimens from which either single coliform strains or coliform strains lacking conjugative R-plasmids are isolated should be excluded, and therefore we have proposed the concept of in-vivo "R-transfer potential" and "R-transfer rate". Although the restrictions imposed by the criteria used to define them minimise the actual values obtained we refrained from the use of the prefix 'minimal" because other biological characters of the plasmids, e.g., surface exclusion, incompatibility, DNA restriction, could further decrease the real transfer potential. Despite these minimising influences the observations that of 116 specimens examined 43 potential transfer events were possible within 22 specimens, and that two such events were realised *in vivo*, suggests that R-transfer is very much more common *in vivo* than was hitherto suggested. This is consistent with recent reports of transfer in individual patients (Datta *et al.*, 1981; Platt *et al.*, 1984*b*).

Further refinements in methodology such as the use of alternative recipients to *E. coli* K12 for plasmids from other genera (Platt and Sommerville, 1981*b*; Sommerville, unpublished data) and the more extensive use of restriction-enzyme fingerprints to compare plasmids that acquire transposons (Kraft *et al.*, 1984) will no doubt necessitate the modification of our proposals. Nevertheless, the cautious use of the suggested terms should lead to a better appreciation of plasmid ecology.

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