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Transferable Drug Resistance Among *Enterobacteriaceae* Isolated from Human Urinary Tract Infections

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Fifteen sulfonamide-resistant cultures isolated from urinary tract infections in eastern Nebraska were screened for transferable drug resistance by three methods. Seven of the 15 resistant cultures could transfer resistance of varying levels to two or more chemotherapeutic agents. Transfer of drug resistance occurred without accompanying transfer of chromosomal traits and required cell to cell contact. In mixed culture, the number of drug-resistant recipients increased exponentially, reaching a plateau 2 hr after mixing. Spontaneous or artificial elimination of resistance was found to be a rare event. In addition, several drug-sensitive isolates from urinary tract infections were shown to be competent recipients of drug resistance determinants. From these data, it appears that the transferable drug resistance observed was mediated by R factors.

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. It is generally agreed that transferable drug resistance is mediated by episomes, termed R factors, which may carry genetic determinants for resistance to various chemotherapeutic agents (7) and heavy metals (8). Transferable drug resistance is characterized by (i) the requirement for cell to cell contact during transfer, (ii) the ability of resistant strains to cure spontaneously or after treatment with acridine dyes, and (iii) the frequent transfer of drug resistance markers without concomitant transfer of chromosomal traits.

Despite the widespread occurrence in many parts of the world of bacteria carrying R factors, surprisingly little is known concerning the occurrence of such strains in the United States. Smith and Armour (9) detected R factors in 53 of 100 enteric bacterial isolates from patients in the Boston area with genitourinary tract infections, and Smith (6) found R factors in *Salmonella* isolated from human infections. In the Chicago area, Kabins and Cohen (3) found 9 of 72 *Shigella*, 3 of 4 *Salmonella*, and 1 of 2 strains of enteropathogenic *Escherichia coli* to carry R factors. Gill and Hook (2) studied 254 *Salmonella* isolates from the eastern United States and observed that 25 of the 35 resistant cultures could transfer resistance to several drugs. Finally, Salzman and Klemm (5) demonstrated the presence of R factors in *E. coli* and *Klebsiella* isolated from nosocomial infections in New York.

Our study, which represents a sampling from eastern Nebraska, was initiated upon finding drug-resistant bacteria among isolates from urinary tract infections being screened for sulfonamide sensitivity. All sulfonamide-resistant isolates were subsequently tested for resistance to other chemotherapeutic agents and for the ability to transfer resistance to sensitive bacteria. Cultures showing transfer of drug resistance were further studied to determine their level of resistance and the level transferred, and to establish the plasmid nature of their drug resistance. In addition, we demonstrated that drug-sensitive pathogens could serve as competent R factor recipients.

MATERIALS AND METHODS

Cultures. All drug-resistant bacteria were isolated from urine samples collected by local physicians from office patients having urinary tract infections. All of the cases considered to be infections of the urinary tract were required to show more than 1,000 bacteria per ml. None of the patients had received antimicrobial agents within 10 days before collection of the urine sample. The predominant organisms were isolated and tested for sulfonamide sensitivity by the disc method. Only *Enterobacteriaceae* showing sulfonamide resistance were screened for the presence of R factors. Several of the drug-sensitive pathogens were tested for their ability to receive R factors in mixed culture.

Recipients used in conjugation experiments were *E. coli* K-12 F⁻ *na*ʳ *trp*⁻ (K-12; obtained through the courtesy of Sidney Cohen, Michael Reese Hospital, Chicago, Ill.) and *E. coli* K-12 F⁻ *thi*⁻ *met*⁻ *thr*⁻ *leu*⁻ *lac*⁻ *sm*ʳ (FW13A; kindly made available to us by David H. Smith, Harvard Medical School). The following abbreviations are used: *F*, F factor; *na*ʳ, nalidixic acid resistance; *trp*, tryptophan; *thi*, thiamine; *met*, methionine; *thr*, threonine; *leu*, leucine; *lac*, lactose; and *sm*ʳ, streptomycin resistance.

Media and antibiotics. The selective medium used in conjugation experiments employing K-12 or FW13A as recipients was Mueller-Hinton Broth (Difco) containing 2% BBL agar (MHA) supplemented with combinations of the following chemotherapeutic agents: nalidixic acid (Sterling-Winthrop), 100 μg/ml; sulfamethizole (Ayerst Laboratories), 100 μg/ml; tetracycline hydrochloride (Lederle Laboratories), 25 μg/ml; chloramphenicol (Parke, Davis & Co.), 25 μg/ml; and streptomycin sulfate (Squibb Institute for Medical Research), 10 μg/ml. The selective medium used in conjugation experiments, employing the drug-sensitive pathogens isolated from urinary tract infections, was minimal medium (1) supplemented with sucrose (0.2%) and tetracycline (25 μg/ml).

Transfer of drug resistance. A 250-ml Erlenmeyer flask, containing 50 ml of Mueller-Hinton Broth (MHB), was inoculated with 1 ml of a 16- to 18-hr MHB culture of either R factor donor or recipient and was incubated for 9 hr on a shaking tray at 37 C. These cultures were diluted in fresh MHB, with spectrophotometric methods, to approximately 5×10^8 cells/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 hr 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, dilutions of the mixed culture and controls were spread with glass rods on selective media (MHA) containing nalidixic acid and either tetracycline, sulfamethizole, or chloramphenicol to detect drug-resistant K-12 (converted recipients), and on media containing streptomycin and either tetracycline, sulfamethizole, or chloramphenicol to detect drug-resistant FW13A. If transfer did not occur by this method, the mixed culture was collected on a membrane (47 mm, 0.45 μm; Millipore Corp., Bedford, Mass.) filter (4), which was then incubated on the surface of an MHA plate. After 18 hr, growth was suspended in 2 ml of MHB and diluted and plated as described above. When cultures could not be filtered because of viscous metabolic products, the donor-recipient mixture was packed in a conical tube by centrifugation, incubated, diluted, and plated as previously described. From each conjugation mixture, 10 converted recipient colonies were confirmed by testing for chromosomal markers on minimal and supplemented minimal medium (1) and for transferred drug resistances by the disc method.

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.

Resistance pattern and level of resistance. R factor donors and converted recipients were screened for drug resistance with the following antibiotic discs (BBL or Difco) on Mueller-Hinton Medium (Difco): sulfamethizole, 1 mg; sulfisoxazole, 1 mg; oxytetracycline, 30 μg; streptomycin, 10 μg; chloramphenicol, 30 μg; neomycin, 30 μg; kanamycin, 30 μg; cephalothin, 30 μg; colistin, 10 μg; nitrofurantoin, 100 μg; and nalidixic acid, 30 μg. Cultures showing little or no zone of inhibition were considered resistant. The minimal inhibitory concentration (MIC) was determined by a serial twofold tube dilution of the chemotherapeutic agent in MHB. Dilutions of the chemotherapeutic agents were inoculated with approximately 10^4 stationary-phase cells and incubated for 12 hr at 37 C.

Curing of resistant cultures. The penicillin method was used to isolate spontaneously cured cells (12) or cells cured by ultraviolet-acriflavine (13) or sodium dodecyl sulfate (10) treatment.

Kinetics of transfer. A 25-ml amount of 20-IR (see below) and K-12, grown and adjusted to approximately 5×10^8 cells/ml as in conjugation experiments, was mixed in a 250-ml Erlenmeyer flask and incubated at 37 C without shaking. Immediately and at 30-min intervals after mixing, 1-ml samples were removed, diluted, and plated on media containing nalidixic acid or tetracycline, or both. At 60 min, 5 ml was agitated in a Waring Blendor for 1 min and then diluted 1:100 and 1:1,000 in MHB. At 30-min intervals, 1-ml samples of these cultures, held at 37 C without shaking, were diluted and plated in a similar manner.

Transfer by culture filtrates. Shake cultures of donors, as described above, were filtered through membrane filters (47 mm, 0.45 μm; Millipore Corp.). Equal quantities (1 ml) of donor culture filtrate and K-12 recipient (diluted to 5×10^8 cells/ml) were mixed, incubated, and plated, as in conjugation experiments, on MHA containing nalidixic acid and tetracycline to detect resistant K-12.

RESULTS

Incidence of sulfonamide resistance. Urine specimens from 100 patients with urinary tract involvement were examined for the presence of bacteria. None of the patients had received antimicrobial agents within 10 days before collection of the first urine sample. Sixty-four of the specimens contained >1,000 bacteria per ml. The predominant organisms in 44 of the specimens were sulfonamide-sensitive, whereas the predominant organisms in the remaining 20 specimens were sulfonamide-resistant. Of the 20 sulfonamide-resistant organisms, 14 were *Enterobacteriaceae*. We examined 12 of these 14 sulfonamide-resistant cultures for transferable drug resistance.

The 44 patients infected with sulfonamide-sensitive organisms were treated with sulfon-

amides for 2 weeks. In three cases, sulfonamide-resistant *Enterobacteriaceae* were isolated from the patients during or after sulfonamide therapy. These cultures (22-III, 45-III, 68-II) were also examined for transferable drug resistance.

The generic classifications and patterns of resistance of the 15 organisms examined for transferable drug resistance are shown in Table 1.

Transfer of resistance. Seven of the 15 cultures transferred their drug resistance upon conjugation with K-12. Those cultures showing no transfer to K-12 in broth were conjugated with K-12 on a membrane filter (Millipore Corp.) or in a centrifuge tube and were conjugated with FW13A in broth. Additional cultures transferring drug resistance were not detected with these methods. Four cultures, 22-III, 65-I, 72-IR, and 95-IR, contained agents lytic for K-12 and were conjugated with mutant strains of K-12 selected for resistance to the lytic agents. Only two of these cultures were able to transfer their drug resistance to the specially selected K-12. Table 2 illustrates the characteristics of the seven cultures which transferred drug resistance and the frequency of transfer of tetracycline resistance to K-12. Transfer frequency varied between 8.17×10^{-5} and 82.5%.

TABLE 1. *Resistance patterns of the sulfonamide-resistant isolates*

Isolate	Generic classification	Chemotherapeutic agents[a]					
		Sm	T	Cm	G	Th	NA
20-IR	*E. coli*	R[b]	R		R	R	
22-III	*E. coli*				R	R	
27-I	*E. coli*		R	R	R	R	
30-I	*E. coli*		R	R	R	R	
31-IR	*E. coli*	R	R		R	R	
36-IR	*E. coli*	R	R		R	R	
45-III	*Klebsiella* sp.			R	R	R	
47-I	*E. coli*				R	R	
65-I	*E. coli*				R	R	
68-II	*Klebsiella* sp.			R	R	R	R
72-IR	*E. coli*	R	R		R	R	
85-IR	*Klebsiella* sp.	R	R		R	R	
87-I	*Proteus* sp.	R	R	R	R	R	
91-IR	*E. coli*	R	R		R	R	
95-IR	*E. coli*	R	R		R	R	

[a] Abbreviations: Sm, streptomycin; T, tetracycline; Cm, chloramphenicol; G, sulfisoxazole; Th, sulfamethizole; NA, nalidixic acid.
[b] Resistant.

Resistance pattern. All of the seven cultures which could transfer resistance showed resistance to sulfonamides, tetracyclines, and streptomycin (Table 2). In all cases, resistance to tetracyclines and streptomycin was transferred to sensitive K-12. Sulfonamide resistance was transferred by five of the seven cultures. Cephalothin resistance was not transferred. All of the 10 converted recipients (resistant K-12) screened from each cross had the same resistance pattern. No segregants were detected.

Level of resistance. The streptomycin MIC varied between 7.8 and 250 μg/ml in R-factor donors and between 2.0 and 3.9 μg/ml in converted recipients (Table 3). R-factor donors always showed sulfonamide resistance at levels >1,000 μg/ml. However, in only one instance was this high level transferred to K-12. The MIC for levels <1,000 μg/ml could not be determined accurately for the sulfonamides. The tetracycline MIC was very constant for both donor and converted recipients, varying between 125 and 250 μg/ml.

Curing of resistant cultures. Attempts were made to eliminate resistance from several of the cultures which could transfer drug resistance. All attempts to eliminate resistance with sodium dodecyl sulfate yielded negative results. One spontaneously cured isolate of 91-IR was ob-

TABLE 2. *Resistance pattern and transfer characteristics of R factor-carrying pathogenic isolates*

Isolate	Generic classification	Resistance[a]	Transferred resistance[a]	No. of K-12 receiving T resistance	Total no. of K-12	Transfer frequency[b]
						%
20-IR	*E. coli*	Su T Sm	Su T Sm	5.35×10^6	1.04×10^9	0.514
31-IR	*E. coli*	Su T Sm	Su T Sm	2.80×10^3	9.15×10^8	3.06×10^{-4}
36-IR	*E. coli*	Su T Sm	T Sm	9.00×10^6	3.72×10^8	2.42
72-IR	*E. coli*	Su T Sm C	T Sm	1.46×10^4	$<10^6$	ND[c]
85-IR	*Klebsiella* sp.	Su T Sm	Su T Sm	4.90×10^2	6.00×10^8	8.17×10^{-5}
91-IR	*E. coli*	Su T Sm	Su T Sm	2.55×10^6	9.75×10^8	0.262
95-IR	*E. coli*	Su T Sm C	Su T Sm	1.65×10^7	2.00×10^7	82.5

[a] Abbreviations: Su, sulfonamides; T, tetracycline; Sm, streptomycin; C, cephalothin.
[b] Transfer frequency = (no. of K-12 receiving T resistance)/(total no. of K-12 in mixture) × 100.
[c] Not determined.

tained by using the penicillin screening technique. Ultraviolet-acriflavine treatment of K-12(20-IR) yielded a very low number of cured cells. These cured cells could again receive resistance determinants upon conjugation with 20-IR. Most attempts to eliminate resistance failed, and, in cases in which cure was obtained, the frequency of cure was very low. However, all drug resistances lost from cured cells were the same as those transferred by cells of the resistant culture.

Transfer of resistance by culture filtrates. Culture filtrates of the seven R factor-carrying cultures were unable to transfer drug resistance to sensitive K-12.

Kinetics of R-factor transfer. Figure 1 illustrates the exponential rate of transfer of R factors in a sensitive bacterial population in vitro. Transfer occurred within 30 min. The number of resistant K-12 cells increased exponentially for 2 hr and then plateaued. The total number of K-12 cells and 20-IR cells increased very little relative to the increase in resistant K-12 during the period of observation. In the 1:1,000 dilution of the mixed culture made after 60 min, the number of converted recipients remained constant throughout the experiment. In contrast, a 1:100 dilution did not inhibit transfer, but resulted in an exponential increase in resistant K-12 after a 30-min lag period.

Transfer of resistance to sensitive urinary isolates.

TABLE 3. *Levels of resistance mediated by R factors in pathogenic strains and in converted K-12 recipients*

Culture	MIC[a]		
	Streptomycin	Thiosulfil	Tetracycline
K-12	<0.12	<1,000	<1.9
20-IR	15.6	>1,000	125
K-12(20-IR)[b]	3.9	>1,000	250
31-IR	250	>1,000	250
K-12(31-IR)	2.0	<1,000	250
36-IR	7.8	>1,000	125
K-12(36-IR)	2.0	<1,000	250
72-IR	31.3	>1,000	125
K-12(72-IR)	3.9	<1,000	125
85-IR	7.8	>1,000	125
K-12(85-IR)	2.0	<1,000	250
91-IR	31.3	>1,000	250
K-12(91-IR)	2.0	<1,000	250
95-IR	62.5	>1,000	250
K-12(95-IR)	2.0	<1,000	125

[a] Minimal inhibitory concentration in micrograms per milliliter.

[b] Converted K-12 cultures are designated as K-12(R-factor donor culture). For example, K-12(20-IR) is K-12 carrying the R factor of 20-IR.

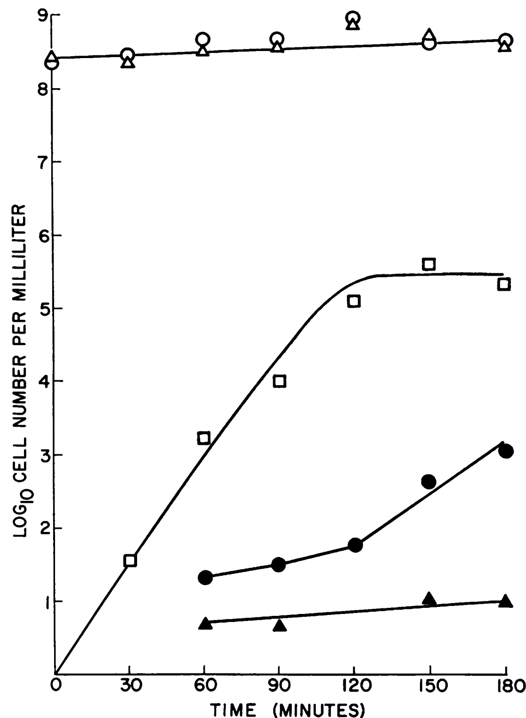

FIG. 1. *Appearance of converted recipients, K-12(20-IR), during conjugation of an R-factor donor, 20-IR, with K-12. Symbols:* ○, *K-12;* △, *20-IR;* □, *K-12(20-IR) undiluted;* ●, *K-12(20-IR) in mating mixture diluted 1:100;* ▲, *K-12(20-IR) in mating mixture diluted 1:1,000.*

An R factor-carrying culture, 20-IR (unable to ferment sucrose), was used as a donor in attempts to transfer resistance to sensitive urinary tract pathogens (sucrose fermenters). Converted recipients were detected on minimal medium containing sucrose as a carbon source for the recipients and tetracycline to inhibit sensitive recipients. Three of four *E. coli* strains (29-I, 97-I, 100-I; 25-I not a competent recipient) and three of three *Klebsiella* strains (45-I, 84-I, 84-III) proved to be competent recipients of the R factor from 20-IR.

DISCUSSION

Infections of the urinary tract are most commonly caused by *E. coli*. Many strains of *E. coli* have been susceptible to sulfonamides, streptomycin, chloramphenicol, and the tetracyclines. Therapy of such infections in this geographical area usually involves a sulfonamide as the drug of choice. Only when sulfonamide treatment fails are other more toxic chemotherapeutic agents employed, such as kanamycin, neomycin, or chloramphenicol.

Our survey indicates that 33.3% of the sulfonamide-resistant *Enterobacteriaceae* causing uri-

nary tract infections in this area harbor transferable sulfonamide resistance. Sulfonamide resistance was shown to be transferred from 5 of 15 resistant isolates to a drug-sensitive strain of *E. coli*.

In addition to episomal transfer, transferable sulfonamide resistance could theoretically be mediated by chromosomal transfer, transformation or transduction. Our observations, as summarized below, indicate that this transferable sulfonamide resistance is mediated by R factors.

First, an R factor will usually mediate resistance to several chemotherapeutic agents. R factors were originally discovered in multiply resistant *Shigella* (11), and, since then, an increasing number of resistance determinants have been found on R factors. Likewise, the transferable sulfonamide resistance dealt with in this paper was only a part of the entire resistance spectrum of these cultures. Transfer of resistance to streptomycin and tetracycline accompanied transfer of sulfonamide resistance. Out of the 15 cultures screened, 7 (47%) were found to contain a transferable type of multiple drug resistance. A statement concerning the general incidence of R factors in all 64 cases of urinary tract infection cannot be made, however, since only sulfonamide-resistant cultures were screened for R factors.

Our findings on the level of resistance mediated by these resistance factors are similar to previous reports (2, 3). R factors usually mediate low-level resistance to streptomycin, as we observed, in contrast to the high level of resistance mediated by chromosomal determinants.

We were unable to detect transfer of resistance to kanamycin, neomycin, or chloramphenicol. Absence of these determinants may be due to infrequent use of these drugs clinically (9).

Second, transfer required cell to cell contact and a certain cell density, as evidenced by the negative results obtained with donor culture filtrates and by the lack of transfer in a 1:1,000 dilution of a conjugation mixture (Fig. 1). Also, the rate of transfer was greater than would be expected if it were mediated by a transducing phage. Thus, transduction or transformation was probably not involved in transfer.

Third, transfer of chromosomal markers did not occur in any cross in frequencies equaling those of resistance transfer. All converted K-12 recipients screened had retained their tryptophan dependence.

Fourth, replication of the resistance factors was independent of host cell chromosome replication, as evidenced by the exponential increase in resistant recipients during conjugation, in contrast to the almost constant number of total recipients during the same period. Our results differ slightly with those of Smith (6), who observed a continuous increase in resistant recipients after mixing exponential-phase cells. It is not known why our results indicated a plateau after 2 hr of conjugation. Increased cell density or loss of competent donors due to repressor formation may be the limiting factor. Frequency of transfer, measured at the end of an 18-hr conjugation period (Table 2), was found to be similar to values determined by Kabins and Cohen (3).

Fifth, the ability to eliminate R factors and other cytoplasmic replicons with acridine dyes has been cited as one criterion for the episomal nature of R factors (13). Workers in Japan (10) have been able to effect up to 100% elimination of resistance with sodium dodecyl sulfate. Spontaneous loss of R factors is reported to approach approximately one cell in 10^3 or 10^4 (7). In our experience, spontaneous or artificial elimination of transferable drug resistance is a very rare event. However, further work is required to determine whether this finding results from an actual low rate of loss of resistance or from an inefficiency in the methods of detecting cured cells. For example, in several cases, penicillin resistance hindered the use of the penicillin technique for isolating cured cells (*unpublished data*).

Clinically, R factors mediating high levels of drug resistance are potentially a serious problem. Its seriousness lies in the fact that this resistance is episomal, having a possible predilection for remaining dormant in intestinal bacteria, which may have the ability to transfer this resistance to many types of incoming enteropathogens. We have shown that drug-sensitive pathogenic *E. coli* and *Klebsiella* can serve as competent recipients upon conjugation in vitro. Others (11) have shown that *Shigella* and *Salmonella* are prone to infection with R factors. Further work is in progress to determine whether the efficiency of such mating systems in vivo is high enough to be clinically significant.

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