

Incidence of Infectious Drug Resistance Among Fecal Coliforms Isolated from Raw Sewage

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Raw sewage was examined for the incidence of antibiotic-resistant coliforms present among both total and fecal coliforms. In both groups, it was found that approximately 3% of the coliform bacteria were resistant to two or more antibiotics. Of these organisms, 48% were capable of transferring all or part of their antibiotic resistance to an antibiotic-sensitive, F⁻, derivative of *Escherichia coli* K-12. Among the R factors identified, those conferring resistance to streptomycin-tetracycline, ampicillin-streptomycin-tetracycline, and ampicillin or ampicillin-streptomycin accounted for 23, 20, and 15%, respectively, of the total R factors detected. The data indicate a significant level of infectious drug resistance among the fecal coliforms of the urban population. The data indicate further that because of the high incidence of coliform bacteria found to be doubly resistant to streptomycin and tetracycline, the inclusion of these antibiotics in selective media used for routine total or fecal coliform counts may serve to identify domestic sources of pollution.

The presence of the coliform group of bacteria in water has long been used as an indication of fecal contamination. Although members of the coliform group are present in large numbers in the feces of warm-blooded animals, they may also be found in association with soil or plants (7). Among many attempts during the past several decades to develop methods which could be applied as means to distinguish between fecal and nonfecal coliforms, those which employ an elevated incubation temperature have found general acceptance (1, 7). A modified method for the direct quantitation of fecal coliforms involves the use of membrane filters for the collection of bacteria, transfer of the filters to an appropriate medium, and the enumeration of typical coliform colonies after 24 hr of incubation at 44.5 C (1, 7).

In a previous communication (12), it was shown that up to 1% of the total lactose-fermenting enteric bacteria recovered from raw or treated sewage were resistant to one or more antibiotics currently used therapeutically or as additives to animal feeds. Furthermore, it was found that a large portion of these antibiotic-resistant bacteria were infectiously resistant due to the presence of R factors which promote transfer of antibiotic resistance from resistant to completely antibiotic-sensitive bacteria. The present study was undertaken to assess the incidence of antibiotic resistance and of R factors among fecal coliforms recovered from sewage by the employment of an

elevated incubation temperature method. For comparative purposes, the incidence of antibiotic resistance and of R factors among total coliforms recovered from the same samples was also determined.

MATERIALS AND METHODS

Sewage samples. Duplicate grab samples of influent sewage were obtained from five sewage treatment plants in Jefferson County, Ala. All samples were collected in sterile bottles and processed in the laboratory within 3 hr of their collection in the field.

Isolation of resistant bacteria. All sewage samples were diluted and plated on MacConkey Agar (BBL) containing the following antibiotic combinations as previously described (12): ampicillin (10 µg/ml), streptomycin (10 µg/ml), and tetracycline (5 µg/ml); and streptomycin (10 µg/ml) and tetracycline (5 µg/ml). One set of plates was incubated at 35 C, and a duplicate set was incubated at 44.5 C. The plates incubated at 44.5 C were sealed in plastic bags to prevent excessive drying during incubation. Lactose-fermenting colonies were quantitated at 24 hr by using a New Brunswick colony counter. Single well-isolated colonies were picked, purified, and identified (5, 12).

Antibiotic susceptibility testing and identification of R factors. Antibiograms were performed by using the methods previously described (12) with the following Sensi-discs (BBL): ampicillin (10 µg), chloramphenicol (5 µg), cephalothin (30 µg), colistin (10 µg), dihydrostreptomycin (10 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (5 µg), sulfachlorapyridazine (250 µg), and tetracycline (5 µg). R factors were

identified by using techniques previously described (8, 12). Briefly, these involved mixing broth cultures with a drug-resistant isolate and a completely antibiotic-sensitive F- derivative of *Escherichia coli* K-12 [WI-A2 (*lac*-)], selecting for drug-resistant recombinants, and identifying the resistance pattern of the recombinant.

Antibiotics. Chloramphenicol was provided by Parke, Davis & Co., ampicillin (Penbritin) was supplied by Ayerst Laboratories, and gentamicin (Garamycin) was provided by Schering Corp. Appropriate concentrations of each antibiotic used in selective media were prepared in sterile distilled water and stock solutions were maintained by storage at -10°C .

TABLE 1. Incidence of antibiotic-resistant, lactose-fermenting bacteria recovered on MacConkey Agar containing antibiotics incubated at 35°C

Sample	No. of lactose-positive colonies/ml		
	No antibiotic	Am + Ds + Te ^a	Ds + Te
A1 ^b	3×10^6	3×10^4	10^5
A2	5×10^6	4×10^4	10^5
B1	2×10^6	7×10^3	10^4
B2	3×10^6	7×10^3	10^4
C1	5×10^5	10^4	2×10^4
C2	5×10^5	10^4	3×10^4
D1	6×10^5	2×10^4	3×10^4
D2	6×10^5	10^4	2×10^4
E1	10^5	7×10^3	10^4
E2	10^5	4×10^3	7×10^4

^a Abbreviations: Am, ampicillin; Ds, dihydrostreptomycin; and Te, tetracycline.

^b Letter designates the particular sewage disposal plant; 1 and 2 are duplicate samples.

TABLE 2. Incidence and transferability of resistance patterns among isolates selected on MacConkey Agar containing multiple antibiotics incubated at 35°C

Isolate	Antibiotic combination used for selection ^a					
	Ds + Te	No.	Transferred resistance	Am + Ds + Te	No.	Transferred resistance
<i>Escherichia coli</i>	Ds, Te	21	5(5) ^b	Am, Ds, Te	25	2(15)
	Am, Ds, Te	8	3(3)	Am, C, Ds, Te	1	0
	C, Ds, Te	2	(2)	Am, Cf, Ds, Te	12	(5)
	Ds, K, Te	3	(2)	Am, Ds, K, Te	2	1(1)
	Am, C, Ds, Te	2	(1)	Am, Ds, Na, Te	1	0
	Am, Cf, Ds, K	1	(1)			
	Am, Cf, Ds, Te	2	(2)			
Total		39	8(16)		41	3(21)
<i>Citrobacter-Klebsiella-Enterobacter</i>	Am, Ds, Te	8	2(2)	Am, Ds, Te	3	0
	Am, Ds, K, Te	1	0	Am, C, Ds, Te	1	0
	Cf, Ds, K, Te	1	(1)	Am, Cf, Ds, Te	4	0
				Am, Cf, Ds, Na, Te	2	0

^a Abbreviations: Am, ampicillin; C, chloramphenicol; Cf, cephalothin; Ds, dihydrostreptomycin; K, kanamycin; Na, nalidixic acid; Sl, sulfachloropyridazine; and Te, tetracycline.

^b Numbers given in parentheses indicate that only a portion of the resistance pattern was transferred.

RESULTS

Recovery of total coliforms. The numbers of lactose-positive colonies which appeared on MacConkey Agar plates without antibiotics after incubation at 35°C were taken to represent the total coliform population of the sewage samples tested. The total coliform populations and the incidence of lactose-positive bacteria exhibiting multiple resistance to the antibiotic combinations streptomycin-tetracycline or ampicillin-streptomycin-tetracycline from each of the sewage samples tested are presented in Table 1. The proportion of total coliforms doubly resistant to streptomycin and tetracycline varied from a low of 0.3% in sample B2 to a high of 6% in samples C2 and D2. The fraction of total coliforms resistant to the combination ampicillin-streptomycin-tetracycline was found to range from 0.2% in sample B2 to 3% in sample D1. Samples E1 and E2 were found to contain significantly higher proportions of antibiotic-resistant coliforms than the other eight samples tested; 10 and 70%, respectively, of the total coliforms present in E1 and E2 were doubly resistant, whereas 7 and 4%, respectively, were capable of growth on media containing three antibiotics.

From the MacConkey plates containing one or the other antibiotic combination, 100 lactose-positive colonies were randomly picked for further study. Of these 100 isolates, 80 were shown to be typical *E. coli* and 20 were shown to be either members of the *Citrobacter* group or the *Klebsiella-Enterobacter* group. For convenience, members of the last two groups were lumped into a single group. The drug-resistance patterns of each of these 100 isolates were determined by using

TABLE 3. Incidence of antibiotic-resistant, lactose-fermenting bacteria recovered on MacConkey Agar containing antibiotics incubated at 44.5 C

Sample	No. of lactose-positive colonies/ml		
	No antibiotic	Am + Ds + Te ^a	Ds + Te
A1 ^b	2 × 10 ⁸	3 × 10 ⁴	8 × 10 ⁴
A2	3 × 10 ⁸	3 × 10 ⁴	8 × 10 ⁴
B1	4 × 10 ⁸	5 × 10 ²	7 × 10 ³
B2	10 ⁸	7 × 10 ²	5 × 10 ³
C1	2 × 10 ⁸	7 × 10 ³	10 ⁴
C2	2 × 10 ⁸	6 × 10 ³	10 ⁴
D1	2 × 10 ⁸	10 ³	8 × 10 ³
D2	3 × 10 ⁸	10 ³	10 ⁴
E1	5 × 10 ⁴	10 ³	5 × 10 ³
E2	5 × 10 ⁴	10 ³	5 × 10 ³

^a See footnote a, Table 1.

^b See footnote b, Table 1.

nine different antibiotics as described above. The drug-resistance patterns observed and the number of strains capable of transferring all or a part of their resistance are shown in Table 2. The resistance patterns did not exhibit as much variation as might have been expected. For example, about 50% of the isolates exhibited resistance patterns identical with the antibiotic combinations used for selection. The remainder included resistance to not more than two additional antibiotics. Of the *E. coli* isolates, 60% were found to be infectious drug-resistant, whereas only 25% of the *Citrobacter-Klebsiella-Enterobacter* group was shown to harbor R factors. In addition to 100% of the isolates being resistant to streptomycin and tetracycline, 72% were resistant to ampicillin, 22%

were resistant to cephalothin, and less than 10% were resistant to chloramphenicol, kanamycin, or nalidixic acid.

Recovery of fecal coliforms. The numbers of lactose-positive colonies appearing on MacConkey Agar without antibiotics after incubation at 44.5 C were taken to represent the fecal coliform population. The fecal coliform populations and the numbers of fecal coliforms found to be capable of growth in the presence of the two antibiotic combinations employed are shown in Table 3. In four of the five sewage treatment plants sampled, it was found that the fecal coliform population ranged from 30 to 60% of the total coliform population. In samples B1 and B2, however, the fecal coliforms represented only 2 to 3% of the total coliform population. The proportion of fecal coliforms found to be doubly resistant to streptomycin and tetracycline ranged from 2 to 5% in all of the samples, except E1 and E2 when this fraction was found to represent 20% of the total fecal coliforms. The incidence of fecal coliforms capable of growth in the presence of the three antibiotics employed ranged from 0.01 to 3% of the fecal coliform populations.

From the selective plates containing one or the other of the antibiotic combinations, a total of 101 lactose-positive isolates were picked for further study. Characterization of these isolates revealed that 92 were typical *E. coli* strains, whereas 9 belonged to either the *Citrobacter* or *Klebsiella-Enterobacter* group. As before, members of the last two groups were pooled for simplification. The resistance patterns were determined for all 101 isolates, and each was grown in mixed culture with the drug-sensitive recipient to assay for infectious drug resistance as described above (Table 4).

TABLE 4. Incidence and transferability of resistance patterns among isolates selected on MacConkey Agar containing multiple antibiotics incubated at 44.5 C

Isolate	Antibiotic combination used for selection ^a					
	Ds + Te	No.	Transferred resistance	Am + Ds + Te	No.	Transferred resistance
<i>Escherichia coli</i>	Ds, Te ^b	34	12(2) ^c	Am, Ds, Te	38	6(12)
	Am, Ds, Te	11	2(2)	Am, C, Ds, Te	1	0
	Ds, Na, Te	1	(1)	Am, Cf, Ds, Te	1	0
	Am, Cf, Ds, Te	1	0	Am, Ds, K, Te	3	(2)
				Am, Cf, Ds, Na, Te	1	(1)
Total		47	14(5)	Am, Ds, K, Sl, Te	1	(1)
				Am, Ds, Te	45	6(16)
<i>Citrobacter-Klebsiella-Enterobacter</i>	Am, Ds, Te	3	1		6	1(1)

^a See footnote a, Table 1.

^b See footnote a, Table 2.

^c See footnote b, Table 2.

TABLE 5. Incidence of R factors identified

Resistance pattern	No.	Resistance patten	No.
Am ^a	14	Ds, Te.....	22
Cf.....	2	Na, Te.....	1
Ds.....	11	Am, Cf, Ds.....	2
Te.....	3	Am, Ds, Te.....	20
Am, Cf.....	1	Ds, K, Te.....	1
Am, Ds.....	15	Cf, Ds, Te.....	1
Am, Na.....	1	Am, Ds, K, Te.....	1
Am, Te.....	2		

^a See footnote a, Table 2.

Of the 92 *E. coli* isolates tested, 41 or 44.5% were shown to be infectious drug-resistant, whereas 33% of the *Citrobacter-Klebsiella-Enterobacter* group was capable of resistance transfer. The incidence of R factors in the fecal coliforms (*E. coli*) was significantly different from that of the total coliforms. As with the total coliforms, the majority of the isolates exhibited resistance only to the antibiotics included in the respective selective medium. This group of coliforms was resistant to the same antibiotics as the total coliform group, although there were significantly fewer resistant to cephalothin in the fecal coliform group.

Comparison of R factors identified. Among the 201 multiply resistant isolates studied, a total of 15 different R factors were identified. A tabulation of the R factors identified among both coliform groups is presented in Table 5. Five R factors comprised 84% of those identified. The patterns streptomycin-tetracycline, ampicillin-streptomycin-tetracycline, ampicillin, ampicillin-streptomycin, and streptomycin accounted for 23, 20, 15, 15, and 11%, respectively, of all of the R factors identified. Resistance to streptomycin, ampicillin, and tetracycline occurred in 76, 57, and 53%, respectively, of the R factors.

DISCUSSION

The results of this study indicate that from 2 to 5% of the total coliform bacteria recovered on initial isolation from raw sewage are doubly resistant to streptomycin and tetracycline and that a smaller, but significant number, are additionally resistant to a third antibiotic, ampicillin. A comparison of the data presented in Tables 1 and 3 reveals that, with the exception of samples B1 and B2, from 30 to 60% of the total coliform counts were recoverable as fecal coliforms by the elevated incubation temperature method employed and that in these instances the percentage of coliforms doubly resistant to streptomycin and tetracycline were similar for both groups. In samples B1 and B2, however, only 2 to 3% of the total coliforms were recoverable as fecal coliforms. Although

only 0.4% of the total coliforms in these samples were found to be doubly antibiotic-resistant, approximately 7% of the fecal coliforms recovered from these same samples were capable of growth on media containing both antibiotics. These observations lead to the conclusion that the source of antibiotic-resistant coliforms detected in untreated sewage is most probably fecal. The exceptionally high incidence of doubly resistant coliforms detected in samples E1 and E2 (Tables 1 and 3) may possibly be attributable to the fact that the particular sewage treatment plant from which these samples were taken receives untreated wastes from several meat packing plants.

After the enumeration of antibiotic-resistant coliform colonies which appeared on MacConkey Agar containing various combinations of streptomycin, tetracycline, and ampicillin after incubation at 35 or 44.5 C, approximately 200 well-isolated colonies were picked for further study. In addition to resistance to the three antibiotics contained in the selective medium, a significant number of strains were found to be additionally resistant to one or more other antibiotics, including chloramphenicol, cephalothin, kanamycin, nalidixic acid, or sulfachloropyridazine. When each of these strains was then utilized as a prospective donor of antibiotic resistance in mating experiments with an antibiotic-sensitive recipient derivative of *E. coli* K-12, 97 or 48% were found to be infectious resistant. These data (Tables 2 and 4) indicate, first, that during the past year there has been no significant change in the overall incidence of infectious antibiotic resistance among sewage coliforms isolated from the same community as those reported in a previous communication (12). On the other hand, an examination of the incidence of specific R factors identified in this study (Table 5) reveals that, although the incidence of R factors containing multiple resistance to various combinations of streptomycin, tetracycline, and ampicillin are similar to those of 1 year ago, the incidence of an R factor conferring single resistance to ampicillin has increased from 2% 1 year ago, to 15% in the present study. The lower incidence of R factors conferring resistance to chloramphenicol in this study, as compared to that observed in our previous study, is most probably due to the exclusion of this antibiotic from the initial selective medium.

Because of the widespread use of streptomycin and tetracycline in both human (11) and animal (2) medicine and the common inclusion of one or both of these antibiotics in animal and poultry feeds (10) over the past several years, it is not too surprising to detect a significant level of resistance to both of these antibiotics among fecal coliforms recovered from untreated sewage in civilized

countries. In contrast, the incidence of antibiotic resistance per se and the level of infectious antibiotic resistance associated with the fecal flora of man and animals in communities where modern antibiotic practices are nonexistent have been found to be extremely low (4, 9). These observations suggest that the inclusion of streptomycin or tetracycline, or both, as a selective device in media commonly employed in the estimation of total coliform numbers or fecal coliform counts may serve as a useful epidemiological marker for studies directed towards the identification of human or domestic animal sources of pollution in contaminated waters.

ADDENDUM IN PROOF

Since the writing of this paper, H. W. Smith [Nature (London) **228**:1286-1288, 1970] has published data which would seem to substantiate the relationship between antibiotic-resistant *E. coli* and fecal coliforms in polluted waters. For example, his study shows that nearly 100% of the *E. coli* isolated from various rivers in England and found to be resistant to chloramphenicol, streptomycin, tetracycline, or neomycin were also found to be fecal coliforms. Since antibiotic-resistant *E. coli* was rarely found in samples taken upstream from heavily populated areas, but were found in significant numbers downstream from such areas, the presence of streptomycin- or tetracycline-resistant fecal coliforms in water may prove to be an indication of domestic pollution.

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