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Transferable Drug Resistance Among *Enterobacteriaceae* Isolated from Cases of Neonatal Diarrhea in Calves and Piglets

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Fecal specimens were collected on 22 different Nebraska ranches and at the Department of Veterinary Science 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 drug. In addition, variation in the frequency of transfer of different resistance determinants in individual R⁺ strains was noted.

Transferable drug resistance was first recognized in Japan in 1959, where it was shown that drug resistance was transferred between strains of *Shigella* and *Escherichia coli* (17). In 1962, Datta (2) provided the first report of this type of drug resistance in Great Britain, and since that time surveys from various parts of the world have shown the widespread existence of transferable drug resistance among the *Enterobacteriaceae*, both in human and animal populations (1). It is generally agreed that transferable drug resistance is mediated by plasmids, termed R factors, which may carry genetic determinants for resistance to various chemotherapeutic agents and heavy metals as well as determinants involving other characteristics (13).

Surprisingly, no interest in transferable drug resistance appears to have been taken in the United States until 1966, when several laboratories reported its existence among *Enterobacteriaceae* isolated from human infections (4, 5, 8, 11, 12). Numerous recent reports confirm that transferable drug resistance is widespread in this country among *Enterobacteriaceae* isolated from

humans. However, no reports have been made concerning the incidence of this type of drug resistance in domestic animals in the United States.

In this study we have shown that transferable drug resistance is common among *Enterobacteriaceae* isolated from cases of neonatal diarrhea in calves and piglets.

MATERIALS AND METHODS

Cultures. Ninety-two *Enterobacteriaceae* cultures were randomly selected from the culture collection of the Department of Veterinary Science, University of Nebraska. These cultures had previously been isolated from fecal specimens obtained from cases of neonatal diarrhea among young 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.

Resistance patterns were determined for these cultures, and those resistant to one or more of the relevant chemotherapeutic agents were used as prospective donors in conjugation experiments to determine whether the resistance was transferable. All of the resistant cultures were sensitive to nalidixic acid (NA) and were tryptophan-independent.

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The recipient used in conjugation experiments was *E. coli* K-12 F⁻ (K-12; obtained through the courtesy of Sidney Cohen, Michael Reese Hospital, Chicago, Ill.). This strain is resistant to 500 µg of NA per ml and is tryptophan-dependent. In some instances, the donor produced a substance which was lytic for K-12. In such cases, K-12 cells resistant to the lytic factors were isolated for use in conjugation experiments.

Media and antibiotics. The selective medium used in conjugation experiments was Mueller Hinton Broth (Difco) containing 2% BBL agar (MHA) supplemented with combinations of the following chemotherapeutic 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.

TABLE 1. Frequency of resistance and R factor-mediated resistance to various chemotherapeutic agents

Chemotherapeutic agent ^a	No. of resistant strains	No. of resistant strains which transferred resistance	Per cent 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 2. 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
<i>Escherichia coli</i>	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)
<i>Proteus</i>	T Sm Su Km	4	3 (75%)	T Sm Su Km (2); T Sm Km (1)
	T	1	1	T
<i>Citrobacter</i>	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.

Minimal medium (3) and minimal medium supplemented with 100 µg of tryptophan per ml were used to confirm converted recipients.

Determination of resistance pattern. Donor cultures were screened for drug resistance with the following antimicrobial discs (BBL or Difco) on MHA: sulfamethizole, 1 mg; oxytetracycline, 30 µg; streptomycin, 10 µg; chloramphenicol, 30 µg; kanamycin, 30 µg; nitrofurantoin, 100 µg; colistin, 10 µg; and NA, 30 µg. Cultures showing little or no zone of inhibition were considered resistant.

Transfer of drug resistance. The mating procedure described in our previous work (10) was used throughout this study. At the end of the conjugation period, dilutions of the mixed culture and controls were spread with glass rods on MHA containing NA and a chemotherapeutic agent to which the prospective donor strain under study was resistant. The number of selective media used for a particular cross corresponded to the number of antimicrobial agents to which the prospective donor was resistant, each medium containing NA and a different antimicrobial agent.

To confirm converted recipients and determine their resistance pattern, 10 colonies from each selective medium were replica-plated to minimal and supplemented minimal medium and to MHA plates containing, singly, the relevant antimicrobial agents.

RESULTS

Incidence of drug resistance. 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 1.

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 2). These 43 R factor-carrying (R⁺) strains were obtained from 17 of the 23 herds studied. The number of strains which transferred resistance to individual drugs is shown in Table 1.

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, and 53.8% of the strains resistant to T only transferred this resistance. With the exception of strains resistant to Su only, strains with other resistance patterns commonly transferred part or all of their resistance (Table 2).

The transfer frequency (10) varied greatly among the 43 R⁺ strains, from 59.2% to $6.7 \times 10^{-4}\%$. Considerable variation was observed between different strains in the frequency of transfer of resistance to a particular drug. In some multiple-resistant strains, the frequency of transfer of different resistance determinants was similar, suggesting a single linkage group. In other strains, the variation in the frequency of transfer of different resistance determinants was great, and not indicative of a single linkage group. Segregation was observed in some of the multiple-resistant strains which transferred individual determinants at about the same frequency. We have not studied the genetic mechanisms involved in the variations described above. Similar observations have been made by others (1, 16), and a provocative discussion is available (1).

DISCUSSION

Our results show that transferable drug resistance is common among *Enterobacteriaceae* isolated from young calves and pigs having neonatal diarrhea. In Great Britain, Smith (14) 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 we studied were not available, and we were unable to relate the presence or absence of R factor-carrying bacteria (R⁺) with the presence or 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 (6) 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 virus only recover, whereas those 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 the 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 (1). The extent of such transfer in vivo is difficult to determine. Thus, the importance of R⁺ *E. coli* as a reservoir of resistance determinants which can be transferred to pathogenic bacteria has not been established. However, early studies in Japan, reviewed by Watanabe (17), demonstrated the transfer of multiple drug resistance in vivo in human volunteers and dogs, and recent studies in microbially defined animals (7, 9) have clearly shown that R factor transfer can occur in vivo.

The high incidence of R⁺ bacteria in farm animals requires that studies be conducted to determine whether these strains can transmit their resistance to human strains in vivo. The recent experiments of Smith (15) are relevant to this question. Smith showed that R factor-mediated antibiotic resistance was transferred to the resident *E. coli* in a human alimentary tract from cultures of *E. coli* of animal and human origin taken in large doses by mouth. The amount of transfer that occurred was small, and the resistant resident organisms did not persist in the alimentary tract for long. However, even such limited transfer might be significant in humans or animals subjected to antibiotic administration.

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