

TRANSMISSION OF THE R FACTOR BY CONJUGATION FROM A NATURALLY-ISOLATED, MULTIPLE DRUG-RESISTANT STRAIN OF *VIBRIO ELTOR* TO *SHIGELLA* AND *SALMONELLA*

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Out of the four multiple drug-resistant strains of El Tor vibrio which were isolated in the Philippines in 1964 and 1965, one strain (D 226) was confirmed to transmit its quadruple drug-resistance to sensitive strains of *Salmonella* and *Shigella*. The recipients maintained their resistance after repeated subcultures in drug-free media, and are able to transfer their resistances to sensitive strains of *Vibrio* and *Aeromonas*. The resistant strains thus obtained lost easily their transmitted resistance after a few subcultures in drug-free media. The remaining three triple-resistant strains of El Tor vibrio failed to transmit their resistance. These strains maintained their resistance quite stably during several years, although the strain D 226 lost its resistance about one year after isolation.

In 1967, it was reported by KUWAHARA and his coworkers¹⁾ that four multiple drug-resistant strains were detected among about 1,500 clinical isolates of El Tor vibrio collected in the Philippines during 1964 and 1965. Out of these resistant strains, one strain was found capable of transmitting its resistance to sensitive strains of *Salmonella* and *Shigella* by conjugation.

This report deals chiefly with the experimental results on the transmission of R factor between this strain and strains of enterobacteria.

Materials and Methods

(1) Strains used: As the donors of R factor four multiple drug-resistant strains of El Tor vibrio were used. The strain D 226 was isolated in Bakolod from a cholera case, and highly resistant to tetracycline (TC), chloramphenicol (CP), streptomycin (SM) and sulfonamide (SA).

Three other strains (DEN 3, E 110 and E 73) were isolated in Manila or its neighboring area, and resistant to CP, SM and SA, but sensitive to TC. As the recipients 8 drug-sensitive strains of *Shigella flexneri* and *Salmonella enteritidis* strain M-11 were used.

In retransmission experiments, a *Shigella* strain which received the resistance from the strain D 226 was used as the donor, and sensitive strain of various species of *Vibrio* and *Aeromonas hydrophila* were used as recipients.

(2) Methods of transmission of resistance: Both donor and recipient strains were cultured overnight in nutrient broth at 37°C. Then 1 ml amounts of donor and recipient were mixed in a test tube, allowed to stand at 37°C for 3 hours, and then 0.1 ml of the mixture was dropped and smeared on the selective plate (SS agar containing 2% sucrose and 10 mcg/ml of CP or TC). After incubation at 37°C for 48 hours, colonies grown were picked up for examination of the biological properties and agglutination test with the

appropriate antisera, and determination of the minimum inhibitory concentrations (MIC) by agar-plate dilution method.

(3) Methods of retransmission of resistance: Retransmission of resistance from the *Shigella* strain receiving R factor from the strain D 226 to sensitive strains of *Vibrio* and *Aeromonas* was performed by the same procedures as described above, except for that a semisynthetic medium of the following composition was used as a selective medium: $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 0.5%, KH_2PO_4 0.15%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01%, NaCl 0.2%, agar 1.5%, sucrose 1.0%, vitamin-free Casamino acids (Difco) 0.5%, BTB 0.002%, CP or TC 5.0 mcg/ml, pH 7.6~7.8.

Table 1. Transmission frequencies of multiple drug-resistance from El Tor vibrio strain D 226 to *Shigella* and *Salmonella*

Donor: El Tor vibrio strain D 226
(resistant to SA·SM·CP·TC)

Selected with		Transmission frequency *	
		CP 10 mcg/ml	TC 10 mcg/ml
Test strain	<i>Shigella flexneri</i> NZ	1×10^{-8}	0
	1	1×10^{-7}	0
	2	0	2×10^{-5}
	3	2×10^{-8}	2×10^{-8}
	4	3×10^{-4}	2×10^{-4}
	5	3×10^{-8}	5×10^{-8}
	6	3×10^{-8}	4×10^{-8}
	9	2×10^{-8}	4×10^{-8}
	<i>Salmonella enteritidis</i> M-11	5×10^{-8}	5×10^{-8}

* Transmission frequency was calculated by
$$\frac{\text{Number of resistantized cells}}{\text{Total number of recipient cells}}$$

Table 2. Drug sensitivity of each recipient colony grown on the selective plate (selected with CP)

Donor: El Tor vibrio D 226

Recipient	No. of colonies on the selective plate	Drug	Sensitivity of original strain	MIC (mcg/ml) of each colony grown on the selective plate				
				1	2	3	4	
<i>Shigella flexneri</i>	M Z	TC	0.78	>100				
		CP	3.12	>100				
		SM	1.56	>200				
	1	1	TC	0.78	>100			
			CP	1.56	>100			
			SM	1.56	>200			
	2	0	TC	0.78				
			CP	1.56				
			SM	1.56				
	3	2	TC	0.78	>100	>100		
			CP	0.78	>100	>100		
			SM	1.56	>200	>200		
	4	3	TC	0.78	>100	>100	>100	
			CP	0.78	>100	>100	>100	
			SM	1.56	>200	>200	>200	
	5	3	TC	0.78	>100	>100	>100	
			CP	0.78	>100	>100	>100	
			SM	1.56	>200	>200	>200	
	6	3	TC	0.78	>100	>100	>100	
			CP	0.78	>100	>100	>100	
			SM	1.56	>200	>200	>200	
9	2	TC	0.78	>100	>100			
		CP	0.78	>100	>100			
		SM	1.56	>200	>200			
<i>Salmonella enteritidis</i> M 11	4	TC	0.78	>100	>100	>100	>100	
		CP	6.25	>100	>100	>100	>100	
		SM	1.56	>200	>200	>200	>200	

Experimental Results

Transmission of R Factor from El Tor Vibrio to *Shigella* and *Salmonella*

When the strain D 226 was used as the donor, the majority of the test strains were found competent recipients of the R factor, and the transmission frequencies were generally low except for *Shigella flexneri* strain No. 4, to which R factor was transferred at the frequency of 10^{-4} level (Table 1).

When the remaining 3 triple-resistant strains (DEN 3, E 110, E 73) were used as the donors, none of the test strains produced any colonies on the selective plates, although quite the same procedures as above were employed. Besides the test strains described above, the *Escherichia coli* strain K-12 was also tested for its competence to serve as a recipient using desoxycholate agar containing 10 mcg/ml of CP, but it failed to give positive results.

Since only a small number of colonies were grown on all the selective plates of positive cases, all of them were picked up to examine for their degrees of resistances to CP, TC, SM and SA. When selected with CP, all the colonies grown were highly resistant to all the four drugs, whereas, when TC was used as a selective agent, there

Table 3. Drug sensitivity of each recipient colony grown on the selective plate (selected with TC)

Donor : El Tor vibrio D 226

Recipient	No. of colonies on the selective plate	Drug	Sensitivity of original strain	MIC (mcg/ml) of each colony grown on the selective plate					
				1	2	3	4	5	
<i>Shigella flexneri</i>	MZ	0	TC CP SM	0.78 3.12 1.56					
	1	0	TC CP SM	0.78 1.56 1.56					
	2	2	TC CP SM	0.78 1.56 1.56	>100 >100 >200	>100 0.78 1.56			
	3	2	TC CP SM	0.78 0.78 1.56	>100 >100 >200	>100 >100 >200			
	4	2	TC CP SM	0.78 0.78 1.56	>100 0.78 1.56	>100 >100 >200			
	5	5	TC CP SM	0.78 0.78 1.56	>100 >100 >200	>100 >100 >200	>100 >100 >200	>100 >100 >200	>100 >100 >200
	6	4	TC CP SM	0.78 0.78 1.56	>100 >100 >200	>100 >100 >200	>100 >100 >200	>100 1.56 1.56	
	9	4	TC CP SM	0.78 0.78 1.56	>100 >100 >200	>100 >100 >200	>100 >100 >200	>100 >100 >200	
<i>Salmonella enteritidis</i> M 11	5	TC CP SM	0.78 6.25 1.56	>100 6.25 1.56	>100 6.25 1.56	>100 >100 >200	>100 >100 >200	>100 6.25 1.56	

Table 4. Maintenance of the transmitted resistances of each recipient clone which received the R factor from El Tor vibrio strain D 226

Recipient	TC-selection					CP-selection					
	No. of colonies grown on the selective plate	Drug	No. of substrains withstanding the drug at 100 mcg/ml			No. of colonies grown on the selective plate	Drug	No. of substrains withstanding the drug at 100 mcg/ml			
			2nd sub-culture	6th sub-culture	8th sub-culture			2nd sub-culture	6th sub-culture	8th sub-culture	
<i>Shigella flexneri</i>	MZ	0	TC CP SM				1	TC CP SM	1 1 1	1 1 1	1 1 1
	1	0	TC CP SM				1	TC CP SM	1 1 1	1 1 1	1 1 1
	2	2	TC CP SM	2 1 1	2 1 1	2 1 1	0	TC CP SM			
	3	2	TC CP SM	2 2 2	2 2 2	1 1 1	2	TC CP SM	2 2 2	2 2 2	2 2 2
	4	2	TC CP SM	2 1 1	2 1 1	2 1 1	3	TC CP SM	3 3 3	3 3 3	3 3 3
	5	5	TC CP SM	5 5 5	5 5 5	2 2 1	3	TC CP SM	3 3 3	3 3 3	2 2 2
	6	4	TC CP SM	4 3 3	4 3 3	4 3 3	3	TC CP SM	3 3 3	3 3 3	3 3 3
	9	4	TC CP SM	4 4 4	4 4 4	4 4 4	2	TC CP SM	2 2 2	2 2 2	2 2 2
<i>Salmonella enteritidis</i> M-11	5	TC CP SM	5 2 2	4 1 1	4 1 1	4	TC CP SM	4 4 4	0 0 0	0 0 0	

were found, besides the quadruple resistant colonies, a small number of clones, which were highly resistant to TC alone, and remained sensitive to CP, SM and SA (Tables 2 and 3).

Each recipient colony was subinoculated into drug-free semisolid nutrient agar, and after three successive, daily subcultures, transfer was made once every 10 days until 10 serial subcultures, and in each subculture drug-resistances were checked by agar-plate dilution method (Table 4). After selection with CP, transmitted resistances were quite stable in *Shigella* recipients, any changes in MICs being noted by repeated subcultures. But, in 4 substrains of *Salmonella enteritidis* strain M-11 resistance was lost after 6 serial subcultures. On the contrary, when TC was used for selection, different results were obtained; in 6 out of 24 substrains of recipient clones, resistance to TC was maintained after 10 subcultures, but that to CP, SM and SA was rapidly lost by successive subcultures. Especially, it was worthwhile mentioning that in 4 out of 5 substrains of *Salmonella enteritidis* resistance to CP, SM and SA was completely lost in the 6th subculture.

Retransmission of the R Factor from Resistantized *Shigella* to Sensitive *Vibrio* and *Aeromonas*

Retransmission experiments were performed using a resistant strain of *Shigella flexneri* as a donor, and sensitive strains of classical cholera and El Tor vibrio, non-agglutinable vibrio (NAG), *V. parahaemolyticus* and *Aeromonas hydrophila* as recipients. Results are summarized in Table 5.

All of the test strains were found competent recipients of the R factor, and the transmission frequencies in strains of NAG and *Aeromonas* tended to be somewhat higher than in other strains. The degrees of resistances in the majority of colonies were generally lower than those in the donor. Only when each recipient clone was successively subcultured in nutrient broth containing 5 mcg/ml of TC or CP, it showed considerably high resistance. As for the patterns of resistances some were found resistant to all the four drugs, others resistant to TC alone, and some others resistant to CP, SM and SA, but not to TC.

All the recipients lost the transmitted resistances after a few successive transfers in drug-free nutrient broth.

Table 5. Frequencies of retransmission of R factor from resistantized *Shigella* to sensitive *Vibrio* and *Aeromonas*

Recipient		Transmission frequency	
		TC	CP
<i>V. cholerae</i>	WLL	2.9×10^{-7}	2×10^{-7}
	35A3	2.4×10^{-8}	3×10^{-8}
El Tor vibrio	D35	2×10^{-8}	1.5×10^{-8}
	D32	3.5×10^{-8}	3.3×10^{-8}
NAG	B3965	4.6×10^{-6}	4×10^{-6}
	13538	2.3×10^{-6}	3.5×10^{-6}
<i>Aer. hydrophila</i>	139	2.2×10^{-6}	6×10^{-7}
	144	1.9×10^{-6}	8×10^{-6}
<i>V. parahaemolyticus</i>	168	4×10^{-8}	7.8×10^{-8}
	194	7×10^{-8}	2.8×10^{-8}

Donor: *Shigella flexneri* No. 3-R₄ which received the quadruple drug-resistance from El Tor vibrio strain D 226

Discussion

The present experiments demonstrated that the transmission of multiple drug-resistance could naturally occur probably between strains of enterobacteria and those of *Vibrio*, since the multiple drug-resistance of a clinical isolates of El Tor vibrio was confirmed to be due to R factor through our present investigations. The transmitted resistance from *Vibrio* to *Shigella* was quite stable, whereas that from resistantized *Shigella* to *Vibrio* as well as *Aeromonas* was easily lost within several successive transfers in drug-free media. Thus, the results in the present investigations were generally in accordance with those we have hitherto reported^{2,3}). PRESCOTT and his coworkers⁴) also reported the emergence of multiple drug-resistant strains of El Tor vibrio in Calcutta. These observations strongly indicate that a gradual increase of El Tor vibrio strains bearing R factor may take place, because of the fact that chemotherapy with tetracycline or streptomycin in cholera-endemic areas might cause the selection of the resistant strains.

The R factor in the El Tor vibrio strain D 226 was relatively unstable, and the successive subcultures of this strain in semisolid nutrient agar resulted the complete loss of the R factor as early as about one year after isolation. The triple drug-resistance of the other 3 strains, which failed to transmit their resistance to enterobacteria, was quite stable, and was still retained after repeated subcultures during several years. The mechanism of resistance of these three strains are now under investigation, and the results will be reported in successive papers.

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