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AMERICAN UNIVERSITY OF BEIRUT

Thesis Title:

DRUG RESISTANCE PATTERNS AND DISTRIBUTION OF R FACTORS AMONG ESCHERICHIA COLI ISOLATED FROM HUMAN FECES AND URINE

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Date of Thesis Presentation: February 3, 1972

DRUG RESISTANCE PATTERNS AND DISTRIBUTION OF R FACTORS AMONG *ESCHERICHIA COLI* ISOLATED FROM HUMAN FECES AND URINE

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A thesis presented in partial fulfillment
of the requirements for the degree of Master of Science

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February, 1972

R FACTORS IN *E. COLI*

ABSTRACT

One hundred eighteen and fifty strains of tetracycline resistant *Escherichia coli* were isolated respectively from feces and infected urine specimens. These isolates were examined by the disk method for sensitivity to 11 antimicrobial agents and were tested for their ability to transfer their resistance to a drug sensitive *Salmonella typhi*.

None of the strains were resistant to erythromycin, furadantin, furoxone or novobiocin but all of them were singly or multiply resistant to one or more of the other drugs. Seventeen different resistance patterns were identified among the fecal strains and 16 patterns among those isolated from the urine specimens.

Fifty five (46.6%) and 21 (42%) of the resistant strains isolated from feces and urine specimens respectively transferred their resistance either totally or partially to the *S. typhi* recipient in mixed culture. Tetracycline resistance was higher in the donors than in the recipients. The minimum inhibitory concentration for tetracycline in the donors and recipients ranged between 65 to 500 μg/ml and 55 to 190 μg/ml respectively.

The eliminatory action of acriflavine on R factors in *S. typhi* recipients was higher (maximum 47.1%) than in *E. coli*

donors (maximum 1.64%). Of 25 substrains of _S. typhi_ recipients, 68% lost their acquired resistance spontaneously after 40 successive transfers in Mueller Hinton broth. None of 10 _E. coli_ donors lost their R factors after similar transfers.

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INTRODUCTION

Until 1959, it was thought that the principal method by which drug resistant organisms arose from a sensitive bacterial population was by a spontaneous genetic mutation prior to the exposure of the sensitive bacteria to the drug. The drug acts only as a selective agent in the isolation of the resistant mutants by the destruction of the susceptible organisms. Such mutants could not transfer their resistance to sensitive strains by mixed cultivation (Mitsuhashi et al., 1960).

In 1959, a new type of drug resistance was discovered in Japan (Akiba). This type of resistance was distinguishable from other forms of resistance by the fact that it could be transferred from a resistant bacterial cell to a sensitive one by cell-to-cell contact. Kagiwada et al. (1960) using human volunteers proved that multiple drug resistant E. coli could transfer their resistance patterns to sensitive shigellae in the intestinal tract. This transferable drug resistance was known to be mediated by genes for drug resistance attached to a transfer factor and could be regarded as a type of infective heredity.

The first transferable drug resistance to be reported outside Japan was found by Datta in 1962 in strains of S. typhimurium causing an outbreak of gastroenteritis in London. Since then, it has become apparent that multiple drug resistance can be transferred to almost

every species of the family Enterobacteriaceae, *Vibrio cholerae*, *Pasteurella pestis*, *Serratia marcescens* and *Pseudomonas aeruginosa* (Mitsuhashi, 1971).

The medical importance of transferable drug resistance is obvious. When individuals ingest multiply drug resistant nonpathogenic bacteria, the resident *E. coli* and other bacterial strains present in their intestines may become resistant to the same drugs. If on a later date the same individuals are infected with pathogenic enteric bacteria, the disease-producing strains may also acquire the multiple drug resistance and become potential sources of serious epidemics. This creates a serious threat to the usefulness of the present antimicrobial drugs used for treatment of these infections.

The present investigations were undertaken to study the incidence of drug resistance patterns and the distribution of resistance transfer factors among *E. coli* strains isolated from human feces and urine. The stability of the resistance factors and the possibility of eliminating them from the *E. coli* donors and *S. typhi* recipients by treatment with acriflavine were likewise studied.

REVIEW OF LITERATURE

Shortly after the World War II, the introduction of sulfa drugs in Japan for the treatment of bacillary dysentery reduced the incidence of the disease greatly. However, since 1949 resistant strains of shigellae to these drugs were isolated. In 1955 Kitamoto et al. isolated a strain of *Shigella* which was resistant to Chloramphenicol, tetracycline, streptomycin and sulfonamide. Since then, many epidemics caused by multiply drug-resistant shigellae were observed by various Japanese workers. In one of these outbreaks, Matsuyama et al. (1957) isolated a strain of *E. coli* which was multiply resistant to the above mentioned four drugs. No acceptable explanation of these findings was presented until 1959 when Akiba suggested that multiple drug resistance could be transferred from a resistant *E. coli* to a senstive *Shigella* strain in the intestinal tract of man.

Ochiai et al. (1959) proved that multiple drug resistance could be transferred *in vitro* from a multiply resistant *E. coli* to a sensitive *Shigella* strain. They also concluded that cell-to-cell contact was necessary for the transfer to occur since they failed to transfer the resistance with cell free filtrates. Kagiwada et al. (1960) using human volunteers proved that multiply drug resistant *E. coli* could transfer their resistance patterns to sensitive shigellae in the intestinal tract. The *in vivo* transfer of multiple

drug resistance from E. coli to sensitive shigellae was also proved to occur in dogs (Mitsuhashi et al., 1960) and in mice (Akiba et al., 1961).

The transfer of the genetic elements responsible for drug resistance is common nearly among all species of the family Enterobacteriaceae and other unrelated Gram - negative bacteria. Mitsuhashi et al. (1960) reported that S. typhosa could transfer its resistance to V. cholerae, which was able to act as a donor of this acquired resistance in crosses with a recipient S. typhimurium strain. The ability of E. coli to transfer its resistance to Pasturella pestis, Serratia marcescens, and Pseudomonas aeruginosa was respectively reported by Ginoza et al. (1963), Rownd et al. (1966) and Mitsuhashi (1969).

The genetic elements controlling drug resistance and transferability were designated as resistance factors (R factors). Watanabe (1963 a) suggested the term resistance transfer factor (RTF) for the genetic elements determining the transferability of the R factors. He also postulated that the genetic elements for drug resistance are distributed on a single circular or rectilinear linkage group and that the RTF is inserted at one point of this linkage group.

Anderson and Lewis (1965) and Anderson (1966) used the term resistance determinants (R determinants) for the genetic materials

which conferred drug resistance and which were carried to the recipient bacteria. They also proved that the R factor consisted of two separate compartments: the RTF and the R determinants, without the R determinants the RTF although transferable, could not transfer drug resistance. Conjugation between a strain carrying an RTF and another carrying the R determinants resulted in passage of the RTF into the resistant strain in which it combined or associated with the R determinants to form the R factor.

According to Novick (1969) the R factor was believed to be an extrachromosomal element (plasmid) which is a stably inherited component of the cell genome when physically separate from the chromosome. Lederberg et al. (1952) and Campell (1962) on the other hand, used the term episome to mean a class of plasmids that could exist in a state of integration into the chromosome of their host cell as well as in the autonomous state. It is clear therefore, that the term episome refers in essence to these activities of the plasmid that are specifically related to chromosomal integration (Pearce and Meynell, 1968). The probability of chromosomal integration has been proved by Harada et al. (1967).

Surveys from various parts of the world have shown a wide distribution and high incidence of R factors among members of the family Enterobacteriaceae. In 1962, Datta studied 509 cultures of _S. typhimurium_ causing an outbreak of gastroenteritis in London.

He found that 15 of the studied strains were multiply resistant to streptomycin, sulfathiazole and tetracycline. This triple resistance could be transferred by growth in mixed broth culture to a sensitive strain of Shigella sonnei and back again to sensitive cultures of S. typhimurium.

Smith and Armour (1966) in the United States studied the incidence of drug resistance in enteric bacteria isolated from urine specimens of 100 patients. The isolates included E. coli, Proteus, Klebsiella and Pseudomonas. Seventy four per cent of the above strains were singly or multiply resistant of which 69% were able to transfer part or all of their resistance patterns to a sensitive E. coli K_{12}.

In 1967, Mitsuhashi et al. studied the distribution of R factors in many isolates, belonging to the family Enterobacteriaceae, from inpatients at geographically scattered hospitals in Japan. Among 2650 Shigella strains, 58% were found to be drug resistant. Of 434 resistant strains, 81% were found to carry R factors. They also examined 160 strains of different species of enteric bacteria and found that 84% of the resistant E. coli, 88% of the resistant Klebsiella and 50% of the resistant Proteus carried R factors.

Schroeder et al. (1968) reported that among 400 strains of Salmonella species isolated in various parts of the United States, 89 (22.2%) were resistant to one or more of the 11 drugs used:

14.2% were resistant to streptomycin, 12.5% to tetracycline, 11.5% to sulfonamide and none to chloramphenicol or polymyxin. S. typhimurium was more often resistant than any other species and accounted for 60% of the resistant strains. Of 52 S. typhimurium resistant strains 41 (79%) were able to transfer their resistance to a sensitive E. coli.

Recently, Gedebou and Eveland (1970) in the United States isolated 131 tetracycline resistant coliforms (43.7%) from 300 stool specimens. Fourteen different resistance patterns were observed and 37.4% of the resistant strains were able to transfer their resistance to a sensitive S. typhimurium.

In 1957, Hirota and Iijima reported that the fertility factor (F^+) of E. coli K_{12} could be eliminated when the F^+ cells were treated with acriflavine. Mitsuhashi et al. (1961) and Watanabe and Fukazawa (1961) found that treatment of multiply resistant Shigella and E. coli with acriflavine and acridine orange converted the resistant cells to drug sensitive ones, indicating that the resistance factors were in an autonomous state. The frequency of elimination was rather low, 4.1% with acriflavine and 2% with acridine orange. In contrast to the total elimination of the R factors by acridine treatment, the same authors reported that a part or all of the resistance factor could be lost spontaneously. Spontaneous loss of resistance occurred to either all of the drugs or to tetracycline alone or to streptomycin,

chloramphenicol, and sulfonamide together. Spontaneous segregants with tetracycline resistance alone or with streptomycin, chloramphenicol and sulfonamide resistance were all able to transfer their resistance factors by conjugation.

Watanabe and Lyang (1962) reported that successive transfers of a S. typhimurium strain (resistant to streptomycin, chloramphenicol, tetracycline and sulfonamide) on penassay broth caused spontaneous segregation of the R factors so frequently as to allow the study of the segregants with replica plating technique. Resistance to streptomycin, chloramphenicol and sulfonamide were spontaneously lost together more frequently than tetracycline resistance alone. The complete loss of the resistance to the four drugs was also noted with low frequencies.

Full information about the mechanisms of transferable drug resistance is not available. Anderson and Datta (1965) showed that transferable ampicillin resistance in S. typhimurium was due to the production of ampicillin (penicillin) destroying enzyme which was proved by Datta and Kontomichalon (1965) and by Egawa et al. (1967) to be a B - lactamase. Okomoto and Suzuki (1965) identified a streptomycinase in cultures of E. coli carrying various R factors conferring resistance to streptomycin. They also claimed that transferable chloramphenicol resistance was associated with the synthesis of chloramphenicolase and established the presence of kanamycinase

in kanamycin resistant strains controlled by R factors. Finally, reduced cell permeability to the drug was demonstrated by Akiba and Yokota (1961) to account for the transferable sulfonamide and tetracycline resistance in cultures of _E. coli_.

MATERIALS AND METHODS

Source of cultures:

Tetracycline resistant E. coli strains isolated from human feces and urine specimens were used in these experiments. Feces specimens were obtained from the parasitology section of the American University Hospital Laboratories. These were randomly picked up and inoculated into MacConkey agar plates supplemented with 25 μg/ml of oxytetracycline. After overnight (18 - 24 hours) incubation at 37°C, tetracycline resistant coliform colonies were picked up and inoculated into Kligler iron agar tubes which were then incubated overnight at 37°C. The E. coli strains were identified by their fermentation of lactose with acid and gas production and were further identified by means of other biochemical reactions as outlined by Edwards and Ewing (1962). A total of 118 tetracycline resistant E. coli strains were isolated from 175 feces specimens. They were maintained in the laboratory at room temperature, in Dorset's egg medium.

Infected urine specimens were obtained from the bacteriology section of the American University Hospital Laboratories. These were streaked on plain MacConkey agar plates and on MacConkey agar plates supplemented with 25 μg/ml of oxytetracycline. The MacConkey agar plates without antibiotic were used to detect the

presence of viable E. coli in the urine specimens. Of the 127 infected urine specimens that were examined, 71 contained E. coli, of which 50 proved to be tetracycline resistant.

A freshly isolated strain of S. typhi which was obtained from the bacteriology diagnostic section of the American University Hospital Laboratories was confirmed as S. typhi by standard serological tests, using polyvalent and monospecific salmonella O and H antisera. This isolate was found to be sensitive to 9 of the 11 drugs(resistant to erythromycin and novobiocin)used in this study and proved to be a good recipient as shown by several preliminary transfer experiments. It was then used as the principal recipient bacterium throughout these investigations. Each E. coli strain was given a serial number equivalent to the number of the feces specimen from which it was isolated. Likewise, each S. typhi recipient was given the same serial number as that of the corresponding E. coli donor.

A sensitive strain of E. coli K_{12}, obtained from the Biology Department at the American University of Beirut, was used as a recipient strain in mating experiments using S. typhi substrains that have acquired drug resistance as donor strains.

Culture Media:

Mueller Hinton broth, Mueller Hintron agar, MacConkey agar, Salmonella-Shigella (SS) agar, Kligler iron agar, all Difco products,

and various phenol red carbohydrate broths were used in these investigations.

Drugs:

The following antimicrobial sensitivity unidisks were obtained from Difco Laboratories, Detroit, Michigan, U.S.A.: chloramphenicol (Cm, 30 μg), erythromycin (Er, 15 μg), kanamycin (Ka, 30 μg), neomycin (Ne, 30 μg), novobiocin (No, 30 μg), penicillin G (Pe, 10 units), streptomycin (St, 10 μg), and tetracycline (Te, 30 μg). Furadantin (Fd, 100 μg) and Furoxone disks (Fu, 100 μg) were obtained from Baltimore Biological Laboratories, Baltimore, Maryland, U.S.A. Ampicillin disks (Am, 25 μg) were obtained from Bristol Laboratories, Syracuse, New York, U.S.A.

Stock standard solutions of oxytetracycline (Chas. Pfizer and Co., INC. New York, U.S.A.) ampicillin (Bristol), chloramphenicol (Park Davis), penicillin (Specia Paris) and streptomycin (Hoechst) were prepared by diluting the drugs in sterile distilled water to a concentration of 25,000 μg/ml for oxytetracycline, chloramphenicol and ampicillin. Penicillin was diluted to a concentration of 10,000 units/ml and streptomycin to 10,000 μg/ml. The diluted drugs were then stored in bijou bottles in 1 ml volumes at -70°C.

Drug sensitivity testing (disk method):

The drug resistance patterns of all the tetracycline resistant

E. coli, the recipient S. typhi, and the substrains of S. typhi which acquired drug resistance by the mating experiments, were determined. Each strain was streaked on a MacConkey agar plate. After overnight incubation at 37°C, 3 to 5 colonies from each plate were picked up with a loop and inoculated into 3 ml Mueller Hinton broth in bijou bottles. The broth cultures were then incubated overnight at 37°C after which each culture was diluted to 10^{-2} using a sterile saline solution as diluent. Mueller Hinton agar plates were then streaked evenly with each culture in 3 planes with a sterile cotton swab that has been immersed in the diluted inoculum and then pressed out against the inside wall of the container. After 3 to 4 minutes drying period at room temperature, drug sensitivity disks were applied with flamed forceps and pressed down to ensure contact with the agar surface. The plates were then incubated overnight at 37°C after which readings were taken by measuring the diameter of zone of inhibition of the growth around each disk. Cultures showing a zone of inhibition less than 10 mm were considered resistant to the drug. This was adopted in order to pick up the highly resistant strains and use them as potential donors of their resistance.

Drug resistance transfer:

A drug resistant E. coli and the sensitive S. typhi were streaked separately on MacConkey agar plates and incubated overnight at 37°C. The next day one colony from each plate was picked up and

inoculated separately into 3 ml Mueller Hinton broth in a bijou bottle and incubated overnight at 37°C. Half ml volumes of each of the donor and recipient cultures (containing approximately 10^{12} organisms per ml) were then added to a universal bottle containing 5 ml Mueller Hinton broth, mixed and incubated overnight at 37°C. The mixed cultures were then subcultured on the following media: SS agar, SS agar + 25 μg/ml Am., SS agar + 25 μg/ml Cm., SS agar + 25 μg/ml Te., and SS agar + 10 μg/ml St. The plates were incubated overnight at 37°C and then examined for the presence of resistant S. typhi. The concentrations of the antimicrobial drugs used in the media were sufficient to inhibit all sensitive strains (as established by the disk method) and insufficient to inhibit resistant strains. When a colony typical of S. typhi appeared on the SS plates containing either Am, Cm, St or Te, it was picked up and streaked on SS agar and it was further identified by its reaction on Kligler iron agar and by slide agglutination test using polyvalent O antiserum.

Fifteen substrains of S. typhi that received resistance factors from different strains of E. coli, were tested for their ability to transfer their acquired resistance patterns to a sensitive strain of E. coli K_{12}.

Frequency of transferable drug resistance:

The frequency of transferability of tetracycline resistance among S. typhi recipients was determined for 10 mating mixtures. Serial tenfold dilutions of each mixture, ranging from 10^{-1} to 10^{-11}, were prepared using sterile saline as the diluent. One tenth of a ml of each dilution was then spread on the surface of 3 plates of plain SS agar and 3 plates of SS agar + 25 μg/ml of tetracycline. The plates were incubated overnight at 37°C. The viable number of tetracycline resistant S. typhi colonies were estimated on the SS plates supplemented with 25 μg/ml of tetracycline. The total number of resistant and sensitive S. typhi colonies were estimated on the plain SS plates. The frequency of the transferability of tetracycline resistance among the recipient cells was then calculated according to the following formula:

$$\text{Frequency of transferability} = 1 : \frac{\text{Total No. of cells/ml of the mating mixture}}{\text{No. of tetracycline resistant cells/ml of the mating mixture}}$$

Level of tetracycline resistance (tube method):

The level of tetracycline resistance was determined for ten resistant strains of E. coli and the corresponding S. typhi recipients. To screw-capped culture tubes containing 4.5 ml of Mueller Hinton broth, 0.5 ml of various concentrations of tetracycline pre-

pared in Mueller Hinton broth was added to make the following final concentrations of the antibiotic : 0.5, 1, 5, 10, 20, 30, 40, 50, 100, 200, 300, 400, and 500 μg/ml. The tubes were then inoculated with 0.1 ml of an overnight Mueller Hinton broth culture of the test strain. Readings were taken after overnight incubation at 37°C and the highest concentration of the antibiotic in the screw capped culture tube showing turbidity was regarded as the concentration resisted by the strain. After determining the approximate level of tetracycline resistance of all the 20 strains, the ranges of the concentrations of the antibiotic in the tubes were then narrowed down to give a 5 μg/ml difference between each two successive concentrations. The minimum inhibitory concentrations (MIC) of tetracycline for each of the donor and recipient strains was then determined.

Elimination of resistance factors with acriflavine:

The elimination of the R factors was studied using 4 substrains of *S. typhi* recipients and 4 strains of *E. coli* donors. Acriflavine (Winthrop) was dissolved in distilled water at a concentration of 1 mg/ml, sterilized by autoclaving and kept as a stock solution in the refrigerator at 4°C. The MIC of acriflavine for each strain was determined and was found to be 25 μg/ml for *S. typhi* substrains, and 35 μg/ml for the *E. coli* strains.

Three concentrations of acriflavine which were below the MIC were used, namely, 10, 15 and 20 μg/ml for *S. typhi* substrains and

20, 25 and 30μg/ml for E. coli strains. Three 500 ml Erlenmeyer flasks each containing 50 ml of Mueller Hinton broth supplemented with the previously mentioned concentrations of acriflavine and a fourth flask without acriflavine, as a control, were used for each strain. The flasks were inoculated with 1 ml of an overnight broth culture of the test strain and were then incubated overnight at 37°C on a shaker with 90 strokes/minute. The next day a loopful of each flask was streaked into each of 4 MacConkey agar plates in a manner to obtain isolated colonies. After overnight incubation at 37°C, 244 isolated colonies from the four plates were transferred with sterile tooth picks into the surface of 4 MacConkey agar plates which were previously marked so that one colony was inoculated on each mark. The plates were incubated overnight at 37°C and were referred to as master plates. The next day the colonies from the master plates were transferred by the replica plating technique (Lederberg and Lederberg, 1952) into MacConkey agar plates supplemented by the drugs to which the test strain was resistant. A control plate of plain MacConkey agar was also used for the transfer of colonies from each master plate. Readings were taken after an overnight incubation of the plates at 37°C. Colonies which grew on the plates of plain MacConkey agar but not on the plates supplemented with the drugs, were considered as cells that have lost their resistance.

Spontaneous loss of resistance factors:

Ten *E. coli* donor strains and 25 *S. typhi* substrains that received R factors from different *E. coli* donors were grown on MacConkey agar plates containing 25 γ g/ml of tetracycline. From each plate a colony which was thus confirmed to be resistant to the drug, was inoculated into a screw-capped culture tube containing 3 ml of Mueller Hinton broth and incubated overnight at 37°C. A 4 mm calibrated loop was used to transfer 0.01 ml of each culture into a fresh Mueller Hinton broth tube which was then incubated overnight at 37°C. Subcultures of each strain was repeated thereafter up to 40 successive similar transfers. After each 5 transfers the foregoing cultures were streaked with the same calibrated loop in a single line on MacConkey agar plates containing the antibiotic to which the test strain was resistant. A known resistant strain was used as a control. After overnight incubation of the plates at 37°C the amount of growth of each strain was compared with that of the control strain. The following reporting scheme was used to indicate the amount of growth and the loss of resistance.

Amount of growth	Loss of resistance
-	Complete (100%) loss of resistance.
+	Approximately 75% loss of resistance.
++	Approximately 50% loss of resistance.
+++	Approximately 25% loss of resistance.
++++	No loss of resistance.

Replica plating technique was also used for the quantitative study of the spontaneous loss of the R factors, using 6 substrains of S. typhi after the 10th, 25th and the 40th transfer.

Biochemical reactions of substrains of S. typhi:

Twenty five substrains of S. typhi, obtained from conjugation experiments between the S. typhi recipients and 25 different strains of E. coli donors, were tested for their biochemical reactions as outlined by Edwards and Ewing (1962).

RESULTS

Tetracycline resistant _E. coli_ were isolated from 118 of 175 feces specimens (67.43%) and from 50 of 71 infected urine specimens (70.4%). Resistance to tetracycline was demonstrated by growth on MacConkey agar containing 25 μg/ml oxytetracycline. The resistant strains were tested by the disk method for sensitivity to 11 antimicrobial agents and were observed for the distribution of the R factors.

None of the strains studied was resistant to erythromycin, furadantin, furoxone or novobiocin, but all of them were singly or multiply resistant to the other drugs. The data presented in Table I show the incidence and transferability of drug resistance patterns among the tetracycline resistant strains of _E. coli_ isolated from feces. Of the 118 resistant strains, 15 (12.7%) were singly resistant to tetracycline, the remaining 87.3% were doubly or multiply resistant. Resistance to Te-Am-Ch-Pe-St accounted for 27.12% of the 17 different patterns followed by Te-Ch-Pe-St (11.01%), Te-Pe (7.62%), Te-Am-Pe-St (6.78%) and Te-Am-Ch-Ne-Pe-St (6.78%). The remaining 11 different resistance patterns accounted for 28% of the total resistant strains.

Table II shows 16 different resistance patterns among the 50 tetracycline resistant strains isolated from urine. Five strains (10%) were singly resistant to tetracycline, while 90% were doubly

or multiply resistant. The incidence of the resistance patterns was as follows: Te-Am-Ch-Pe-St (22%), Te-Am-Ch-Ka-Ne-Pe-St (18%), Te-St (6%) and the other 12 resistance patterns accounted for 44% of the studied strains.

When tested for transferability of drug resistance 55 (46.6%) of the resistant strains isolated from feces and 21 (42%) of those isolated from urine specimens were able to transfer all or part of their resistance patterns to the S. typhi recipient (Tables I & II).

Tables III and IV show the resistance patterns of the E. coli active donors isolated from feces and urine specimens respectively and the patterns of the acquired resistance in S. typhi substrains. Tetracycline resistances in all of the active donors were transferred to the recipient strain, whereas resistance to the other drugs among the doubly or multiply resistant donors was transferred either as a whole or in different combinations. An example is the resistance pattern to Te-Am-Ch-Pe-St for E. coli isolated from feces. Fifteen strains with such a pattern were able to act as active donors. Seven (46.66%) of them were able to transfer the whole pattern to the recipient strain. The patterns of the transferred resistance in the remaining 8 R factors were to Te alone, Te-Am-Pe, Te-Ch-Pe-St, and Te-Am-Ch-Pe accounting for 3 (20%), 2 (13.3%), 2 (13.3%) and 1 (6.66%) respectively.

The ability of 15 S. typhi substrains to transfer their

acquired resistance patterns to a sensitive strain of E. coli K 12 was studied. Table V shows that 14 of the substrains tested were able to transfer their whole acquired resistance patterns to E. coli K 12 recipient. However, one of the substrains posessing the resistance pattern Te-Ch-Pe could transfer only tetracycline resistance.

The frequency of the transfer of tetracycline resistance was determined for 10 mating mixtures using the replica plating technique. The results shown in table VI indicate that the frequency of transfer of the R factors depends on the donor strain. The best efficient donor for tetracycline resistance was E. coli strain in the mating mixture No. 110. The frequency was $1 : 3 \times 10^2$ that is in each 3×10^2 recipient cells present in the mating mixture, one cell acquired tetracycline resistance. The least frequency ($1 : 6.7 \times 10^6$) was obtained in the mating mixture No. 100.

Table VII shows the MIC of tetracycline in 10 E. coli donors and their corresponding S. typhi recipients. Tetracycline resistance was always higher in the donors than in the recipients except in strain 117 in which case the MIC of both donor and its corresponding recipient was the same. The MIC for tetracycline in the donors and recipients ranged between 65 to 500 μg/ml and 55 to 190 μg/ml respectively.

The results of treating 4 S. typhi substrains and their corresponding E. coli donors with different sublethal concentrations of

acriflavine are presented in Tables VIII and IX. The frequency of elimination of R factors was determined by the replica plating technique(Fig. 1). R factors were eliminated from 3 substrains of S. typhi and 2 strains of E. coli. The frequencies of the loss of R factors differ greatly from one strain to the other and also among different drugs of the same R factor. Elimination of Te resistance alone in S. typhi substrain 34 (resistant to Te-Ch-St) was at a higher frequency than that of Ch-St resistances which were eliminated together. In the case of S. typhi substrain 42 (resistant to Te-Am-Ch-Pe), elimination of Te resistance alone was at a lower frequency than Am-Ch-Pe resistances which were eliminated together.

The frequency of elimination of drug resistance in the S. typhi substrains increased with the increase of acriflavine concentrations. Elimination of the R factors from E. coli donors was at a much lower frequency than from S. typhi substrains (Table IX). Unlike the recipient, E. coli 34 lost resistance to all the three drugs (Te-Ch-St) with the same frequency (1.32%) when 30 γ g/ml of acriflavine was used and no elimination was noted at 25 and 20 γ g/ml of acriflavine.

Spontaneous loss of R factors by successive transfer in Mueller Hinton broth was determined for 25 resistant substrains of S. typhi and for 10 E. coli donors. After successive transfers

each of the foregoing cultures was streaked on MacConkey agar plates containing the drugs to which the original culture was resistant and the amount of growth was read after overnight incubation of the plates (Fig. 2). Of the 25 S. typhi substrains, 8 (32%) showed ++++ growth (growth equivalent to resistant control) after the 40th transfer (Table X). This indicates that these strains did not lose the R factor by successive transfers. Eight (32%) of the original 25 substrains lost their R factors completely after 20, 30, 35 or 40 transfers while the remaining 9 cultures showed only partial loss of their R factors. On the other hand all the tested 10 E. coli donors continued to show ++++ growth after the 40th transfer indicating that none of them lost its R factors.

Replica plating technique was used to study the frequency of loss of the R factors of 6 substrains of S. typhi which showed partial loss of R factors by the previous technique. Two hundred and fourty four colonies of each substrain after the 10th, 25th and 40th transfers were replicated on MacConkey agar plates containing the drugs to which the substrains were resistant. Table XI shows that tetracycline resistance was more stable than other drugs in the multiply resistant substrains 80 and 117. Substrain 65 showed an equal loss of resistance to all the drugs to which it was resistant Te-Ch-Pe-St-Am. The frequencies of loss of resistance for this substrain were 4.92%, 6.55% and 9.01% after the 10th, 25th and 40th transfers respectively.

The results of testing the biochemical reactions of the original _S. typhi_ strain and 25 substrains that acquired drug resistance, revealed that all the biochemical reactions of the recipient substrains were identical to those of the original _S. typhi_ strain.

TABLE I

Incidence and transferability of drug resistance patterns among 118 *E. coli* strains isolated from feces

Antibiotics tested[1]	Resistant strains		Strains transferring resistance	
	No.	%	No.	%
Te	15	12.71	8	53.33
Te-Ch	3	2.54	0	0
Te-Pe	9	7.62	5	55.5
Te-St	5	4.32	0	0
Te-Am-Pe	2	1.61	1	50.0
Te-Ch-St	7	5.93	6	85.71
Te-Ne-St	1	0.85	0	0
Te-Pe-St	6	5.08	2	33.3
Te-Am-Ch-Pe	2	1.61	0	0
Te-Am-Pe-St	8	6.78	4	50.0
Te-Ch-Pe-St	13	11.01	3	23.07
Te-Am-Ch-Ka-Pe	1	0.85	1	100.0
Te-Am-Ch-Pe-St	32	27.12	15	46.87
Te-Am-Ch-Ka-Pe-St	2	1.61	2	100.0
Te-Am-Ch-Ne-Pe-St	2	1.61	0	0
Te-Ch-Ne-Pe-St	2	1.61	0	0
Te-Am-Ch-Ka-Ne-Pe-St	8	6.78	8	100.0
Total	118	100	55	46.61

1) Abbreviations: Te, tetracycline; Am, ampicillin; Ch, chloramphenicol; Pe, penicillin; Ka, kanamycin; Ne, neomycin; St, streptomycin

These abbreviations are used also in subsequent Tables.

TABLE II

Incidence and transferability of drug resistance patterns among 50 *E. coli* strains isolated from infected urine

Antibiotics tested	Resistant strains		Strains transferring resistance	
	No.	%	No.	%
Te	5	10	2	40
Te-Ch	2	4	1	50
Te-Pe	2	4	0	0
Te-St	3	6	0	0
Te-Am-Pe	1	2	0	0
Te-Ch-St	2	4	1	50
Te-Pe-St	2	4	0	0
Te-Am-Ch-Pe	1	2	1	100
Te-Ne-Pe-St	2	4	0	0
Te-Ch-Ka-Ne	1	2	0	0
Te-Ch-Pe-St	1	2	1	100
Te-Am-Ch-Pe-St	11	22	5	45.45
Te-Am-Ne-Pe-St	1	2	0	0
Te-Am-Ch-Ka-Pe-St	2	4	1	50
Te-Am-Ch-Ne-Pe-St	5	10	2	40
Te-Am-Ch-Ka-Ne-Pe-St	9	18	7	77.7
Total	50	100	21	42

TABLE III

Drug resistance patterns of E. coli donors isolated from feces and the corresponding S. typhi recipients

Donors		Recipient	
Resistance pattern	No.	Resistance pattern	No.
Te	8	Te	8
Te-Pe	5	Te Te-Pe	3 2
Te-Am-Pe	1	Te-Am-Pe	1
Te-Ch-St	6	Te-Ch-St	6
Te-Pe-St	2	Te Te-Pe-St	1 1
Te-Am-Pe-St	4	Te-St Te-Am-Pe-St	1 3
Te-Ch-Pe-St	3	Te Te-Ch-Pe-St	1 2
Te-Am-Ch-Ka-Pe	1	Te-Am-Ch-Ka-Pe	1
Te-Am-Ch-Pe-St	15	Te Te-Am-Pe Te-Am-Ch-Pe Te-Ch-Pe-St Te-Am-Ch-Pe-St	3 2 1 2 7
Te-Am-Ch-Ka-Pe-St	2	Te-Am-Ch-Pe-St Te-Am-Ch-Ka-Pe-St	1 1
Te-Am-Ch-Ka-Ne-Pe-St	8	Te-Pe Te-Ch-Ne-St Te-Am-Ch-Pe-St Te-Am-Ch-Ka-Ne-Pe-St	1 1 2 4
Total	55		55

TABLE IV

Drug resistance patterns of *E. coli* donors isolated from infected urine and the corresponding *S. typhi* recipients

Donor		Recipient	
Resistance pattern	No.	Resistance pattern	No.
Te	2	Te	2
Te-Ch	1	Te-Ch	1
Te-Ch-St	1	Te-Ch-St	1
Te-Am-Ch-Pe	1	Te-Am-Ch-Pe	1
Te-Ch-Pe-St	1	Te-Ch-Pe-St	1
Te-Am-Ch-Pe-St	5	Te-Am-Ch-Pe-St Te-Am-Ch-St Te-Am-Pe Te	2 1 1 1
Te-Am-Ch-Ka-Pe-St	1	Te-Am-Ch-Ka-Pe-St	1
Te-Am-Ch-Ne-Pe-St	2	Te-Am-Ch-Ne-Pe-St Te-Am-Pe	1 1
Te-Am-Ch-Ka-Ne-Pe-St	7	Te-Am-Ch-Ka-Ne-Pe-St Te-Am-Ch-Pe-St Te-Am-Pe Te	3 1 1 2
Total	21		21

TABLE V

Ability of *S. typhi* recipients to transfer their acquired resistance patterns to a sensitive strain of *E. coli* K 12

S. typhi recipients (donors)		*E. coli* K 12 (recipient)	
Resistance pattern	No.	Resistance pattern	No.
Te	5	Te	5
Te-St	2	Te-St	2
Te-Am-Pe	2	Te-Am-Pe	2
Te-Ch-Pe	1	Te	1
Te-Ch-St	1	Te-Ch-St	1
Te-Am-Ch-Pe	1	Te-Am-Ch-Pe	1
Te-Ch-Pe-St	1	Te-Ch-Pe-St	1
Te-Am-Ch-Pe-St	1	Te-Am-Ch-Pe-St	1
Te-Am-Ch-Ka-Ne-Pe-St	1	Te-Am-Ch-Ka-Ne-Pe-St	1
Total	15		15

TABLE VI

Frequency of the acquired tetracycline resistance among the recipient *S. typhi* cells in 10 mating mixtures

Serial No. of mating mixture	*S. typhi* viable count/ml		Frequency of Te resistant colonies among the recipients
	Total colony count	Count of Te resistant colonies	
34	2.2×10^{13}	3.3×10^{10}	$1 : 6.7 \times 10^{2}$
40	5.2×10^{13}	2.6×10^{8}	$1 : 2 \times 10^{5}$
42	3.6×10^{12}	1.2×10^{9}	$1 : 3 \times 10^{3}$
65	2×10^{14}	8×10^{10}	$1 : 2.5 \times 10^{3}$
69	7.5×10^{12}	5×10^{7}	$1 : 1.5 \times 10^{5}$
71	2.8×10^{12}	4×10^{8}	$1 : 7 \times 10^{3}$
100	5.6×10^{14}	8.4×10^{7}	$1 : 6.7 \times 10^{6}$
102	2.8×10^{13}	1.6×10^{11}	$1 : 3.5 \times 10^{2}$
110	3.8×10^{13}	1.14×10^{11}	$1 : 3 \times 10^{2}$
125	1.6×10^{11}	6.4×10^{7}	$1 : 2.5 \times 10^{3}$

TABLE VII

Tetracycline resistance levels of 10 E. coli donors and the 10 corresponding S. typhi recipients

Serial No. of recipient and donor	Minimum inhibitory concentration of tetracycline $\mu g/ml$	
	E. coli donor	S. typhi recipient
34	170	110
40	140	100
42	180	55
65	150	65
100	85	50
102	400	55
110	215	55
117	65	65
124	340	130
125	500	190

TABLE VIII

Elimination of R factors of S. typhi recipients with acriflavine

Serial No. of recipient	R factors	Frequency of cells losing resistance out of 244 replicated colonies							
		µg of acriflavine/ml of Mueller Hinton broth*						Control (no acriflavine)	
		10 µg		15 µg		20 µg			
		No.	%	No.	%	No.	%	No.	%
34	Te	81	33.19	93	38.1	107	43.8	68	27.86
	Ch	7	2.86	12	4.9	16	6.55	3	1.32
	St	7	2.86	12	4.9	16	6.55	3	1.32
42	Te	0	0	1	0.4	3	1.32	0	0
	Am	11	4.5	112	45.9	115	47.1	0	0
	Ch	11	4.5	112	45.9	115	47.1	0	0
	Pe	11	4.5	112	45.9	115	47.1	0	0
71	Te	0	0	0	0	0	0	0	0
125	Te	66	27.5	69	28.68	71	29.1	57	23.36

* The cells were incubated in broth containing the above concentrations of acriflavine at 37°C for overnight before replica plating.

TABLE IX

Elimination of R factors of *E. coli* donors with acriflavine

Serial No. of *E. coli*	R factors	Frequency of cells losing resistance out of 244 replicated colonies							
		μg of acriflavine/ml of Mueller Hinton broth*						Control (no acriflavine)	
		20 μg		25 μg		30 μg			
		No.	%	No.	%	No.	%	No.	%
34	Te	0	0	0	0	3	1.32	0	0
	Ch	0	0	0	0	3	1.32	0	0
	St	0	0	0	0	3	1.32	0	0
42	Te	0	0	0	0	0	0	0	0
	Am	0	0	0	0	0	0	0	0
	Ch	0	0	0	0	0	0	0	0
	Pe	0	0	0	0	0	0	0	0
71	Te	0	0	0	0	0	0	0	0
125	Te	0	0	1	0.41	4	1.64	0	0

* The cells were incubated in broth containing the above concentrations of acriflavine at 37°C for overnight before replica plating.

TABLE X

Spontaneous loss of R factors of S. typhi recipients after successive transfers in Mueller Hinton broth

Serial No. of S. typhi recipient	R factors	No. of transfers						
		10	15	20	25	30	35	40
6	Te	++++[1]	+++[2]	+++	+++	+++	+++	+++
9	Te	++++	+++	+++	+++	+++	+++	+++
	Pe	++++	+++	+++	+++	++[3]	++	++
	St	++++	+++	+++	+++	++	++	++
20	Te	++++	+++	++	+[4]	-[5]	-	-
34	Te	++	+	-	-	-	-	-
	Ch	++	+	-	-	-	-	-
	St	++	+	-	-	-	-	-
40	Te	+++	++	++	+	+	+	-
	Ch	++	++	+	+	+	-	-
	St	++	++	+	+	+	-	-
65	Te	++++	+++	+++	+++	+++	+++	++
	Am	+++	+++	+++	+++	+++	+++	++
	Ch	+++	+++	+++	+++	+++	+++	++
	Pe	+++	+++	+++	+++	+++	+++	++
	St	+++	+++	+++	+++	+++	+++	++
80	Te	++	++	++	+	+	+	+
	Ch	+	+	-	-	-	-	-
	Pe	+	+	-	-	-	-	-
	St	+	+	-	-	-	-	-

TABLE XI

Spontaneous loss of R factors of 6 S. typhi recipients after successive transfers in Mueller Hinton broth

Serial No. of S. typhi recipient	R factors	No. and percentage of cells losing resistance among 244 replicated colonies after successive transfers					
		10 transfers		25 transfers		40 transfers	
		No.	%	No.	%	No.	%
65	Te	12	4.92	16	6.55	22	9.01
	Am	12	4.92	16	6.55	22	9.01
	Ch	12	4.92	16	6.55	22	9.01
	Pe	12	4.92	16	6.55	22	9.01
	St	12	4.92	16	6.55	22	9.01
80	Te	78	31.96	232	95.08	244	100.0
	Am	225	92.67	244	100.0	244	100.0
	Ch	225	92.67	244	100.0	244	100.0
	Pe	225	92.67	244	100.0	244	100.0
	St	225	92.67	244	100.0	244	100.0
100	Te	22	9.01	244	100.0	244	100.0
101	Te	56	22.95	187	76.22	244	100.0
117	Te	56	22.95	98	40.16	244	100.0
	Ch	88	36.05	221	90.57	244	100.0
	St	88	36.05	221	90.57	244	100.0
125	Te	48	19.67	241	98.77	244	100.0

DISCUSSION

During the past 15 years there has been an increasing recognition of the importance of R factors in clinical and epidemiological work. This was reported first from Japan and later from other countries. The work reported in this thesis constitutes the first observation on the incidence of drug resistance and distribution of R factors among tetracycline resistant E. coli strains isolated from human feces and infected urine specimens in Lebanon.

None of the 168 strains tested was resistant to erythromycin, furadantin, furoxone and novobiocin, but, all of them were singly or multiply resistant to one or more of the other drugs used in this study (Tables I and II). The frequency of tetracycline resistance among E. coli isolates from feces and infected urines was significantly high, accounting to 67.45% and 70.42% respectively. About 15% of the fecal strains and 10% of the urine strains were singly resistant to tetracycline. The rest of the strains were doubly or multiply resistant to the drugs used in this investigation. This study, however, excluded all tetracycline sensitive strains, some of which could be resistant to other drugs. It may be assumed, therefore, that the actual number of E. coli resistant to any one or more drugs other than tetracycline must be greater than the numbers appearing in Tables I and II.

Seventeen different resistance patterns were identified among the E. coli isolated from feces and 16 patterns among those isolated

from infected urine specimens (Tables I and II). Among the different resistance patterns of the E. coli isolates, Te-Am-Ch-Pe-St resistance was the most frequently encountered and accounted for 27.12% and 22% of E. coli isolated from feces and urine specimens respectively. This indicates that multiply drug resistant E. coli may be easily recovered from feces and urines of persons who may or may not be receiving antimicrobial drugs. However, it is reported that E. coli isolated from persons receiving such drugs, give a greater incidence of infectious drug resistance than E. coli recovered from persons not receiving antibiotic treatment (Sturtevant et al., 1971). This is due to the fact that the major selective force favoring the emergence of drug resistant bacteria is the use of antimicrobial drugs.

Our results show that 46.61% of the resistant strains isolated from feces and 42% of those isolated from urine specimens were able to transfer all or part of their drug resistance to a sensitive S. typhi recipient. Gedebou and Eveland (1970) found that 43.7% of tetracycline resistant coliforms isolated from feces were able to transfer their resistance. A transferability frequency of 50% among drug resistant lactose fermenting bacteria isolated from untreated sewage was reported by Sturtevant and Feary (1969). The percentage of transferability reported in this study among the different E. coli donors ranges from 0 to 100% (Tables I and II). The efficiency of the transfer, therefore, depends on the donor strain. Watanabe (1963 b) and Anderson (1968) reported that certain species

or even certain strains were better donors than others and that some recipient species or strains were more efficient than others. A strain, therefore, could be a good recipient for a particular donor and a poor one for another.

Partial or complete transfer of resistance patterns were observed in our study (Tables III and IV). The partial transfer of the resistance patterns may be due to the possibility that the donor strains carry one or more R factors and only one of them is transferred during conjugation. Another possibility for the segregation of the resistance patterns is that if replication starts at one end of the R factor and for some unknown reason stops somewhere between the two ends, different segregant types could be produced (Watanabe and Layang, 1961).

In this study 15 S.typhi substrains proved to be able to transfer their acquired resistance to a sensitive E. coli K 12 recipient. The frequency of the transfer was 100%. This high efficiency of transferability may be attributed to the fact that E. coli K 12 is the best known recipient for R factors (Anderson, 1968; Mitsuhashi, 1969). It is also possible that R factors replicate at a high rate in cells that have just received the R factors. Such recipients will act as highly competent donor cells (Watanabe et al., 1964).

The degree of resistance to tetracycline in the recipients was lower than in the donors (Table VII). This indicates that tetra-

cycline resistance is transferred in part and not _in toto_. This is in agreement with the results reported by Mann and Gedebou (1967). Since the mechanism of tetracycline resistance is to reduce cell permeability to the drug (Anderson, 1968), it may be assumed, therefore, that lower concentrations of tetracycline are needed to generate the active tetracycline transport system in the recipient _S. typhi_ substrains than in the _E. coli_ donors.

Tables VIII and IX show that resistance factors can be eliminated by treating the resistant cells with sublethal concentrations of acriflavine. This confirms the previously reached conclusion that the R factors are in autonomous state (Watanabe and Fukazawa, 1961). Two of the tested _E. coli_ donors did not lose their R factors upon treatment with acriflavine. The other 2 strains lost their R factors with very low frequency (Maximum 1.64%). On the other hand, 3 of the 4 _S. typhi_ recipients lost their R factors at a much higher frequency (maximum 47.1%) than the _E. coli_ donors. The lower frequencies of elimination from _E. coli_,suggests that these R factors are in a state of chromosomal integration which cannot be eliminated with acriflavine.

In the present work we have found that the spontaneous segregation and loss of R factors in _S. typhi_ substrains, upon successive transfers in Mueller Hinton broth, take place with high frequency. This enabled us to conduct clone analysis of the segregation and loss of the R factors with replica plating technique. Resistance to all

the drugs were lost together in some instances (strain 65) but segregated loss of R factors was also noted in others. During segregation the R factors were divided into Te alone and Am-Ch-Pe-St as a group in substrain 80, or into Te alone and Ch-St as in substrain 117 (Table XI).

It is well established that the distribution of the R factors is widely spread among the different members of the family Enterobacteriaceae which can transfer in vitro their R factors to a number of bacterial species. Early studies in Japan reviewed by Watanabe (1963 à) demonstrated the transfer of multiple drug resistance in human volunteers, in dogs and mice. Recent studies in microbialy defined animals (Reed et al., 1969; Salzman and Klemm, 1968) have clearly shown that the transfer of R factors can occur in vivo at a low frequency. This is due to the presence of fatty acids and other unknown inhibitors in the intestinal tract. Such limited transfer which occur in vivo may be significant in humans and animals subjected to antimicrobial drug treatment.

It can be concluded that singly and multiply drug resistant _E. coli_ occur in significant numbers in feces and infected urine specimens and that high percentage of these resistant _E. coli_ could act as the source of transferable drug resistance to pathogenic bacteria such as salmonellae, shigellae, and _V. cholerae_ which share the same intestinal habitat. The transfer of the R factors occurs at a lower frequency in the intestine than in vitro. However, this

frequency may be increased by the use of selective drugs. Such drugs will eliminate the sensitive strains allowing the resistant strains to multiply freely. These findings give a direct and strong evidence to warrant strict control on the indiscriminate use of antimicrobial drugs. Minimal use of drugs in human and veterinary medicine is suggested as the only practicable measure at present to minimize the spread of resistance factors.

Fig. 1. Replica plating technique showing the elimination of R factors from *E. coli* donors (plates A and B) and from *S. typhi* recipients (plates C and D).

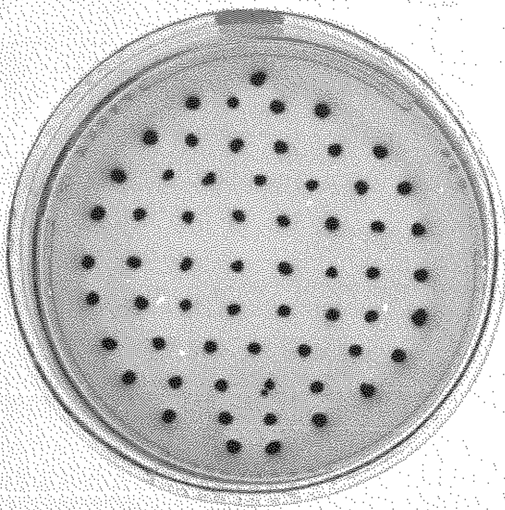
Plate A : E. coli colonies on plain MacConkey agar.

Plate B : *E. coli* colonies on MacConkey agar + 25γ g/ml. oxytetracycline, two out of 61 colonies lost their resistance.

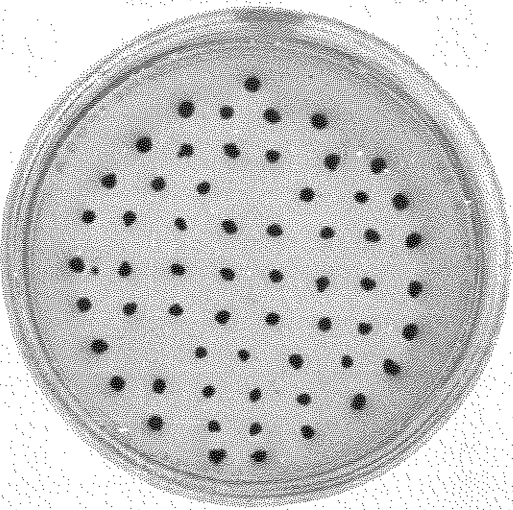
Plate C : *S. typhi* recipient colonies on plain MacConkey agar.

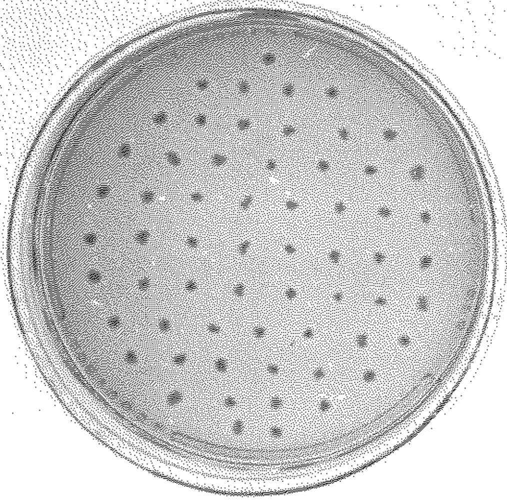
Plate D : *S. typhi* recipient on MacConkey agar + 25γ g/ml. oxytetracycline. Fourteen out of 61 colonies lost their resistance.

A

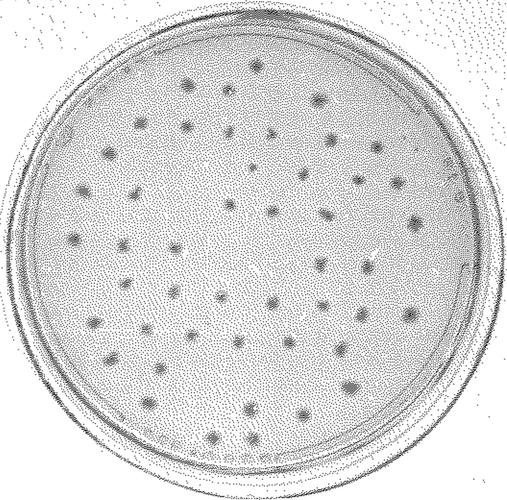


B





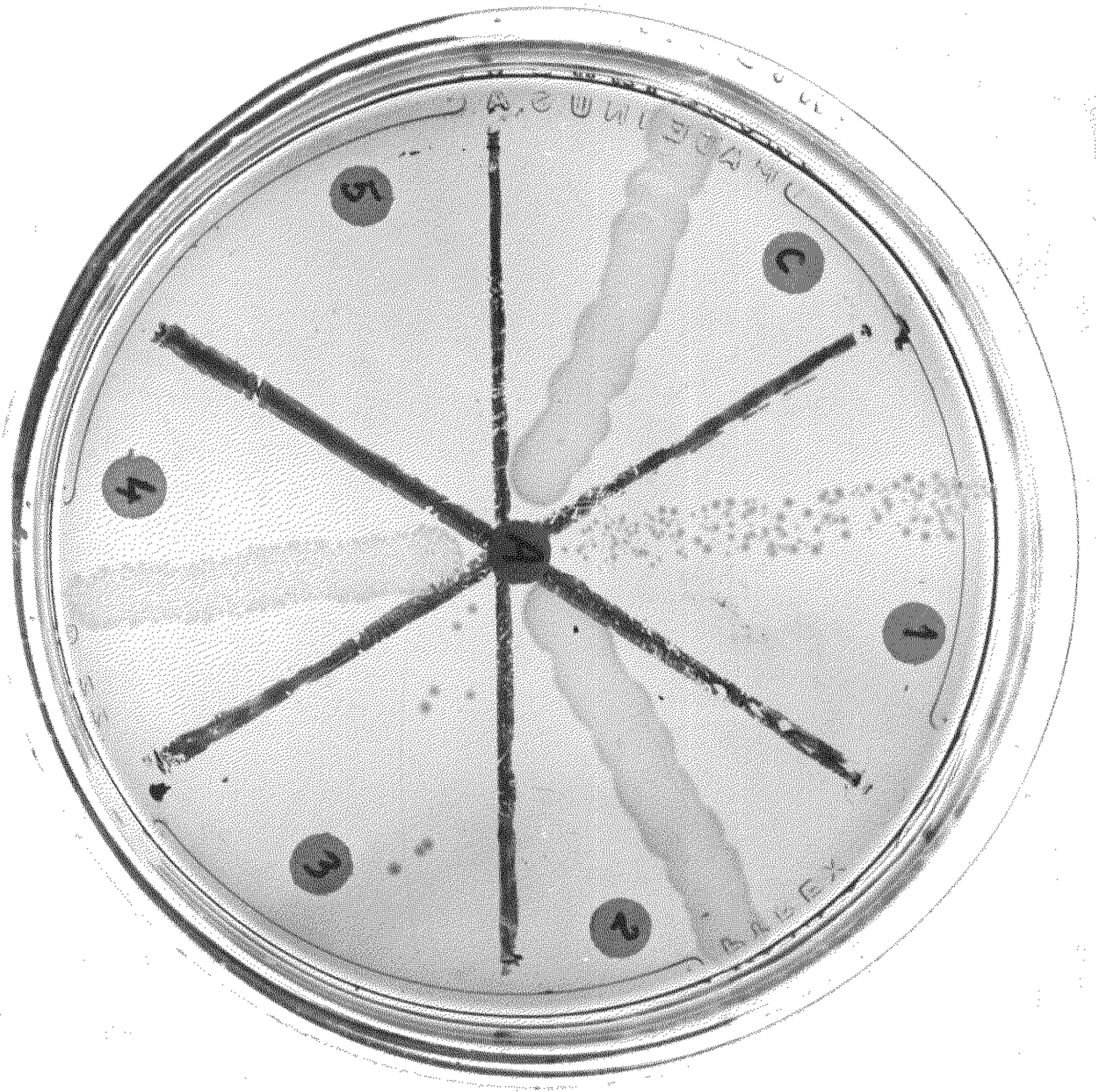
C



D

Fig. 2. Spontaneous loss of tetracycline resistance after successive transfers of the recipient *S. typhi* substrains in Mueller Hinton broth.

No. of substrain	Amount of growth	Loss of resistance
1.	++	Approximately 50% loss of resistance.
2.	++++	No loss of resistance.
3.	+	Approximately 75% loss of resistance.
4.	+++	Approximately 25% loss of resistance.
5.	-	Complete (100%) loss of resistance.
6.	++++	Resistant strain (control).


5
C
4
1
3
2

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