In vitro and in vivo studies on transferable drug resistance in the Enterobacteriaceae
by David Paul Aden

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Microbiology
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Abstract:
Fecal specimens were collected on 22 different Nebraska ranches and at the Department of Veterinary Science, University of Nebraska, from young calves and pigs with neonatal diarrhea. Enterobacteriaceae isolated from these fecal specimens were screened for resistance to tetracycline, streptomycin, sulfamethizole, kanamycin, chloramphenicol, colistin, nitrofurantoin, and nalidixic acid. Of the 92 strains studied, 57 were resistant to one or more of these antimicrobial agents. Resistant strains were obtained from all herds involved in the study. The two most common resistance patterns were tetracycline streptomycin sulfamethizole (22 of 57) and tetracycline (13 of 57). None of the strains were resistant to chloramphenicol, colistin, nitrofurantoin, or nalidixic acid. The 57 resistant strains were studied to determine whether the resistance was transferable. Forty-three of the 57 resistant strains could transfer part or all of their resistance to a drug sensitive recipient. The 43 R strains were obtained from 17 of the 23 herds studied. Considerable variation was observed between different R strains in the frequency of transfer of resistance to a particular antimicrobial agent. In addition, variation in the frequency of transfer of different resistant determinants was noted in individual strains. In vivo mating of R strains with recipients was performed utilizing the gastrointestinal tract of germfree mice. Antimicrobial agents were then administered to determine if a selective effect was observed for organisms mediating resistance to the antimicrobial agent or agents given. Administration of antimicrobial agents was not observed to select for higher numbers of organisms resistant to the agent or agents given. Various chemical and biological means were then investigated as to their ability to inhibit mating and transfer of drug resistance. Inhibition of in vivo transfer of drug resistance was not observed with the methods studied.

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Signature David P. Alden

Date July 29, 1970

IN VITRO AND *IN VIVO* STUDIES ON TRANSFERABLE DRUG RESISTANCE IN THE *ENTEROBACTERIACEAE*

by

DAVID PAUL ADEN

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

of

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in

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Approved:

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ABSTRACT

Fecal specimens were collected on 22 different Nebraska ranches and at the Department of Veterinary Science, University of Nebraska, from young calves and pigs with neonatal diarrhea. Enterobacteriaceae isolated from these fecal specimens were screened for resistance to tetracycline, streptomycin, sulfamethizole, kanamycin, chloramphenicol, colistin, nitrofurantoin, and nalidixic acid. Of the 92 strains studied, 57 were resistant to one or more of these antimicrobial agents. Resistant strains were obtained from all herds involved in the study. The two most common resistance patterns were tetracycline streptomycin sulfamethizole (22 of 57) and tetracycline (13 of 57). None of the strains were resistant to chloramphenicol, colistin, nitrofurantoin, or nalidixic acid. The 57 resistant strains were studied to determine whether the resistance was transferable. Forty-three of the 57 resistant strains could transfer part or all of their resistance to a drug sensitive recipient. The 43 R^+ strains were obtained from 17 of the 23 herds studied. Considerable variation was observed between different R^+ strains in the frequency of transfer of resistance to a particular antimicrobial agent. In addition, variation in the frequency of transfer of different resistant determinants was noted in individual strains. In vivo mating of R^+ strains with recipients was performed utilizing the gastrointestinal tract of germfree mice. Antimicrobial agents were then administered to determine if a selective effect was observed for organisms mediating resistance to the antimicrobial agent or agents given. Administration of antimicrobial agents was not observed to select for higher numbers of organisms resistant to the agent or agents given. Various chemical and biological means were then investigated as to their ability to inhibit mating and transfer of drug resistance. Inhibition of in vivo transfer of drug resistance was not observed with the methods studied.

INTRODUCTION

History

Kitamoto et al. (21) in 1955 isolated a multiply drug resistant strain of Shigella in Japan. The patient from which the strain was isolated had dysentery, which was believed incidental to a trip to Hong Kong. This strain was resistant to four antimicrobial agents: sulfonamides, streptomycin, chloramphenicol, and tetracyclines. No such multiply resistant strains of *Shigella* had previously been isolated in Japan despite extensive nationwide surveys of antibiotic sensitivities.

In 1959, Akiba et al. (2) reported that the development of multiply drug resistant organisms was due to transfer between organisms. That this transfer could occur between species was shown by transfer between *Shigella* and *Escherichia coli* in a mixed culture. Cell-free filtrates were unsuccessfully used in attempts to transfer resistance, showing that cell-to-cell contact was probably essential.

Mechanism

An organism may become resistant to an antimicrobial agent by spontaneous mutation or by transfer of genetic information from a resistant bacterium to a sensitive one. Transfer of genetic material may occur by processes of transformation (DNA mediated) transduction (bacteriophage mediated), and sexual recombination (conjugation). In the case of multiple drug resistance, the later was suggested.

though it was later shown that transmission did not depend on the presence of an F factor. Thus it was reasoned that the bacteria had acquired an independent mechanism for conjugation.

Several unusual features were found in the epidemiological studies of the multiply drug resistant Shigella that suggested that a different genetic mechanism was responsible. Both drug sensitive and multiply drug resistant strains of the same antigenic type were at times isolated from the same patient. Patients carrying multiply drug resistant strains of Shigella usually carried multiply drug resistant strains of E. coli. When patients who had previously been excreting drug sensitive Shigella, were treated with a single drug, chloramphenicol for example, they suddenly started excreting multiply drug resistant Shigella of the same antigenic type.

Transferable drug resistance (R factors) was then shown to exist in the bacterial cytoplasm. Evidence for their plasmid state was seen from curing with acridine dyes (7) and from the use of recipient strains that have various chromosomal markers, that show that transfer of the chromosome is not required.

Enterobacteriaceae possessing R factors are found to have two outstanding characteristics. The R factors render their hosts resistant to antimicrobial agents, and at the same time enable them to transmit resistance to other bacteria. The recipient of the R factor

thus becomes drug resistant and can then serve as a genetic donor in its turn.

Molecular Models

R factors are self-replicating units that carry the genetic information for drug resistance and their conjugal transferability. It seems safe to assume that R factors are composed of DNA. In support of this one finds that the decay of incorporated ^{32}P inactivates the R factor, and that ultraviolet light causes inactivation. Both of these lines of evidence strongly suggest that R factors are comprised of nucleic acids.

Biophysical studies show that R factors do not differ greatly in size from F (sex factor) or even the smallest F^1 factor, having a molecular weight of 2×10^7 daltons. This is equivalent to about 1% of the *E. coli* chromosome. Density gradient centrifugation (36) in CsCl indicates that 90% of the DNA of F has a guanine-cytosine (GC) content similar to the chromosome of *E. coli*, *Shigella*, and *Salmonella*. The remaining 10% has a GC content of 44%. R factors studied contain some DNA with a 50-52% GC content, which is thought to point to an evolutionary relationship (36), but the size of this fraction varies considerably from R factor to R factor. The loss of resistance genes by certain R factors is accompanied by changes in density that indicate the gene for tetracycline resistance is comprised of 50% GC.

Where as the resistance to chloramphenicol alone, or to a complex of sulfonamide, streptomycin, and chloramphenicol consists of DNA with a 56% GC content. This ratio is similar to the normal chromosome of such species as Aerobacter, Klebsiella or Serratia (14). The presence of 56% GC being correlated with chloramphenicol resistance was found to be true for a number of wild type appearing R factors. This is surprising, in that it is recognized that chloramphenicol resistance is present only on fi^+ (defined in section on R factor types) R factors, while the GC content is so unlike that of F. However, the correlation did not hold for the case of one R factor, for all of its known functions, including chloramphenicol resistance, were retained after loss of the 56% GC fraction (36).

Watanabe (53) postulates a linear model or map for the linkage group of R factors. Evidence for this model was obtained from transduction experiments. Anderson (5) interprets his data to represent a circular linkage map, in which the transfer factor (TF), consisting of genes that confer the ability to conjugate the transfer, is capable of existing in a state free of the resistance determinants. When a resistance determinant and a suitable transfer factor co-inhabit the same cell, association may occur, resulting in the transfer of both entities. Under other conditions, dissociation may occur with either the TF being transferred without resistant determinants, or resistance

determinants being transferred without the TF, which makes the recipient cells resistant but unable to transfer their resistance. Segregation of the resistance pattern has also been observed (1, 49). When this occurs, the resistance patterns of the recipient bacteria vary.

R factor types

When transmissible drug resistance was first discovered an analogy with F factors was at once apparent. For here were genetic elements which brought about their own transfer by conjugation (50) and occasionally transfer of chromosomal segments. Thus both R and F act as sex factors, but in many other ways their differences were also apparent. Conjugation frequency is far less with R than F, and chromosomal recombinants with R are seldom observed. Moreover, the majority of R factors, termed fi^+ for fertility inhibition, inhibit F. Introduction of an fi^+ R factor into an F^+ organism reduces its ability to conjugate, and abolishes agglutination by F specific antiserum, and lysis by F specific phage (52).

Egawa and Hirota (13) attribute the inhibition of F expression to the production of a cytoplasmic repressor produced by R that acts on F and R. Meynell and Datta (27) suggest that this reflects not a difference, but a close relationship between fi^+ R and F^+. They conclude that the observation was in fact precisely as expected if

the fi^+ sex factor was under the control of a repressor determined by a regulator gene in the R factor which also acted on F. The ability of fi^+ sex factor to repress F would simply reflect its ability to repress its own sex factor.

The fi^- R factors do not suppress the function of F and do not seem to determine the production of an F like pilus. The pilus determined by fi^- R^+ bacteria is similar to the pilus of certain colicin positive strains. A filamentous phage was then isolated that was specific for the fi^- pilus (28).

It was then reasoned that a repressed fi^- R factor should yield derepressed mutants analogous to the derepressed mutants of fi^+. That is, mutants that have the ability to transfer their resistance at a much higher frequency than normally observed. Such mutants were isolated (27) by selecting clones of R^+ cells which transferred drug resistance at greatly increased frequency. These mutants were able to transfer their resistance in ten minutes, and though most cells were expressing their conjugation function, the cultures remained insensitive to F specific phage.

Romero et al. (35) have recently shown that a number of fi^+ R^+ cells also carry an fi^- R factor, which they attribute to a second R factor in the cell. It is shown that with these doubly infected cells, that fi^+ and fi^- are transferred independently at conjugation.

Biochemical mechanisms of R factor mediated drug resistance.

Decreased permeability of R factor-carrying cells to antimicrobial agents was initially thought to be the physiological mechanism of resistance. The possibility that a single gene could effect the permeability of cells to several antimicrobial agents was also considered. More recent evidence has shown that permeability is not the biochemical basis for the resistance to most antimicrobial agents, but may be the case with sulfonamides and tetracyclines (53). Chloramphenicol, streptomycin, kanamycin, neomycin, and paromycin are inactivated by enzymatic modification, such as by acetylation, adenylation, or phosphorylation, while penicillin is hydrolyzed by B-lactamases (9, 54).

The biochemical evidence, plus additional evidence from transduction studies, has shown that R factors carry genetic determinants for each similar type of chemotheropeutic agent. It would thus seem more reasonable to postulate the existance of a separate genetic determinant for each enzymatic inactivation reaction. All the inactivating enzymes are apparently produced constitutively, for bacteria grown in the absence of the antimicrobial agents have a full complement of these enzymes.

The levels of antimicrobial resistance conferred by R factors differ from host cell to host cell and from R factor to R factor.

The mechanism for this variation is not known. Mutations can also occur in the R factor to give higher resistance to antimicrobial agents. This can occur as the result of successive mutations in genes or in a single mutation (31).

Host range.

Many gram negative organisms can harbor R factors. Included are Salmonella, Escherichia, Proteus, Klebsiella, Shigella, Pseudomonas, Pasteurella, Vibrio, Serratia, and Citrobacter. Transfer to gram positive organisms has not been reported to occur. Staphylococci, though they can not transfer DNA by cell-to-cell contact, can accomplish gene transfer through extrachromosomal elements termed plasmids. For example, some strains of staphylococci carry plasmids for the production of penicillinase.

The large host range of R factors includes important pathogenic bacteria for man and animals. R factors do not usually effect the virulence of organisms, however a decrease in virulence has been observed to occur in some Salmonella strains (19). That the existence of R factors may play an important role in public health and clinical medicine is a foreseeable possibility.

Distribution

The first R factors reported outside of Japan were found by Datta (10, 11). The presence of R factors was related to an outbreak of gastroenteritis in London. Since this time, it has become apparent that R factors exist and are prevalent in strains of Enterobacteriaceae throughout the world.

Until 1963, R factors found were resistant to the four antimicrobial agents previously mentioned. Then Lebek (23), in West Germany, discovered an R factor that mediated resistance to kanamycin and neomycin, in addition to the afore mentioned agents. Anderson and Datta (4) then isolated R factors that were resistant to penicillin and its derivatives. An interesting note is that resistance to kanamycin and neomycin by R factors is widely spread in Europe, but relatively rare in Japan. This may well be connected with drug use, for selection by multiply drug resistant bacteria may occur with the wide-spread or indiscrimate use of a drug.

Origin

Despite some differences R factors are similar to other sex factors and it is reasonable that a common ancestry may be shared. The origin may have occurred with a transfer factor picking-up chromosomal genes from its host. Thus genes for drug resistance may have originated from the chromosome of some unknown bacterium.

Evidence that argues against this line of reasoning is that biochemical mechanisms of resistance differ between R factor-mediated resistance and chromosomally-mediated resistance (10, 11).

Similarity with other sex factors is seen in that information other than resistance to antimicrobial agents can be transferred by R factors. Smith (41) has documented at least 15 different genes of R factors. These include separate resistances to heavy metals (mercury, cobalt, nickel, etc.), to bacterial viruses, to certain bactericidal substances produced by other bacteria, and to ultraviolet light (25).

It is not known whether drug resistance genes have been picked-up in a single step or successively. A four antimicrobial agent resistant R factor was first observed, but this does not preclude that a progression did not occur. The mechanism of the development of resistance for new drugs is not known. Evidence for pre-existence is found in that cultures obtained from a drug-free community were found to contain R factors for tetracycline and streptomycin (15). Additional evidence is reported by Smith (40), who observed R factors for the same two antimicrobial agents among cultures lyophilized in 1946. This date is prior to the introduction of these drugs. It is not possible, however, to draw any definite conclusion from these or any other observations at present, concerning the mechanism of the

development of R factors. The final answer may come from the production of R factors *in vitro*.

This literature review is by no means comprehensive of the work published in this area. Review articles in this area include those of Watanabe (53, 54), Anderson (5, 6), Kiser et al. (20), Mitsuhashi (29) and Meynell (28).

Introduction to thesis problem.

The immediate importance of infectious drug resistance is that it provides a mechanism for the rapid dissemination of inherted resistance to antimicrobial agents, between organisms of similar or diverse genotype. That antimicrobial agents do have a selective effect when used is not debated. The question is whether the presence of R factors causes complications in the treatment of disease. The people working in this area of research can usually be divided into two groups. One camp, headed by Smith (42, 43) contends that the problem of drug resistance may well be a major hazard to health. Jarolmen (19), head spokesman for the other group, contends that there is little or no evidence that drug resistance causes complications in the treatment of infectious diseases.

The majority of research involving transferable drug resistance has been a study of the incidence in human populations, especially hospitalized groups. The second most widely studied area is that of

the genetics involved in transfer and characterization of R factors.

Relatively little work has been concentrated on the study of the incidence of R factors in diseased and healthy animals, and the process of *in vivo* transfer. Most of the information and data in this area is from England.

In England, the continuous feeding of low "nutritional" levels of antimicrobial agents has been permitted and practiced for more than 15 years. The two antibiotics most commonly used are tetracyclines and penicillin. Smith et al. (43) have observed that this use of antimicrobial agents selects for the emergence of resistant strains of bacteria. Among these resistant strains the percentage of R factors is high. Smith has worked mainly with poultry and pigs, but Walton (48) has also found that the frequency of infectious drug resistance among *E. coli* isolated from healthy farm animals (swine and cattle) is also high. The only reported study of the frequency of R factors among domestic animals in the United States is work reported from our laboratory (1).

A report of interest is that of Morrhouse (30) who has reported the isolation of R^+ bacteria from uncooked and cooked sausages in Ireland. She concludes that it is probable that the strains originated in the intestines of pigs. This may demonstrate a possible means of transmission to man.

The study of *in vivo* transfer has mainly utilized germfree animals, or animals in which antimicrobial agents have been used to create a reduced flora. Reed et al. (32), using microbially defined mice, studied the frequency of transfer and the kinetics involved. From these experiments, it was observed that transfer does occur in the intestinal tract of animals, and R factors will persist therein. The shortest time for the collection of fecal samples after adding bacteria was 5.5 hours, and this was sufficient time for transfer to occur. The number of converted recipients in all cases was found to be lower than the number of donor cells. The low frequency was thought to occur because of repressed R factor-carrying donors, and secondly to the ability of the donor strain to establish in higher numbers in the intestinal tract than the recipient strain. Thus, limiting the number of recipients available to receive R factors.

Smith (44) has reported that it is possible for animal strains of *E. coli* with R factors to transfer their resistance within the alimentary tract of man. Large doses of donor cells were required for transfer to be observed, and still the frequency of transfer was low, and did not persist, or colonize the alimentary tract. The criticisms of this experiment are that only one individual was involved, the donor strains may not have been suitable for establishing themselves, and no selective pressure, as seen in antimicrobial

therapy or "nutritional" feeding procedures, was involved. That people who work with animals, or their carcasses do have a high incidence of R factors has been reported by Moorhouse (30).

Approaches for controlling R factors have seldom been reported. It would seem desirable that means of curing episomes, such as R factors,or preventing their transferring resistance would be found. A small number of chemical agents have been reported to either eliminate or inhibit transfer of R factors. Among the compounds used are acridines (7), sodium dodecyl sulfate (46), mepacrine hydrochloride (24) and clindamycin and its analogues (34). All of these reports involve in vitro studies and no known reports of the in vivo effects have been published. The successful use of these compounds for curing is questionable, for elimination frequency is low for most compounds. Attempts in this lab (33) have not been uniformly successful in the curing of cells of R factors by the methods and procedures reported.

Since little was known of the frequency of transferable drug resistance among domestic animals in the United States, a study was undertaken in this area. Aspects of interest were the frequency of resistance, the frequency of R factors, the frequency of transfer, the resistance patterns of the donor strains, the resistance patterns transferred, and the occurrence of segregation. Upon completion of

this aspect of the study, part of the results were published (1).

Since antimicrobial agents are commonly used as feed additives, and in treatment of diseased animals, it was decided to study the effect of their use on the flora of microbially defined mice. It was hypothesized that antimicrobial agents would creat a selective advantage for organisms resistant to the antimicrobial agent or agents used.

The third area of interest was to determine if there was a means of inhibiting transfer from occurring *in vivo*, again utilizing germ-free mice converted to microbially defined mice. From previous *in vitro* attempts in our laboratory (33) it did not seem feasible to attempt *in vivo* curing of cells of R factors. Areas and means selected to be investigated were the use of chemical agents, mepacrine hydrochloride and mitomycin-C, virulent phage, and colicin production.

MATERIALS AND METHODS

Cultures

Part of the following has previously been reported (1). Ninety-two *Enterobacteriaecae* cultures were randomly selected from the culture collection of the Department of Veterinary Science, University of Nebraska. Biochemical tests determined that 90 of the cultures were *Escherichia coli*, 1 a *Proteus*, and 1 a *Citrobacter*. These cultures had previously been isolated from fecal specimens obtained from cases of neonatal diarrhea among calves and pigs. The fecal specimens were collected between 24 March 1967 and 2 June 1967, from animals on 22 different Nebraska ranches, and animals maintained at the facilities of the Department of Veterinary Science, University of Nebraska.

Resistance patterns for these cultures were determined by methods described in a later section, and those resistant to one or more of the relevant antimicrobial agents were used as prospective donors in conjugation experiments, to determine whether the resistance was transferable. All the resistant cultures were sensitive to nalidixic acid (NA) and were tryptophan-independent, which are presumably chromosomally determined.

The recipient used in all *in vitro* and most *in vivo* conjugation experiments was *E. coli* K-12 F^+ nal-R (K-12; obtained through the courtesy of Sidney Cohn, Michael Reese Hospital, Chicago, Illinois).

This strain is resistant to 500 μg of NA per ml and is tryptophan-dependent. For cases in which the donor cells produced a colicin or phage to which K-12 was sensitive, resistant cells were isolated for use in conjugation experiments (see compatibility testing). A second culture used in conjugation experiments was E. coli K-12 F^- thi^- met^- thr^- leu^- lac^- str-R (FW13A; obtained through the courtesy of David H. Smith, Harvard Medical School). The following abbreviations are used: F, F factor; nal-R, nalidixic acid resistant; thr, threonine; leu, leucine; try, tryptophan; thi, thiamine; met, methionine, lac, lactose; and str-R, streptomycin resistance.

Phage MS2, used in experiments to inhibit mating and transfer of R factors in vivo, is a male specific RNA phage (MS2; obtained through the courtesy of Elinor Meynell, Lister Institute of Preventive Medicine, London, England). A stock preparation, with a final phage concentration of 3.1×10^{11} particles per ml was prepared. The phage stock was then frozen in small quantities and used for all experiments. No loss of viability was seen throughout the duration of the experiments.

Other cultures used in various experiments were selected from the original 92 obtained from the Veterinary Science Department, University of Nebraska. These cultures are NU60-2 (Nebraska University and culture number) used in segregation studies, NU5 R^+ (T) (R^+ for

tetracycline), used in experiments selecting for resistant organisms by antimicrobial agents, and NU73-3 T-R (resistant to tetracycline, non-transferable).

Mice.

Mice of the CF 1 strain were used throughout this study. Germ-free (GF) CF 1 mice were originally obtained from Carworth, Inc., New City, N.Y., and have been maintained by breeding in a GF environment in our laboratory.

Gnotobiotic methods.

Part of the following has previously been reported by Reed et al. (32). GF mice were housed in plastic cages in Trexler flexible film isolators and were maintained by routine gnotobiotic procedures selected for use in this laboratory. All mice received sterile water and sterile Wayne Sterilizable Lab-Blox ad lib. GF mice were examined weekly by established procedures (47) for microorganisms and macro-parasites. It is not claimed that these mice are virus-free; however, serum samples were assayed for antibodies to 12 common murine viruses and antibodies were not detected. All attempts to isolate *Mycoplasma* from these mice have yielded negative results.

For most experiments, mice were removed from the GF units and placed in sterile plastic cages with individual filter tops (Isocage

system, Carworth, Inc.). Using gnotobiotic techniques, mice were maintained on sterile feed, water, bedding, and handled with sterile gloves. Using this system, mice were maintained without noticeable cross-contamination for the duration of the experiments.

To associate GF mice with drug resistant donor or drug sensitive recipient bacteria, the contents of 2.5 ml Mueller Hinton Broth (Difco) (MHB) overnight cultures were added to 150 ml of sterile drinking water. To avoid possible interaction of donor and recipient bacteria in the drinking water bottle, the bacterial strains, and phage were given to the mice sequentially. After establishment of the selected strains, the microbially defined (MD) mice were given sterile water for the duration of the experiment.

Phage cultures were given in a manner identical to that of bacterial cultures, after a F^+ recipient strain was established, and prior to association of the mice with R factor donor strains.

In experiments to select for converted recipients (recipient cells that have received an R factor from the donor strain) in vivo, mice in which mating and transfer had already occurred were given antimicrobial agents using a gavage tube. The gavage tube had an internal diameter of 0.38 mm, and an outside diameter of 1.08 mm (PE 20, Intramedic polythylene tubing, Clay Adams), and for injections purposes, a 27 gauge needle was fitted into the tubing thus providing

an adapter for attachment of a syringe. This method insured a constant and controlled dosage.

In experiments to inhibit mating and resulting transfer of R factors, various chemicals were given in the manner described in the previous procedure. This method insured a constant and controlled dosage, and allowed chemicals to be used that the mice would not drink if contained in the drinking water.

Media, antimicrobial agents, and chemicals.

Part of the following has previously been reported (1). The selective medium used in conjugation experiments was Mueller Hinton Broth (Difco) containing 2% BBL agar (MHA) supplemented with combinations of the following antimicrobial agents: NA (Sterling-Winthrop), 100 µg/ml; sulfamethizole (Ayerst Laboratories), 100 µg/ml; tetracycline hydrochloride (Lederle Laboratories), 25 µg/ml; streptomycin sulfate (Squibb Institute for Medical Research), 10 µg/ml; and Kanamycin sulfate (Bristol Laboratories), 25 µg/ml (see appendix B for preparation procedures of antibiotics used). Stock solutions of tetracycline (T) were stored and used for a maximum of 10 days. Plates of MHA containing antimicrobial agents were stored for no longer than 7 days. This was to insure high antimicrobial activity and minimize variation between experiments.

Mitomycin-C (Calbiochem) and mercaprine hydrochloride (Sterling-Winthrop) were used in experiments to inhibit mating and resulting transfer of R factors from occurring *in vivo*. (See appendix C for the preparation procedures for these two chemicals.)

Minimal medium and supplemented minimal medium (see appendix D for preparation procedures) were used to confirm the converted recipients from mating experiments, as recipient cells that had received an R factor, and to show that chromosomal transfer had not occurred.

Determination of resistance pattern.

Donor cultures were screened for antimicrobial agent resistance with the following antimicrobial discs (BBL of Difco) on MHA: Sulfamethizole, 1 mg; oxytetracycline, 30 µg; streptomycin, 10 µg; chloramphenical, 30 µg; kanamycin, 30 µg; nitrofurantoin, 100 µg; colistin, 10 µg; and NA, 30 µg.

The procedure for sensitivity testing was to culture the donor strains in MHB for 9 hours at 37° C. One drop of this broth culture was then added to 5.0 ml of fresh MHB, mixed, and then spread using a sterile cotton swab, on MHA plates containing 20 ml of medium. Antimicrobial discs as listed above were then added 6 per plate, using a sterile forcep. Plates were then incubated for 15-18 hours at 37° C. Cultures showing little or no zone of inhibition to an

antimicrobial disc at this time were considered resistant.

Compatability testing.

Donor cultures were screened for the production of a colicin or phage to which the recipient was sensitive. Donor strains were grown in colonies by making stabs into nutrient agar (Difco) plates, and incubated for 36 hours. The colonies were then killed by placing the plates, with lid ajar, within an anaerobe jar with chloroform. Vaccum was applied and jar sealed off. After 1 hour of exposure to the chloroform at room temperature, the plates were removed and allowed to dry for 15 minutes at 37° C. The plates were then overlayed with trypticase soy broth (BBL) containing 0.9% BBL agar, to which 0.05 ml of recipient (K-12 or FW13A) had been added from a culture in logarithmic growth phase. Plates were then incubated at 37° C for 24 hours. Donor cells producing a colicin or phage resulted in an area of lysis or inhibition surrounding the killed colonies. Strains that were compatable did not inhibit growth of the recipient. Strains producing an inhibitory substance were incubated for additional time at 37° C, upon which a few colicin or phage resistant colonies would usually appear in the zone of inhibition. These colonies were then cultured and the above procedure was repeated to determine compatability. For colicin or phage producing strains in which colonies did not appear upon additional incubation, recipient cells

from the edge of the zone of inhibition were selected to repeat the above procedure until a compatable recipient was obtained for conjugation experiments. For some donor strains it was necessary to repeat the above procedure up to 6 times, before obtaining a recipient cell line that was entirely resistant to the inhibitory substance produced.

From the compatability screening, donor cultures were chosen that were highly lytic or inhibitory for the recipient FW13A. One of these cultures, NU73-3, was then used in experiments to determine if the inhibition observed *in vitro* would be active *in vivo*, and thus act as a natural means of selection for colicin or phage producers over colicin or phage sensitive strains.

Transfer of drug resistance.

Part of the following has previously been reported (38). A 125 ml Erlenmyer flask, containing 25 ml of MHB, was inoculated with 1 ml of a 16-18 hour MHB culture of either R factor donor or recipient, and was incubated for 9 hours on a shaking tray at 37° C. These cultures were then diluted in fresh MHB, with spectrophotometric methods, to approximately 5×10^{8} cells per ml. Diluted donor (1 ml) and recipient (1 ml) were mixed in a culture tube (25 by 200 mm) and were incubated without shaking for 18 hours at 37° C. To serve as controls for spontaneous mutation to drug resistance, 2 ml of diluted donor and recipient was incubated separately under the same conditions. At the

end of the conjugation period, 0.2 ml of dilutions of the mixed culture and controls were spread with sterile glass rods on selective media (MHA) containing nalidixic acid and either tetracycline, sulfamethizole, kanamycin, or streptomycin to detect K-12 cells having received drug resistance (converted recipients), and on media containing singly the above antimicrobial agents to determine the numbers of parental cells present in the mating mixture. The number of selective media used for particular mating corresponded to the number of antimicrobial agents to which the prospective donor was resistant, each medium containing NA (Sm if FW13A used as recipient) and a different antimicrobial agent. For each mating, and each combination of antimicrobial agents, in which transfer of drug resistance was observed, ten colonies were picked and used to confirm that they were converted recipients, that is genetically K-12 with the addition of drug resistance from the donor strain. Minimal medium and minimal medium (see appendix D for preparation procedure) supplemented with 100 µg/ml of required amino acids was used to show that transfer of drug resistance did not involve chromosomal transfer.

Segregation studies.

Donor strains that were multiply resistant to antimicrobial agents and were capable of transferring resistance to one or more of these agents, were studied to determine if segregation of resistance

determinants occurred. Ten colonies of converted recipients were selected from each successful mating medium and replica plated to MHA plates containing singly the antimicrobial agents to which the donor was resistant. This determined if selection on different antimicrobial agents resulted in the transfer of the same or varying resistance patterns.

Assay procedure for in vivo studies.

Part of the following has previously been reported by Reed et al. (32). Fresh fecal specimens were collected in sterile screw-cap tubes, at various times, from mice intentionally associated with various strains of bacteria and bacteriophage. One fecal pellet from each mouse was macerated, by using a blunt glass rod, in 4.0 ml of Mueller Hinton Broth (MHB). Portions (0.2 ml) of such suspensions and various dilutions of the suspensions were spread on selective media. Plates were incubated at 37° C until colonies were suitable for counting. Counts are expressed as the number of viable cells or phage per milliliter of fecal homogenate.

RESULTS

Incidence of drug resistance.

The following has previously been reported (1). Of the 92 strains studied, 57 were resistant to one or more of the relevant antimicrobial agents. Resistant strains were obtained from all herds involved in the study. The number of strains resistant to individual drugs is shown in Table I.

The 57 resistant strains were considered prospective donors and were mated with K-12 to determine if the resistance was transferable.

Incidence of R factors among resistant cultures.

Of the 57 resistant strains, 43 could transfer part or all of their resistance pattern upon conjugation with K-12 (Table II). These 43 R factor-carrying strains were obtained from 17 of the 23 herds studied. The number of strains which transferred resistance to individual drugs is shown in Table I.

The most common resistance patterns for the 57 strains were T Sm Su (22 of 57, or 38.6%) and T (13 of 57, or 22.8%). Of the strains with the resistance pattern T Sm Su, 95.5% were found to transfer part or all of their resistance pattern to the recipient in mixed culture. With the exception of strains resistant to Su only, strains with other resistance patterns commonly transferred part or all of their resistance (Table II).

Table I. Frequency of resistance and R factor-mediated resistance to various antimicrobial agents.

Chemotherapeutic agents	Number of resistant strains	Number of resistant strains which transferred resistance	Percent of resistant strains which transferred resistance
T	49	34	69.4
Sm	31	19	61.3
Su	36	20	55.6
Km	5	4	80.0
Cm	0		
Nf	0		
Co	0		
NA	0		

[a] Abbreviations: T, tetracycline; Sm, streptomycin; Su, sulfamethizole; Km, kanamycin; Cm, chloramphenicol; Nf, nitrofurantoin; Co, colistin; NA, nalidixic acid.

Table II. Resistance pattern and transfer characteristics of drug resistant strains.

Strains	Resistance pattern[a]	No. resistant	No. which transferred part or all of resistance pattern	Resistance patterns transferred[b]
Escherichia coli	T	13	7 (53.8%)	T
	Sm	2	2	Sm
	Su	5	0	
	Km	1	1	Km
	T Sm	3	3	T Sm (2); Sm (1)
	T Su	5	4 (80%)	T Su (1); T (3)
	T Sm Su	22	21 (95.5%)	T Sm Su (10); T Sm (1); T Su (2); T (3); Su (5)
	T Sm Su Km	4	3 (75%)	T Sm Su Km (2); T Sm Km (1)
Proteus	T	1	1	T
Citrobacter	T	1	1	T
Total		57	43 (75.4%)	

[a] Abbreviations: T, tetracycline; Sm, streptomycin; Su, sulfamethizole; Km, kanamycin

[b] Numbers in parenthesis indicate the number of strains showing a particular resistance pattern.

The transfer frequency, calculated as: (no. of K-12 receiving resistance)/(total no. of K-12 in mixture) X 100, varied greatly among the 43 R^+ strains, from 59.2% to 10^{-4}% (Table III).

To confirm that the colonies observed on the selective media were K-12 that had received an R factor (converted recipients) and chromosomal transfer had not occurred, ten colonies were randomly selected and replica plated on minimal medium, supplemented minimal medium, and on plates containing antimicrobial agents to determine segregation patterns. Though the number of chromosomal markers using K-12 was minimal, in all cases the converted recipients randomly selected required supplemented minimal medium for growth, as does K-12 the recipient, and would not grow on minimal medium alone. The donor strains were all capable of growing equally well on minimal or supplemented minimal media. From these results, it was concluded that transfer of drug resistance had occurred without transfer of the chromosome.

Segregation.

Considerable variation was observed between different strains in the frequency of transfer of resistance to a particular antimicrobial agent. This was observed by plating dilutions of the mating mixture (0.2 ml) on MHA plates containing NA and another antimicrobial agent to which the donor strain was resistant (see materials and methods).

Table III. Transfer characteristics of several R factor-carrying strains of *E. coli*.

Strain of *E. coli*	Resistance pattern[a]	Resistance transferred	Frequency of transfer[b] (%)			
			T	Sm	Su	Km
NU5	T	T	9.6			
NU61-1	T	T	3.0×10^{-2}			
NU65-1	T Sm Su Km	T Sm Su Km	0.39	0.56	2.4	2.3
NU60-2	T Sm Su Km	T Sm Su Km	10.0	1.0×10^{-4}	10.0	1.6×10^{-3}

[a] Abbreviations: T, tetracycline; Sm, streptomycin; Su, sulfamethizole; Km, kanamycin.

[b] Transfer frequency = (no. of K-12 receiving resistance)/(total no. of K-12 in mixture) x 100.

The transfer frequency was then calculated from the formula previously given (see incidence of R factors or Table III) for each antimicrobial agent to which resistance was transferred. Table III gives the frequency of transfer observed for four representative strains of E. coli observed and studied. Cultures NU5 and NU61-1, though having the same resistance pattern, show typical variation in their ability to transfer their resistance to recipient K-12. Cultures NU65-1 and NU60-2 have the same resistance pattern and transfer the same pattern, and variation in frequency of transfer is observed in these two multiply resistant strains. Ability to transfer resistance to different antimicrobial agents at different frequencies is observed with NU60-2.

For NU65-1 the variation observed between the transfer frequency for the four antimicrobial agents involved is not large, and a single linkage group could be postulated. In NU60-2, T and Su may be linked, for their transfer frequencies are identical, and Sm and Km may be linked, but comparing the transfer frequencies of the four antimicrobial agents, the evidence does not support a single linkage group. Data from cultures like NU60-2 make it difficult to construct linkage maps for the models proposed (see introduction: Models).

With culture NU60-2, other interesting results were observed upon replica plating (Table IV). Selection on T results in a high frequency of transfer for T, while the frequency for Sm was much lower selecting

Table IV. Segregation pattern of NU60-2.

Selected on[a]	Frequency of transfer[b] selected on agent listed in column one	Replica plated on[c]	Number of colonies resistant to agent in column three over the number of colonies replica plated
T	10.0%	T	10/10
		Sm	10/10
		Km	0/10
Sm	1.0×10^{-4}%	Sm	10/10
		Km	2/10
		T	10/10
Km	1.6×10^{-3}%	Km	10/10
		T	5/10
		Sm	10/10

[a] Abbreviations: T, tetracycline; Sm, streptomycin; Km, kanamycin.

[b] Transfer frequency = (no. of K-12 receiving resistance)/(total no. of K-12 in mating mixture) X 100.

[c] Replica plating to sulfamethizole was not possible due to background growth.

on Sm. Thus it was quite unexpected when all ten of the colonies taken from the T selection plates were able to mediate resistance to Sm. Additional unexpected results were observed upon replica-plating Km selected colonies, for only 5 of the 10 colonies mediated resistance to T which was transferred at a much higher frequency. Some, but doubtfully all, of the above results may be explained by the possible existence of two R factors within the same cell. That two R factors can exist and transfer separately within the same cell has recently been shown by Romero and Meynell (39).

Selection for resistant organisms by antimicrobial agents.

In vivo mating of R^+ bacteria with recipient strains was performed as described in materials and methods. The order of establishment of strains within the gastrointestinal tract was recipient K-12, followed two days later by NU5, the R factor donor. NU5 is resistant to tetracycline only and transfers this resistance to a suitable recipient with an *in vitro* transfer frequency of 9.6% (Table III).

Two days after the addition of the donor strain, NU5, the drinking water containing the donor was removed, and it was confirmed that transfer had occurred within the gastrointestinal tract of the microbially defined mice. Cell concentrations present could be determined, for donor cells were resistant to T only, recipient cells were resistant to NA only, and converted recipient cells were resistant to

both NA and T, and possessed the genetic dependency markers of the recipient. These mice in which transfer of drug resistance had occurred, were then divided into four groups depending upon the antimicrobial agents given by gavage. Mice received 1) no antimicrobial agents; 2) nalidixic acid, 5.0 or 10.0 mg per dose; 3) tetracycline, 2.5 or 5.0 mg per dose; and 4) both tetracycline, 2.5 mg, and nalidixic acid, 5.0 mg, per dose.

Antimicrobial agents were given daily for the first week, and every other day for the duration of one month. Figure 1 presents a representative graph of the cell numbers found in the absence of antimicrobial agents, expressed as the number of cells per ml of fecal homogenate. No increase in the numbers of any of the three cell types present was observed in the mice receiving antimicrobial agents singly or in combination over the control mice receiving no antimicrobial agents. Figure 1 is thus not only representative of cell populations observed in control animals, but similar to the results observed in animals receiving antimicrobial agents, for there was little variation in the cell concentrations observed in animals treated with antimicrobial agents over controls.

Colicin selection.

From compatability testing (see materials and methods) various cultures were observed to produce a colicin or phage to which

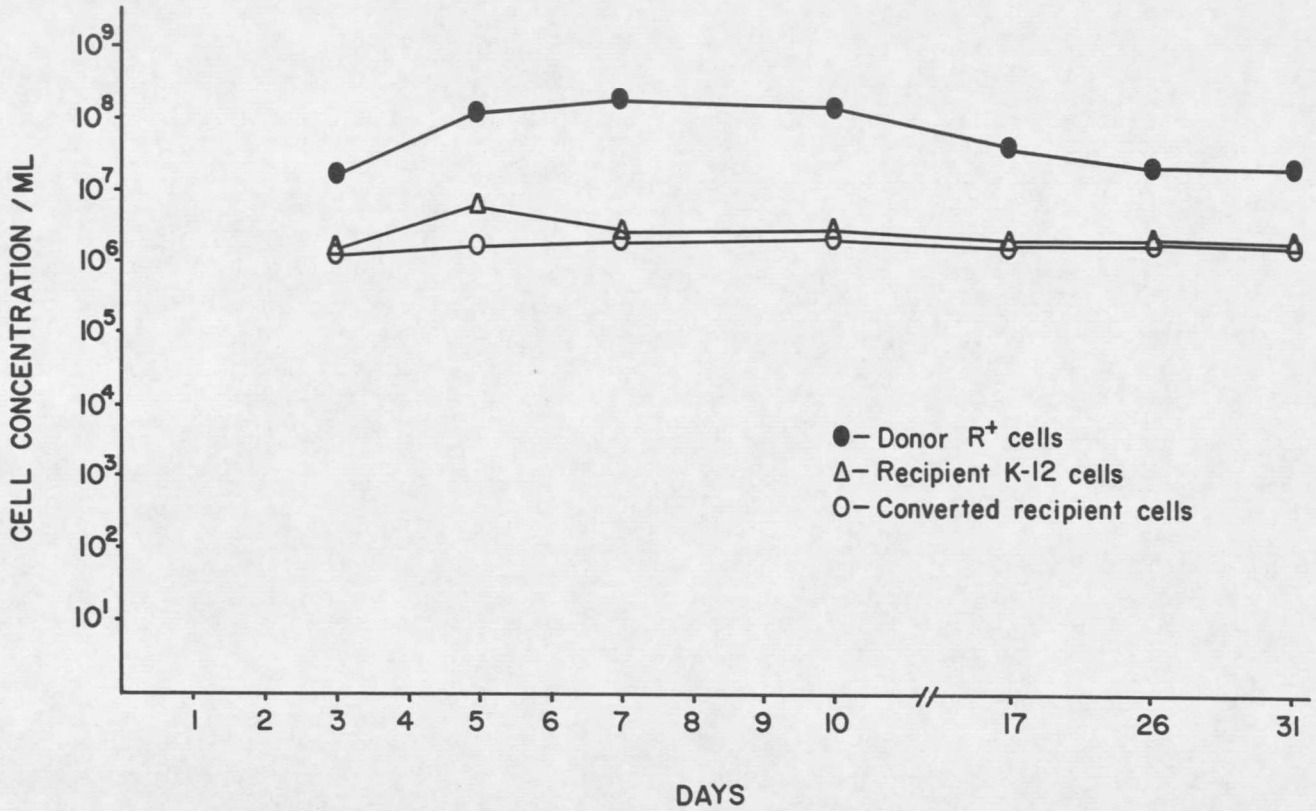

Figure 1. Cell concentrations observed in vivo. Mating control. K-12 F^+ nal-R, the recipient, was associated with GF mice on day -1. On day 1, water containing the K-12 culture was removed, and the R factor donor, NU5 R^+ (T), was given in the water, and removed on day 3. Fecal samples were obtained on the days indicated and assayed for cell concentrations per ml of fecal homogenate. The results plotted are from one of the many control animals observed, and very similar results were observed with all other control animals.

recipient strains K-12 and FW13A were sensitive, and a large zone of inhibition was observed. Upon mating two of these strains that produced an inhibitory substance, no converted recipient cells were observed, and the number of viable recipient cells in the mating mixture was considerably decreased from the 5 X 10^8 cell initially added (Table V). The number of donor cells was not noticeably effected. The supernatant from these two cultures was studied to determine if a virulent phage was responsible for the inhibition of the recipient. Plaquing ability was not observed with the supernatant from these strains, and from this preliminary evidence these strains were considered to be colicin producing. Culture NU73-3 which possesses non-transferable tetracycline resistance was selected for further study.

It was postulated that if the colicin was active *in vivo* this could be a means of natural selection for colicin producing strains over colicin sensitive strains, and that colicin producing strains might be used to alter the frequency of *in vivo* mating and transfer of R factors.

On day -3, a GF mouse was given FW13A str-R as described in materials and methods. Three days later, the water containing this culture was removed and replaced by water containing the colicin producing strain, NU73-3 T-R. Two days later the water containing

Table V. *In vitro* colicin and phage observations.

Culture	Transfer frequency[b]	Cell concentrations per ml of mating mixure		
		Donor	Recipient	Converted recipient
NU82[a]	0	1.6×10^9	$<10^6$	0
NU73-3[a]	0	3.5×10^8	$<10^4$	0
NU5 & MS2[c]	0.91%	6.5×10^8	5.5×10^7	5.5×10^5
NU5	14.3%	1.9×10^9	1.4×10^8	2.0×10^7

[a] Colicin producing strains.

[b] Transfer frequency = (no. of recipients receiving resistance)/(total no. of recipients in mating mixture) X 100.

[c] Phage MS2 added to give a final concentration of 1.8×10^{10} particles per ml.

this culture was removed, and sterile water given for the duration of the experiment. Fecal samples were observed as to the relative cell numbers during the next 17 days by plating dilutions of the fecal homogenate on MHA plates containing either Sm, to select for FW13A, or T, to select for NU73-3. Both strains were found to be established in near equal numbers in the gastrointestinal tract of the MD mouse (Figure 2).

To determine whether the original colicin sensitive cells (FW13A) were the ones established or non-sensitive cells had been selected for *in vivo*, colonies were randomly selected from the fecal homogenate platings on day 17. Five colonies of each strain were selected and compatibility testing was preformed. In all cases, FW13A recovered from the fecal sample remained highly sensitive and inhibited by NU73-3 cells recovered from the same fecal sample Comparison of zone size and characteristics to original compatibility tests showed no variation.

Phage inhibition of transfer of R factors.

MS2, which is a male specific RNA phage, was used to determine if its presence during mating would inhibit transfer of R factors from occurring by lysing F^+ recipient cells and donor cells expressing F. *In vitro* studies were performed by mating donor and recipient strains as detailed in materials and methods, with the addition of

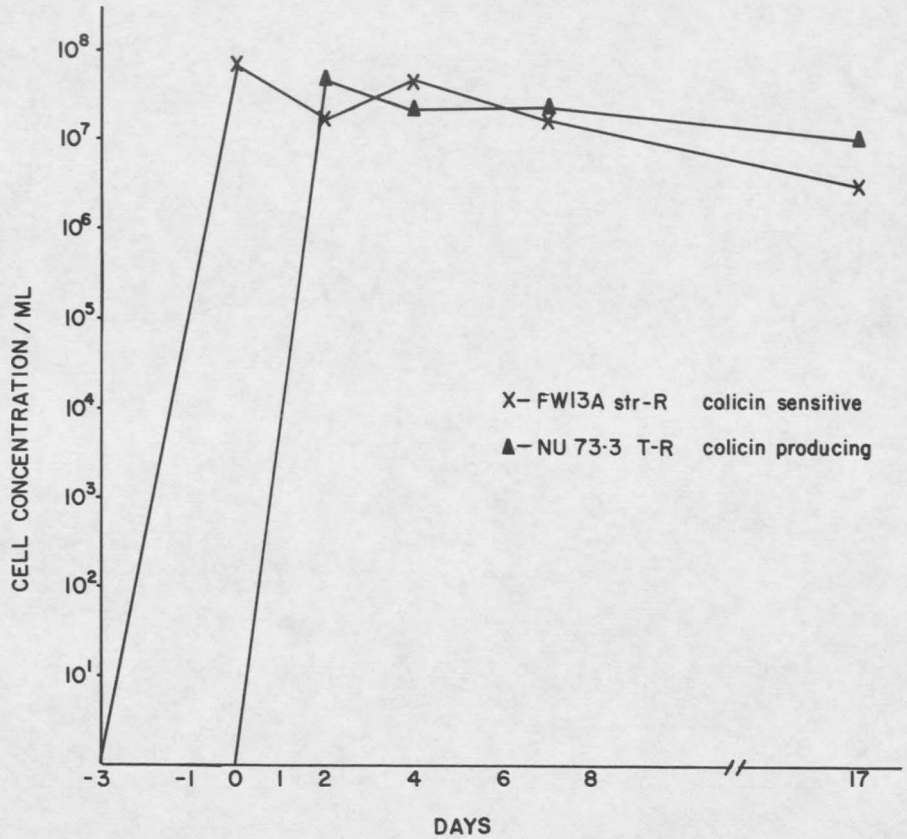

Figure 2. In vivo interaction of colicin producing and colicin sensitive strains. On day -3, FW13A str-R was associated with a GF mouse. On day 0, the water containing this culture was removed, and NU73-3 T-R was given in the drinking water. Water containing the culture NU73-3 was removed on day 2, and sterile water given for the duration of the experiment. Fecal samples were obtained on days indicated and assayed for the cell concentrations of the two strains, and expressed as cells per ml of fecal homogenate.

phage MS2 to a second set of mating tubes. The *in vitro* procedure was to add K-12 F^+ cells (5×10^8 per ml) to the mating tube, followed by MS2 in a small volume, to give a final phage concentration of 1.8×10^{10} particles per ml, ten minutes were then allowed for phage absorption prior to addition of donor cells (5×10^8 per ml). Controls were normal matings with no phage added. The results were a decrease in the number of converted recipient cells observed for the four cultures studied, and a smaller decrease in the total number of recipient cells present. The results for culture NU5 R^+ (T), which was selected for additional *in vivo* studies, were a decrease in transfer frequency from 14.3% (control) to 0.91% when mated in the presence of MS2 (Table V).

It was then determined if the passage of MS2 through the gastrointestinal tract of mice would inactivate the phage or whether virulence would be retained in the gastrointestinal tract was determined. Germfree mice were associated with phage MS2 in a concentration of 1.5×10^7 particles per ml via the drinking water for two days. The water containing phage was then removed (day 1) and sterile water given. Fecal samples were obtained daily and assayed for the presence of virulent phage by plaquing on a lawn of succeptible bacteria (Figure 3). The phage titer per ml of fecal homogenate was high showing that virulence was retained after passage

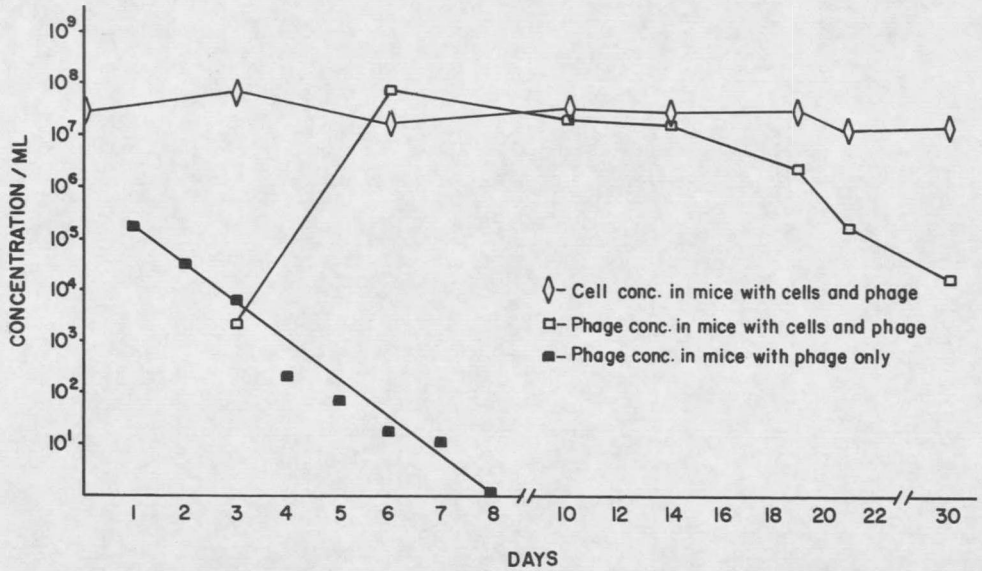

Figure 3. Phage and cell concentrations *in vivo*. On day -1 GF mice (closed rectangles) were associated with phage MS2 in the drinking water containing 1.5×10^7 particles per ml. On day 1, the water containing the phage culture was removed and sterile water given. Fecal samples were obtained daily and assayed for virulent phage particles and expressed as phage particles per ml of fecal homogenate. Results are the average of two mice.

On day 0, GF mice were associated with K-12 F^+ nal-R (open diamonds) in the drinking water. On day 3, water containing the cell culture was removed, and phage MS2 in a concentration of 6.2×10^7 particles per ml was given in the water. On day 4, the water containing the phage culture was removed, and sterile water given. Fecal samples were obtained on the days indicated and assayed for phage (open rectangles) and cell (open diamonds) concentrations per ml of fecal homogenate. Results are the average of two mice.

through the gastrointestinal tract of the GF animal. A logarithmic decline to zero in the number of virulent particles was observed during the 6 days following removal of the phage contained in the drinking water (Figure 3).

It was next determined whether MS2 could be maintained within the gastrointestinal tract of a microbially defined (MD) animal containing F^+ host bacterial cells. On day 0, K-12 F^+ was associated with a GF mouse. Day 3 the drinking water containing the F^- cells was removed, and MS2 in a concentration of 4.4×10^8 particles per ml was given in the drinking water. On day 4, the phage water culture was removed and sterile water given for the duration of the experiment. Fecal samples were collected at various times and assayed for F^+ cell concentration and phage concentration (Figure 3). Though phage concentration declined during the month of the experiment, for 21 days the phage concentration remained above 10^5 particles per ml of fecal homogenate.

The preliminary work completed, experiments were performed to determine if the presence of MS2 would inhibit transfer of R factors from occurring <u>in vivo</u>. Germfree mice were associated with recipient K-12 F^+ nal-R on day -4 and two days later (day -2) the water containing the cell culture was removed and MS2 given in a concentration of 6.2×10^7 particles per ml in the water. On day 0, the phage

contained in the water was removed, and culture NU5 R^+ (T) was given in the water. Day 2, water containing NU5 was removed and sterile water given for the duration of the experiment. Starting day 1, fecal samples were collected at various times and assayed for the number of donor, recipient and converted recipient cells present. This was accomplished by plating fecal homogenate samples on media containing antimicrobial agents, either singly or in combination. Phage concentration was also assayed at the same time for each fecal sample. Control mice for the experiment received the same strains of bacteria, on the same days, but no MS2 was given.

The presence of phage MS2 did not reduce the number of converted recipients observed over the control animals not receiving phage. Cell concentrations observed were essentially the same as seen in Figure 1, and the phage concentration maintained during the duration of the experiment was similar to the preceding results as seen with MS2 and F^+ cells (Figure 3).

Chemical inhibition of R factor transfer.

Various chemicals have been reported (7, 24, 34, 46) as having an inhibitory effect on *in vitro* R factor transfer. Two of the chemicals, mitomycin-C and mepacrine hydrochloride, were selected to determine if they would be effective in inhibiting *in vivo* transfer of R factors. The procedure used was identical for both chemicals.

Germfree mice were associated with K-12 str-R on day -2. On day 0, the drinking water containing the recipient was removed and mitomycin-C (30 or 50 μg) or mepacrine HCl (5.0 or 10.0 mg) was given by gavage tube. Water was deprived for one hour, then donor culture NU5 R^+ (T) was given in the drinking water. Water containing the donor culture was removed on day 2 and sterile water given for the duration of the experiment. Chemical doses were given daily for the first week and every other day for the duration of the experiment. Fecal samples were obtained on days 1, 2, 5, 8 and 19. By day 5, mepacrine HCl was observed to have an adverse effect on mice when given directly into the stomach. Weight loss resulted, and death occurred on day 26, though drug administration was halted on day 19. Mitomycin-C did not appear to have any adverse effects on mice.

Neither of the chemicals used resulted in a decrease in the number of converted recipients observed over control animals not receiving either of the compounds. Mice receiving mitomycin-C or mepacrine HCl and control animals were observed to have cell concentrations very similar to those seen in Figure 1.

DISCUSSION

Results show that transferable drug resistance is common among Enterobacteriaceae isolated from young calves and pigs having neonatal diarrhea. In Great Britain, Smith (42) reported a high incidence of drug resistance, most of which was transferable, among E. coli strains isolated from animals suffering diarrhea, and discussed the effect of animal feeds which contain antimicrobial drugs on the emergence of drug-resistant strains of bacteria. Reliable feeding records of the herds studied in this paper were not available, and it is not possible to relate the presence or absence of R factor-carrying bacteria with the presence of absence of antimicrobial drugs in animal feeds. Some animals in the herds studied had been treated with chemotherapeutic agents; most commonly tetracycline, a sulfonamide or neomycin. However, the individual animals used in this study had not been treated with chemotherapeutic agents.

Recent evidence (26) suggests that invasive strains of E. coli are not the primary etiologic agents in neonatal calf diarrhea. Studies utilizing hysterectomy-derived, colostrum-deprived calves have shown that a virus, isolated from fecal material obtained from herds having neonatal diarrhea, can produce neonatal calf diarrhea typical of that seen in field cases. The disease was not readily reproduced with bacterial cultures alone. However, it appears from these experimental studies that calves inoculated with both virus and

invasive strains of E. coli are subject to intestinal overgrowth of E. coli and later septicemia and death. Thus, drug resistance among enteric bacteria can clearly complicate the therapy and management of this economically important disease.

It is well established that R^+ Enterobacteriaceae can, in vitro transfer drug resistance to an impressive number of bacterial species (5). The extent of transfer in vivo is more difficult to determine. Thus, the importance of R^+ Enterobacteriaceae such as E. coli as a reservior of resistance determinants which can be transferred to pathogenic bacteria has not been established. However, early studies in Japan, reviewed by Watanabe (53), demonstrated the transfer of multiple drug resistance in vivo in human volunteers, and dogs, and recently studies in microbially defined animals (32, 37) have clearly shown that R factor transfer can occur in vivo. Anderson and Lewis (3) have reported finding Salmonella R^+ strains with resistance to penicillin and its derivatives arise soon after the administration of penicillin to livestock. They assume that the origin of the R factors is from bacteria already in the intestinal tract of the animals.

The question of whether antimicrobial agents added to feed or given directly into the stomach as in this investigation provide a selective force for multiply resistant bacteria is debatable. For the antimicrobial agents, the methods, and the times of administration used in the previous experiments, these multiply resistant strains

were not selected for. Many reasons for this may exist. The length of administration of antimicrobial agents was never longer than one month, while animals diets may contain levels of antimicrobial agents continuously for the life of the animals. The antimicrobial agents used may be another factor, for tetracycline and nalidixic acid are both bacteriostatic in action, while penicillin, which is lytic, is more commonly used as a feed additive. The antimicrobial agents may thus have been absorbed or inactivated within the stomach and intestine, prior to effecting the replication of sensitive bacteria present. The numbers of frequency of R factors may be of little importance. For theoretically, one R factor-carrying bacteria could transfer its resistance to a pathogenic organisms, which could result in complications of the treatment of the disease. This may be an important aspect to consider, for it is observed that once established, R factors can persist. This is seen from the persistance of R factors in the control mice of the previous experiments, and from the high incidence of R factors found in strains of Enterobacteriaceae associated with neonatal diarrhea in calves and pigs. From this evidence it is seen that a reservior of R factors may become established even with limited use of antimicrobial agents, for the animals from which the specimens had been obtained had not received antimicrobial therapy.

In the cultures studied the frequency of transfer varied considerably. Segregation was noted with a number of strains, with the resistance to some antimicrobial agents being transferred at a much higher frequency than others. The high frequency of transfer observed with some cultures shows that repression is not occurring as commonly observed with most strains several generations after receiving an R factor. These continually derepressed cultures, such as NU5 (Table III), could be especially effective in causing complications in treatment of infectious diseases, due to the ability of these derepressed strains to transfer their resistance at a high frequency to other strains.

One culture in particular, NU60-2, was observed to have an unusual segregation pattern upon replica-plating (Table IV). It is possible to explain part of this unusual segregation pattern on the existence of two R factors within the same cell. Other explanations might include the level of resistance that is mediated in the recipient by the R factor. For an unexplainable reason, the newly acquired R factor may not be able to express resistance to streptomycin until growth and division have occurred. This might explain why streptomycin is observed to transfer at a very low frequency when selected on medium containing streptomycin, yet all of the cells selected from tetracycline selected plates, where T resistance is

transferred at a much higher frequency, are capable of mediating resistance to streptomycin.

The curing of cells of R factors has been reported (7, 34, 45), but only *in vitro* and with low frequency. The low frequency of curing obtained, and the negative *in vivo* attempts to inhibit transfer of R factors previously described, show the stability and persistance of R factors once they become established. Foreseeable complications could exist in the treatment of infectious diseases if the resistance range carried by R factors should increase and include antimicrobial agents which are yet effective. One hint as to the effect may have already been observed in the cases of chronic urinary tract infections which have a high frequency of R factor-carrying organisms present. In these cases, infection is persistent and recurring.

Colicin, an extracellular antibiotic-like protein synthesized by some enteric bacteria, is lethal for colicin sensitive bacteria *in vitro*. From the action of colicins observed *in vitro*, it is tempting to postulate the occurrance of an evolutionary relation between colicins and the selective advantage they could bestow in competing with colicin sensitive neighbors *in vivo*. If this was true, this would be a possible means of controlling R factors, by finding strains of bacteria that produced colicins to

which the R factor-carrying strains were sensitive. The recent work of Ikari et al. (18) and results of experiments described in this paper, show that colicin production for the cases studied does not contribute to a selective advantage in vivo. It is possible that the colicins produced are susceptible to proteolytic enzymes and are inactivated in the gastrointestinal tract of the animal.

A vast amount of research has been devoted to the possible therapeutic applications of bacteriophages. In most cases the results have been disappointing, but in some diseases such as cholera, there has been some evidence for a favorable effect of phage therapy (45). Interest in this application has subsided in recent years, but since the transfer of drug resistance is dependent upon the expression of a specific philus, unlike the virulence of bacteria, it was postulated that a virulent phage might be used to inhibit transfer of R factors from occurring.

Virulent phage MS2 when present in the gastrointestinal tract of MD mice did not effect cell numbers present to any degree, and did not inhibit the ability of R factor transfer to occur. The most surprising observation from in vivo experiments with MS2 phage was the continued high concentration of F^+ cells, most of which retained their sensitivity to MS2. With continual presence of the virulent phage MS2 it seems surprising that sensitive cells were not lysed, and

resistant cells selected for.

Due to the high incidence of R factors it therefore seems necessary to evaluate the influence of antimicrobial agents on the dynamics of microbial ecology, deciding whether their use therapeutically, and more importantly for growth promotion in feeds for short term meat production is beneficial or detrimental. It must also be assessed whether the current feeding practices allow for the emergence of drug resistant and R factor-carrying microbial populations, and if so, whether these are in turn transmitted to man. Means of eliminating, or at least inhibiting transfer of R factors *in vivo* need to be found. Continued increase in the frequency of R factors within microbial populations may result in health problems of major importance.

SUMMARY

Fecal specimens were collected on 22 different Nebraska ranches and at the Department of Veterinary Science, University of Nebraska, from young calves and pigs with neonatal diarrhea. Enterobacteriaceae isolated from these fecal specimens were screened for resistance to tetracycline, streptomycin, sulfamethizole, kanamycin, chloramphenicol, colistin, nitrofurantoin, and nalidixic acid. Of the 92 strains studied, 57 were resistant to one or more of these antimicrobial agents. Resistant strains were obtained from all herds involved in the study. The two most common resistance patterns were tetracycline streptomycin sulfamethizole (22 of 57) and tetracycline (13 of 57). None of the strains were resistant to chloramphenicol, colistin, nitrofurantoin, or nalidixic acid. The 57 resistant strains were studied to determine whether the resistance was transferable. Forty-three of the 57 resistant strains could transfer part or all of their resistance pattern to a drug sensitive recipient. The 43 R^+ strains were obtained from 17 of the 23 herds studied. Considerable variation was observed between different R^+ strains in the frequency of transfer of resistance to a particular antimicrobial agent. In addition, variation in the frequency of transfer of different resistance determinants was noted in individual strains.

In vivo mating of R^+ strains with recipients was performed utilizing the gastrointestinal tract of germfree mice. Antimicrobial

agents were then administered to determine if a selective effect was observed for organisms mediating resistance to the antimicrobial agent or agents given. Administration of antimicrobial agents was not observed to select for higher numbers of organisms resistant to the agent or agents given. Various chemical and biological means were then investigated as to their ability to inhibit mating and resulting transfer of drug resistance. Inhibition of *in vivo* transfer of drug resistance was not observed with the methods studied.

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APPENDIX

Appendix A

Abbreviations

CR	-	converted recipient
F	-	F factor
fi^+	-	fertility inhibition positive
fi^-	-	fertility inhibition negative
GF	-	germfree
Km	-	kanamycin
lac	-	lactose
leu	-	leucine
MD	-	microbially defined
met	-	methionine
MHA	-	Mueller Hinton Broth (Difco) + agar (BBL)
MHB	-	Mueller Hinton Broth
NA	-	nalidixic acid
nal-R	-	nalidixic acid resistant
R^+	-	R factor positive
R^-	-	R factor negative
Sm	-	streptomycin
str-R	-	streptomycin resistant
Su	-	sulfamethizole
T	-	tetracycline
thi	-	thiamine
thr	-	threonine
try	-	tryptophan

Appendix B

Preparation of Antimicrobial Agents

Nalidixic Acid (Neg Gram)

Sterling-Winthrop Research Institute
Rensselaer, New York 12144
(Dr. S. Archer, Associate Director)

1. Prepare a 0.1 N solution of KOH.
2. Dissolve nalidixic acid in 0.1 N KOH to a final concentration of 10.0 mg/ml.
3. Filter* through sintered glass filter. Collect and store in sterile glass vial at 4°C. Stock solution is useable for 20 days.
4. In the selection media of the previous experiments, nalidixic acid was used at a concentration of 100 µg/ml. To achieve this desired final concentration, 10.0 ml of stock solution (10.0 mg/ml) were added to 990 ml of MHA medium. For *in vivo* experiments, various amounts of stock nalidixic acid solution, depending upon the desired dose.

* Wash filter using 1.0 N KOH, followed by copious quantities of distilled water.

Tetracycline HCl (Achromycin)

Lederle Laboratories
Pearl River, New York

Appendix B (Cont'd)

(Dr. A.C. Dornbush)

1. Add 0.4 ml of concentrated HCl (37%) to 99.6 ml of distilled water, or mix in a similar ratio.
2. Dissolve tetracycline HCl in above acid solution to concentration of 10.0 mg/ml.
3. Filter* through sintered glass filter, collect and store in sterile glass vial, at 4° C in absence of light. Stock solution is useable for 10 days.
4. In the selection media of the previous experiments, tetracycline HCl was used at a concentration of 25 μg/ml. To achieve the desired final concentration, 2.5 ml of stock tetracycline (10.0 mg/ml) was added to 997.5 ml of MHA medium.

* Wash filter using 1.0 N HCl, followed by copious quantities of distilled water.

Streptomycin Sulfate

Squibb Institute for Medical Research
New York, New York

1. Streptomycin sulfate for injection was purchased and reconstituted according to directions in package. Then was diluted and added to medium to give desired concentration. Shelf life of reconstituted streptomycin sulfate at 4° C is given

Appendix B (Cont'd)

by the company.

2. In the selection media of the previous experiments a final concentration of 10 μg/ml was used in MHA.

Sulfamethizole (Thiosulfil)

Averst Laboratories, Inc.
685 Third Avenue
New York, New York 10017
(Dr. John B. Jewell, Medical Director)

1. Prepare stock solution by adding thiosulfil to sterile Mueller Hinton broth to give a concentration of 2 mg/ml.
2. Filter through a 0.45 μ Millipore Filter, collect and store in a sterile glass vial at 4° C. Useful life of stock solution was not determined, and in all cases it was prepared within 36 hours of use.
3. In the selective media of the previous experiments a final concentration of 100 μg/ml was used. To achieve this desired concentration, 50 ml of stock sulfamethizole (2 mg/ml) was added to 950 ml of medium (MHA).

Appendix B (Cont'd)

Kanamycin Sulfate (Kantrex)

Bristol Laboratories
Syracuse, New York 13201

1. Kanamycin sulfate for injection was purchased. Already in a liquid suspension, it was diluted and added to give final desired concentration. Stock solutions were stored at 4° C.
2. In the selective media of the previous experiments, kanamycin sulfate was used at a concentration of 25 µg/ml of MHA.

Appendix C

Chemical Preparation

Mitomycin-C

Calbiochem
Los Angeles, California 90054

1. Sterile powder was reconstituted and diluted to give a stock solution with a concentration of 50 µg/ml. The stock solution was then frozen and stored in small sterile glass vials.
2. The dosage used in experiments to inhibit mating and resulting transfer of R factors *in vivo* was 30 or 50 µg.

Mepacrine Hydrochloride (Atabrine)

Sterling-Winthrop Research Institute
Rensselaer, New York 12144
(Dr. S. Archer, Associate Director)

1. Mepacrine hydrochloride was dissolved in sterile distilled water to give a final concentration of 10 mg/ml.
2. Filter through a 0.45 µ Millipore filter, collect and store in a sterile glass vial at 4° C in absence of light.
3. The dosage used in experiments to inhibit mating and resulting transfer of R factors *in vivo* was 2.5 or 5.0 mg.

Appendix D

Minimal Medium

The following composition was used:

	Per liter
H_2O	975 to 985 ml
K_2HPO_4	7.0 g
KH_2PO_4	3.0 g
$(NH_4)_2SO_4$	1.0 g
Na citrate	0.5 g
Agar	20.0 g

Directions:

1. Add water to flask.
2. Dissolve each compound completely before adding next.
3. Add agar last.
4. Autoclave 15 minutes.
5. Cool to 50° C.
6. Then add:

Sterile 20% glucose solution	10.0 ml
Sterile 2% $MgSO_4 \cdot 7H_2O$ solution	5.0 ml

For supplemented minimal medium, add 100 mg of sterile amino acids to each liter of media, to obtain a final concentration of 100 μg/ml for each amino acid added.

For example:

Sterile L-tryptophan (10 mg/ml) ---- 10.0 ml per liter

Aden, David Paul
In vitro and *in vivo* studies on transferable drug resistance in the *Enterobacteriaceae*