

R plasmids conferring multiple drug resistance from shigella isolated in Korea

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(Received 16 May 1983; accepted 26 September 1983)

SUMMARY

The majority (85%) of shigella isolated in 1980 and 1981 in Korea were *Shigella flexneri*, the others were *Sh. sonnei* (14%) with only a small number of *Sh. dysenteriae*. Only 14 of the 459 strains of shigella isolated were susceptible to all 12 drugs tested, and 445 were resistant to three or more drugs. Strains multiply resistant to the six drugs, chloramphenicol (Cm), tetracycline (Tc), streptomycin (Sm), sulfisomidine (Su), ampicillin (Ap) and trimethoprim (Tp) were most frequently encountered, followed by those resistant to Cm, Tc, Sm, Su and Tp. The complete patterns of resistance to drugs except nalidixic acid and rifampin in approximately 73% of drug-resistant strains were co-transferred to *Escherichia coli* by conjugation, indicating that the resistance was R plasmid-mediated. Randomly selected R plasmids conferring various patterns of resistance markers were tested for the incompatibility groups, and almost all of them were classified into *Inc FII*. Two of three R plasmids conferring resistance to Cm, Tc, Sm and Su were classified into *Inc B* and one to *Inc FII*. Two R types with resistance markers of Cm, Tc, Sm and Ap were not classified with our standard plasmids used.

INTRODUCTION

The emergence of multiply drug-resistant shigella strains has been widely recognized and become one of the most serious problems in clinical medicine. It is particularly of interest in Korea since shigellosis is still prevalent and large numbers of strains are multiply resistant to chloramphenicol (Cm), tetracycline (Tc), streptomycin (Sm), sulphonamides and ampicillin (Ap) (Seol, 1980). Trimethoprim (Tp) has been reported to be active against almost all shigella (Barada & Guerrant, 1980), but large numbers of shigella strains isolated in Korea in recent years were found to be highly resistant to Tp (Seol, 1980). In most strains resistance

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to these drugs, including Tp, was transmissible to *Escherichia coli* by conjugation, suggesting that the resistance is mediated by plasmids (Chun, Seol & Suh, 1981).

The incompatibility grouping of R plasmids in shigella isolated from various places was reported. The common group of R plasmids carried by shigella isolated in Mexico, U.S.A., Central America and the United Kingdom was Inc B (Datta & Olarte, 1974; Frost & Rowe, 1983). On the other hand, both in Japan (Yoshikawa, Nagashima & Mitsuhashi, 1971) and South Africa (Watson, 1967), F-like R plasmids predominate. We studied the incompatibility group of our isolates to compare the results from other parts of the world.

MATERIALS AND METHODS

Isolation procedures of shigella

Stool samples collected in 1980 and 1981 in Taegu area were inoculated directly on SS and MacConkey agar plates. After incubation at 37 °C for 20 h, colonies resembling shigellae were purified on MacConkey agar plates, transferred in KIA agar slants, and confirmed to be shigella biochemically and serologically (Edwards & Ewing, 1972).

Antimicrobial susceptibility tests

A plate dilution method was used throughout the study, using brain heart infusion (BHI) broth and BHI agar. For the test of resistance to sulfisomidine (Su) and Tp, Mueller-Hinton (MH) agar was used. The following drugs were tested at the final concentrations ($\mu\text{g/ml}$): Cm 25; Tc 25; Sm 25; Su 400; Ap 25; Tp 8; nalidixic acid (Na) 25; rifampin 50; kanamycin (Km) 25; gentamicin 12.5; amikacin 32; and cephaloridine (Cr) 32. A strain was recorded as resistant when the growth was not inhibited at these drug concentrations.

Detection conjugative R plasmids

R plasmids were detected by the procedure described by Chun *et al.* (1977). Na-resistant *E. coli* ML 1410 (Ishiguro, Oka & Sato, 1978) and Rf-resistant RG 488 (Grant, Bannatyne & Shapley, 1976) were used as recipient. Single colonies of donor and recipient strains were cultured overnight in BHI broth. One drop of each culture was inoculated in 5 ml of BHI broth and incubated at 37 °C for 3.5 h with gentle shaking. One millilitre of donor and four ml of recipient cultures were mixed and conjugated for 18 h at 37 °C, and then spread on selective agar plates (BHI or MH agar) containing 50 $\mu\text{g/ml}$ of Na or Rf and one of the drugs to which the donor strain was resistant. After incubation at 37 °C for 24 h, plates were inspected for colonies of resistant *E. coli*. Five to ten colonies were picked, purified on MacConkey agar plates, confirmed to be *E. coli*, and tested for resistance patterns.

Incompatibility tests

The incompatibility was tested by colony test (Datta & Olarte, 1974; Uhlin & Nordstroem, 1975). A list of standard R plasmids used was shown in Table 1. *E. coli* carrying R plasmid from shigella was mated to *E. coli* carrying standard R plasmid of each incompatibility group whose resistance markers were distinguish-

Table 1. Standard R plasmids used in test for incompatibility

Incompatibility group	Plasmid	Host Strain*	Marker	Source†
C	R40a	ML1410	SuApKm	Sato
FI	R386	ML1410	TC	Sato
FII	R1	J53	CmSmSuApKm	Datta
FIV	pOH3145	SG3	CmSuKm	Sato
H1	pOH3123	SG8	TcKm	Sato
I _α	R144	ML1410	Km	Sato
I ₂	TP114	# 361	Km	Lederberg
J	R391	ML1410	Km	Sato
N	RN3	J53	TcSmSu	Datta
B	R16	ML1410	TcSmSuAp	Sato
P	RP4	ML1410	TcApKm	Sato
T	Rts1	RG172	Km	Grant
W	RS-a	SG3	CmSmSuKm	Sato

* Plasmids carried by *E. coli* other than ML1410 were transferred to ML1410 before use.

† Plasmids were provided by: G. Sato, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan; N. Datta, Royal Postgraduate Medical School, London, W. 12, England; E. M. Lederberg, Plasmid Reference Center, Stanford University School of Medicine, Stanford, Calif., and R. B. Grant, The Hospital for Sick Children, Toronto, Ontario, Canada.

able from those of the donor. Ten transconjugant clones obtained on selective media were picked and purified by successive single colony isolations and tested, by double ditch plates, for the presence of incoming and resident plasmids. When a resident plasmid was eliminated from all transconjugant clones, the cross was made in the opposite direction. Elimination of the resident in both directions was taken as evidence that two plasmids belong to the same group. When the resident was eliminated from most but not all of the tested transconjugants, the apparent doubles were tested for stability. After overnight growth in drug-free media, they were inoculated on agar and 20 colonies were tested for the continued presence of markers of each plasmid. If the doubles were stable, they were used as donors to another strain of *E. coli*, each R plasmid being selected separately in transconjugants. If one or both R plasmids was separately transferred from the doubles, the two were recorded as being compatible. When both plasmids were always transferred simultaneously, they were considered as the recombinant of both plasmids.

RESULTS

Isolation frequency of shigella and drug-resistant strains

The majority of the 459 strains isolated in 1980 and 1981 in our laboratory were classified as *Sh. flexneri* and the others were *Sh. sonnei*, including six of *Sh. dysenteriae* (Table 2). No *Sh. boydii* was found. Many *Sh. flexneri* and *Sh. sonnei* were resistant to Cm, Tc, Sm, Su, Ap and Tp, except Ap, for which only two among 65 strains of *Sh. sonnei* were resistant. Strains resistant to Na, Rf and Km were less than 12%, and no strain was resistant to gentamicin, amikacin and cephaloridine.

Table 2. *Number of shigella strains resistant to drugs tested*

Drug	No. (%) of resistant strains		
	<i>Sh. dysenteriae</i> (6)*	<i>Sh. flexneri</i> (388)*	<i>Sh. sonnei</i> (65)*
Chloramphenicol	2 (33.3)	374 (96.4)	64 (98.5)
Tetracycline	2 (33.3)	377 (97.1)	64 (98.5)
Streptomycin	2 (33.3)	376 (96.9)	62 (95.4)
Sulfisomidine	2 (33.3)	370 (95.4)	64 (98.5)
Ampicillin	1 (16.7)	338 (87.1)	2 (3.1)
Trimethoprim	1 (16.7)	355 (91.5)	63 (96.9)
Nalidixic acid	0	45 (11.6)	1 (1.5)
Rifampin	0	11 (2.8)	6 (9.2)
Kanamycin	0	13 (3.4)	0

*No. of strains isolated.

Resistance patterns and detection of conjugative R plasmids in shigella

As listed in Table 3, only 14 among 459 strains were susceptible to all the drugs tested, and the others were multiply resistant to three or more drugs. Among 17 patterns of drug resistance observed, the most predominant R-type was Cm, Tc, Sm, Su, Ap, Tp, followed by Cm, Tc, Sm, Su, Tp, and Cm, Tc, Sm, Su, Ap, Tp, Na. The other patterns included less than 20 strains. Four hundred and forty drug-resistant strains, except five strains which were resistant to both Na and Rf and other drugs, were tested for the transmissibility of their resistance to *E. coli* by conjugation, and the resistance of 322 (73.1%) strains was found to be conjugative. However, autotransferability was very poor in *Sh. flexneri* with R-type Cm, Tc, Su, Ap, Tp, Na, and in *Sh. sonnei* with R-type Cm, Tc, Sm, Su, Tp. The complete patterns of original resistance to drugs except Na and Rf were always co-transferred, when recipient strains were selected with any of the drugs except Na and Rf. The resistance to Na and Rf was never transferred by conjugation to *E. coli*.

Incompatibility of R plasmids

Mating of R plasmids having different resistance markers derived from shigella and standard plasmids produced three kinds of results (Table 3).

pKY201

This plasmid which represents the most frequent patterns of resistance markers among those carried by shigella was transferred in *E. coli* RG488 (Rf^r) and mated to standard plasmids having distinguishable markers from donor (Table 4). The results of colony test showed that both donor and resident plasmids except R1 (*Inc FII*) were present in recipient cultures after selection with appropriate markers, and both plasmids in recipient cultures were always separately transferable to another strain of *E. coli*. When the donor plasmid was transferred to *E. coli* carrying R1, 18 colonies among 20 tested had incoming plasmid only in recipient cultures and the other two had both plasmids. These two doubles were considered to carry recombinant plasmids since all resistance genes were transferred together when they were used as donors to another strain of *E. coli*, no matter which drug

Table 3. Drug resistance patterns and transferable drug resistance of shigella strains isolated in Taegu area of Korea in 1980 and 1981

Drug resistance pattern*	Species†	No. of strains	No. of strains transferred resistance	Pattern of resistance transferred to RG 488	Plasmid no. (pKY)‡	Incompatibility group
CmTcSmSuApTpNaRf	B	5	Nt§			
CmTcSmSuApTpNa	B	40	15	CmTcSmSuApTp	101,102,103	FII
CmTcSmSuApTpRf	B	5	5	CmTcSmSuApTp	161,162	FII
CmTcSmSuApTp	B	269	236	CmTcSmSuApTp	201-215	FII
	D	2	0	None		
CmTcSmSuTpKm	B	13	13	CmTcSmSuTpKm	501,502,503	FII
CmTcSmSuTpRf	D	6	6	CmTcSmSuTp	511,512	FII
CmTcSmSuTpNa	D	1	0	None		
CmSmSuApTpRf	B	1	1	CmSmSuApTp	601	FII
CmTcSmSuAp	A	1	1	CmTcSmSuAp	621	FII
	B	6	4	CmTcSmSuAp	651,652	FII
CmTcSmSuTp	A	1	1	CmTcSmSuTp	701	FII
	B	19	18	CmTcSmSuTp	711-715	FII
	D	52	14	CmTcSmSuTp	761,762	FII
CmSmSuApTp	B	1	0	None		
CmTcSuApTp	B	2	2	CmTcSuApTp	771,772	FII
CmTcSmSu	B	4	3	CmTcSmSu	801,802	B
				CmTcSmSu	803	FII
	D	1	0	None		
CmTcSmAp	B	8	2	CmTcSmAp	851,852	UC
CmTcSuTp	D	2	1	CmTcSuTp	861	FII
TcSmSu	B	5	0	None		
CmTcAp	B	1	0	None		
Subtotal	A	2	2			
	B	379	299			
	D	64	21			
Total		445	322 (73.1%)¶			

Susceptible to all drugs tested. 14

*Drug resistance symbols: see text.

† A = *Sh. dysenteriae*, B = *Sh. flexneri*, D = *Sh. sonnei*.

‡ Plasmids tested were selected at random and numbered with heading pKY.

§ Not tested.

|| Unclassified.

¶ Percentage of 440 strains tested.

was used for selection. Mating in reversed direction showed similar results and this plasmid was considered to belong to *Inc* FII. Other plasmids tested, such as pKY101, 102, 103, 161, 162 and 202 through 215 which encode the same resistance markers with 201 produced similar results, and were also classified into *Inc* FII.

pKY501, 511 and 621

These three plasmids encode resistance markers Cm, Tc, Sm, Su, and Tp, Km, or Ap respectively. When they were mated to R1, a majority of them among 20 daughter colonies tested contained the incoming plasmid only and one to three contained both plasmids. In these doubles, both plasmids were always co-

Table 4. *Incompatibility between a plasmid pKY201 derived from shigella and standard plasmids*

Donor	Recipient	Selection	Daughter colonies containing		
			Incoming plasmid only	Resident plasmid only	Both
pKY201	R40a (C)	Tc+Na	0	0	20
	R1 (FII)	Tc+Na	18	0	2*
	pOH3145 (FIV)	Tc+Na	0	0	20
	pOH3123 (HI)	Cm+Na	0	0	20
	R144 (I α)	Cm+Na	0	0	20
	TP114 (I $_2$)	Cm+Na	0	0	20
	R391 (J)	Cm+Na	0	0	20
	RP4 (P)	Cm+Na	0	0	20
	Rts1 (T)	Tc+Na	0	0	20
	RS-2 (W)	Tc+Na	0	0	20

After mating, 20 recipient colonies selected as described in the text were subjected for colony test. The other standard plasmids were not tested without having appropriate selecting markers.

* Two colonies contained stable recombinant of incoming and resident plasmids.

transferred to the other *E. coli*, and considered to be recombinants. Mating in the reverse direction showed similar results and these three plasmids were classified into *Inc FII*. pKY 502, 503, 512, 601, 651, 652, 701, 711 through 715, 761, and 762 which encode the same markers with one of 501, 511, and 621 showed similar results, indicating that they belong to *Inc FII*.

pKy 771, 772 and 861

These plasmids have different resistance markers, and all them were also classified into *Inc FII*.

pKY801, 802 and 803

These plasmids encode resistance markers of Cm, Tc, Sm and Su, but they had different results in compatibility tests. When pKY801 and 802 were mated to R16 (*Inc B*), the incoming plasmid only was found in recipient cultures. Mating in the reverse direction also showed the same results, and these two plasmids were classified into *Inc B*. pKY803 was classified into *FII*.

pKY851 and 852

These two plasmids encoding the resistance genes of Cm, Tc, Sm and Ap were not classified with our standard plasmids tested, since they were compatible with *FII*, *B*, and other plasmids.

DISCUSSION

Shigella strains isolated in Korea were multiply resistant to four drugs, Cm, Tc, Sm and Su, those resistant to Ap formed not more than 10%, with no strains resistant to Tp until 1977 (Chun & Seol, 1978). Strains resistant to Ap and Tp suddenly increased in large numbers from 1978 and thereafter continued to

maintain almost the same levels (70–80%) of resistant strains (Seol, 1980). The increase in the resistance to Ap, Tp and other drugs is most probably a reflexion of the abuse of drugs, since antimicrobial drugs are purchased at drug stores without physician's prescription. Na is also a drug which has been used frequently in recent years without physician's prescription, and since 1978 approximately 10% of strains are Na-resistant strains (Seol, 1980). Since the resistance to Na was always not conjugally transferred to *E. coli*, the resistance is considered not to be mediated by R plasmids, but is probably of chromosomal origin. Fortunately, the number of Na-resistant strains is not increasing with time. The complete pattern of resistance to drugs except Na and Rf was conjugally co-transferred to *E. coli*, suggesting that resistance is mediated by R plasmids (Chun, Seol & Suh, 1981).

Even though about 73% of strains transferred their resistance to *E. coli*, in 25 of the 40 strains of *Sh. flexneri* with R-types Cm, Tc, Sm, Su, Ap, Tp, Na the linkage group was not transferable. Similarly in 38 of 52 strains of *Sh. sonnei* with R-types Cm, Tc, Sm, Su, Tp, the linkage group was not transferable. This is in contrast to our previous report that almost all of these two linkage groups of R-type were autotransferred to *E. coli* by conjugation (Chun, Seol & Suh, 1981). We do not know why the autotransferability has decreased in these plasmids. As we observed that the transferability of R plasmids differs according to the recipient strains used (Chun & Seol, 1979), the autotransferability of R plasmids in our study may be improved using some other *E. coli*. We did not attempt to mobilize non-autotransferable plasmids, and the transferability of the plasmids may be improved by mobilization study (Anderson, 1965) which is not possible here at present.

Olarte, Filloy & Galindo (1976) noted two R plasmids in *Sh. dysenteriae* 1; one was responsible for resistance to Cm, Tc, Sm, Su and the other caused resistance to Ap, but resistance to Ap, Tp and other drugs was always transferred together with other resistance in our study. Our result suggests that the close linkage of genes governing resistance to Ap, Tp, and other drugs, and the linkage of genes in *Sh. dysenteriae* 1 isolated in Mexico differs from our strains. The difficulty of isolating segregants lacking one or more R plasmid markers by the method of Bochner *et al.* (1980) (not shown in the results) also suggests the close linkage of genes governing resistance in our strains.

The incompatibility group B plasmids in shigella predominate in Central America, including Mexico, and in the United Kingdom (Datta & Olarte, 1974; Frost & Rowe, 1983), but we identified only two of *Inc* B having markers of Cm, Tc, Sm and Su, suggesting that this plasmid is rare in Korea. Incompatibility group FII and related groups are common in Asia, Africa, and other parts of the world (Yoshikawa, Nagashima & Mitsuhashi, 1971; Watson, 1967; Frost & Rowe, 1983). A majority of our plasmids in shigella were classified into *Inc* FII and was considered the most predominant group in Korea. Our report is the only one for Korea, and further studies are necessary to know the detailed distribution of incompatibility groups in shigella.

During incompatibility studies we found, in most cases, numbers of recombinants of both incoming and resident plasmids in recipient cultures. This fact may be a cause of the broadening of resistance patterns and the yearly increase of multiply drug-resistant shigella strains. The predominant multiplicity of drug resistance

increased from four drugs in 1977 to six or more drugs after 1977 (Chun & Seol, 1979; Chun, Seol & Suh, 1981).

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