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Transferable plasmid-mediated drug resistance among non-O1 *Vibrio cholerae* and rough strains of *Vibrio cholerae* from Tamilnadu, India

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SUMMARY

A total of 289 non-O1 *Vibrio cholerae* (NVC) strains and 20 rough *V. cholerae* (RVC) strains isolated in an endemic area were tested for antibiotic resistance and for transferable R-plasmids. Twenty three per cent of NVC and 40% of the RVC isolates were found to be resistant to one or more drugs. Eight NVC and four RVC strains possessed multiple drug resistance, varying from four to eight drugs. The common spectrum found in NVC isolates were chloramphenicol and streptomycin (CS) or chloramphenicol, streptomycin, tetracycline and ampicillin (CSTA). Resistance to sulphamethoxazole (Su) and to trimethoprim (Tm) was encountered infrequently. In RVC isolates in addition CSTASuTm determinants, resistance markers to aminoglycosides kanamycin, gentamicin and neomycin were also found. Eighteen of the 27 *V. cholerae* strains with two or more resistance determinants transferred them en bloc to *Escherichia coli* K12. The level of resistance in the recipient strain was equal to or greater than that of the donor vibrio strains. Most of the strains possessing solitary resistance markers were unable to transfer them. β-lactamase production could be demonstrated in 92·8% of the ampicillin resistant strains. None of the strains was resistant to nalidixic acid or furazolidone. The results emphasize the importance of antimicrobic susceptibility determination of *V. cholerae* isolates, regardless of the serotypes, before commencing chemotherapy.

INTRODUCTION

In recent years vibrios biochemically similar to cholerae vibrios but which do not agglutinate with *Vibrio cholerae* O-group 1 antiserum – now referred as non-O1 *V. cholerae* (NVC) – have attracted increasing attention, since they may cause illness indistinguishable from cholera. These vibrios have also been involved in outbreaks in some areas (El-Shawi & Thewaini, 1969; Aldova *et al.* 1968).

Shimada & Sakazaki (1973) observed that R (rough) antigens of *V. cholerae* were serologically identical among the strains regardless of the serotypes of S (smooth) parent cultures; rough *V. cholerae* strains (RVC) have been detected in longterm carriers following clinical illness and occasionally in the acute stage of the illness (Barua, 1974).

Although stable transferable drug resistance in *V. cholerae* is rare, resistant strains have been reported in the past (Prescott, Datta & Datta, 1968; Hedges *et*

al. 1977). Recently outbreaks due to plasmid-mediated multiple drug resistant strains have been reported from Tanzania (Mhalu, Mmari & Ijumba, 1979) and Bangladesh (Glass *et al.* 1980). However, only very few of the earlier workers considered it important to study the antimicrobic susceptibility of NVC strains (Kuwahara *et al.* 1967; Misra *et al.* 1970; and Sil, Sanyal & Mukherjee, 1974). Very little is known about the susceptibility pattern of RVC strains.

In view of these findings and increasing role of NVC and RVC strains in human disease in an endemic area, this study was designed to determine the antibiotic susceptibility pattern of NVC and RVC strains and screen for naturally occurring R (resistance) plasmids in any resistant strains isolated.

MATERIALS AND METHODS

Bacterial strains

A total of 251 NVC strains isolated in our laboratory, between 1977 and 1982 through public health centres distributed throughout the State of Tamilnadu, India, known to be an endemic area for cholera, (Sundaram, Shanthakumari & Murthy, 1980) were used in this study. In addition, nine NVC strains isolated from urine (human) and 29 strains from marine fishes were also included. Twenty strains identified as RVC by their agglutinability in acriflavine solution and with antiserum raised against a known *V. cholerae* strain (B-53-3) which possesses R antigen were also tested. The known strain was obtained from Central Research Institute, Kasauli, India. RVC isolates were from human stool except one from urine. Identity of the isolates were confirmed and NVC serovars were serotyped by referring the cultures to Dr R. Sakazaki, National Institute of Health, Tokyo. Some of the isolates were confirmed by Dr J. V. Lee and Dr T. J. Donovan, Public Health Laboratory, Maidstone, Kent.

Determination of antibiotic susceptibility

Antibiotic susceptibility testing was initially by the disc diffusion method (Matsen & Barry, 1974) against chloramphenicol (C), streptomycin (S), tetracycline (T), gentamicin (G), ampicillin (A), neomycin (N), co-trimoxazole (Ct) and nalidixic acid (Nx). Furazolidone (Fz), 200 μg disc, was used to assess susceptibility against this drug. Subsequently the susceptibility was confirmed by the agar dilution method employing the following single concentration of the antibiotics incorporated in Mueller-Hinton agar (MHA) plates. C(0·5 μg/ml), S(15 μg/ml), T(2 μg/ml), G(3 μg/ml, A(4 μg/ml), N(10 μg/ml), Nx(0·5 μg/ml) and Fz(2 μg/ml). The inoculum was approximately 10^6 cfu/ml. Similarly, minimal inhibitory concentrations (MIC) for the resistant strains were determined by employing MHA plates containing doubling dilutions of appropriate antibiotic solutions; MICs of sulphamethoxazole (Su) and trimethoprim (Tm) were determined individually for Ct resistant strains. MHA supplemented with lysed horse blood or Wellcotest agar was substituted whenever testing against Ct, Su or Tm. The lowest concentration that did not permit visible growth of bacteria after overnight incubation at 37 °C was considered as the MIC. Kanamycin (K) susceptibility was also determined for isolates resistant to two or more drugs. *Escherichia coli* NCTC 10418 was used as sensitive control throughout the study.

Transfer experiments

E. coli K12 F^- lac^+ nal^r (nalidixic acid resistant) plasmid free recipient strain and *E. coli* K12 lac^+ X^+ and *E. coli* K12 $lac^+\Delta^+$ cultures possessing standard transfer factors were received from Dr K. B. Sharma, Lady Hardinge Medical College, New Delhi.

Test strains resistant to two or more drugs were subjected to mating experiments. Representative strains possessing solitary resistance determinants were also included in the mating experiments. One ml of young nutrient broth culture of the resistant strain was mixed with an equal amount of the recipient strain grown with shaking. After overnight incubation at 37 °C, 0·1 ml of conjugation mixture was plated on to the appropriate selective media-MacConkey agar or Wellcotest agar plates containing Nx, 40 μg/ml alone or with one of the following selective agents: C(20 μg/ml), S(20 μg/ml), T(10 μg/ml), K(20 μg/ml), G(20 μg/ml), A(25 μg/ml), N(20 μg/ml), Su(100 μg/ml) or Tm(10 μg/ml); the plates were incubated overnight at 37 °C. In all successful mating experiments, transconjugants were purified and tested for their entire resistance pattern. If there was no evidence of transfer on the selection plates the conjugation mixture was subsequently incubated at room temperature after the addition of fresh broth to facilitate transfer if any, at lower temperature. When there was no direct transfer of drug resistance, the strains were examined for determinant mobilization in a triparental cross using *E. coli* K12 lac^+ X^+ and *E. coli* K12 lac^+ Δ^+ (WHO, 1978).

β-lactamase production

Ampicillin resistant strains were screened for the production of β-lactamase by an acidometric method employing phenol red-penicillin agar (Lucas, 1979).

RESULTS

Antibiotic resistance pattern

Sixty seven (23·1 %) of the total NVC isolates were resistant to one or more of the drugs tested. Relatively more strains were resistant to A(16·9 %); followed by S(9·3 %), C(5·8 %), T(3·8 %). Ct resistant strains have been encountered occasionally.

MICs for NVC strains possessing solitary resistant determinants against S or A are shown in Table 1. Six of the 39 A only resistant strains were isolated from marine fishes and belonged to the non-O1 serotypes O:6, O:41 and O:82; two further strains were untypable with the present scheme and the remaining one was not typed. Unlike human isolates no other resistant markers were found in fish isolates.

Altogether eight NVC and four RVC strains were multiply drug resistant varying from four to eight drugs (Table 2). The common R-spectrum found in NVC isolates were CS and CSTA. In addition to CSTASuTm, resistance markers to aminoglycosides K, N and G were also found in some of the RVC isolates but not in any of the NVC strains. MIC ranges of the multiply resistant isolates were as follows: C: 4–32 μg/ml, S: 64–> 256 μg/ml, T: 8–64 μg/ml, K: > 256 μg/ml, G: 256 μg/ml, A: 32–> 256 μg/ml, Su: ⩾ 256 μg/ml and Tm: > 256 μg/ml.

Table 1. *MICs for the NVC strains with solitary resistance determinants*

	MIC (μg/ml) (n = 289)							Total no. of resistant strains
Antibiotic	8	16	32	64	128	256	> 256	
Ampicillin	2	—	7	11	14	3	2	39 (13·4 %)
Streptomycin	—	—	2	3	3	—	—	8 (2·7 %)

n, Total no. of strains tested.

Table 2. *Resistance spectrum and transfer pattern of NVC and RVC strains*

Resistance spectrum	Transfer pattern
NVC strains	
CSTASuTm (1), STA (1), SA (1), TA (1)	Transferable en bloc (4)
CSTA (6)	Transferable en bloc (5) No-transfer (1)
CS (8)	Transferable en bloc (4) No-transfer (4)
CSTSuTm (1), CST (1), S (2)	No-transfer (4)
A (9)	Transferable (1) No-transfer (8)
RVC strains	
CSKANSuTm (2), CSTASuTm (1), GSKGANSuTm (1), SuTm (1)	Transferable en bloc (5)
SA (2), A (1)	No-transfer (3)

Figures in parentheses indicate number of strains.

All the strains screened were sensitive to Nx and Fz. Four of the typable strains resistant to two or more drugs belonged to the serotype O :54; other serovars were less frequent.

β-lactamase producing strains

β-lactamase production could be demonstrated in 52(92·8 %) of the 56 A resistant strains.

Transfer of resistance markers

The complete R-pattern was directly transferable to *E. coli* K12 in 13 out of the 20 NVC strains. In the remaining seven strains the determinants could not even be mobilized by triparental crosses. None of the strains tested possessing solitary determinants for S or A resistances could initiate transfer except one which had apparently low frequencies. Direct transfer of R-determinants have taken place en bloc in 62·5 % of the resistant RVC strains (Table 2). The level of resistance, especially C resistance, in the transconjugants was equal to or markedly greater than that shown by the donor *V. cholerae* strains; the increase was as much as 32-fold in some of the strains.

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DISCUSSION

The increasing prevalence of plasmid-mediated transferable antibiotic resistance has serious implications. Occurrence of plasmid determined resistance has been reported in *V. cholerae*-O1 serovars by some workers (Prescott, Datta and Datta, 1968; Threlfall, Rowe & Huq, 1980); but is not well documented in the cases of NVC serovars and RVC strains.

In the present survey of antibiotic resistance patterns in these two groups of organisms in our region we were able to identify several NVC (23·1 %) and RVC (40 %) strains demonstrating resistance to one or more commonly used drugs. A considerable proportion of them were able to directly transfer their resistance determinants en bloc to the recipient *E. coli* strain in transfer experiments (Table 2) thus indicating their plasmid origin.

In some of the previous studies (Kuwahara *et al.* 1967; Misra *et al.* 1970; Sil, Sanyal & Mukherjee, 1974) no multiply drug-resistant NVC strains have been encountered, which suggests that occurrence of resistant strains now might be due to extensive and wide spread use of antibiotics in the treatment and prophylaxis of cholera. However a single strain showing CS resistance was isolated in Kerala as early as 1965 (Sil, Sanyal & Mukjerjee, 1974).

The predominent R-type shown by NVC strains in this study were CS and CSTA; they were not quite identical to the R-pattern displayed by *V. cholerae*-O1 strains of earlier outbreaks in Tanzania (CSTKASu) and Bangladesh (TKASuTm, TASuTm TKGASu, SSpTKASuTm) (Towner, Pearson & O'Grady, 1979; Threlfall & Rowe, 1982). However some of the resistance determinants are common in both.

No correlation between antibiotic resistance and serotypes of NVC strains could be made in this survey.

Relatively more resistant strains (40 %) were noticed in RVC isolates; and multiple drug resistance was seen in four of them. This gives the impression that RVC strains might be more prone to carry R-determinants, since this group of vibrios are often isolated from carriers (Barua, 1974) who have been treated with several antibacterial agents. Unfortunately the history of previous attack amongst the patients could not be ascertained in the current study.

Nine strains resistant to two or more drugs were unable to transfer their R-determinants either directly or by triparental crosses. Similarly resistant strains possessing solitary A or S markers were not able to mediate transfer except in one case (Table 2). It is noteworthy that six A resistant strains were isolated from marine fishes but the conferred resistance was not transferred when representative strains were tested. The above observations might suggest that these resistance markers may be chromosomal mediated.

Earlier reports (Hedges & Jacob, 1975; Hedges *et al.* 1977; Threlfall & Rowe, 1982) strongly emphasize the domination of plasmids of the compatibility group C in *V. cholerae*. Hence there is every reason to suspect that plasmids conferring resistances in NVC and RVC isolates in the present study could also be one of the group C plasmids. Genetic and molecular characteristics of these plasmids are to be investigated further.

Resistance conferred by R-plasmids in the recipient strain was found to be markedly greater than that shown by donor *V. cholerae*-O1 strains examined by

Hedges *et al.* (1977). A similar increase in resistance in *E. coli* K12 with the current isolates suggests that host cell might lack essential structures or suitable co-factors for the optimal expression of the resistance genes.

It is quite surprising to detect G resistance in a RVC strain and it is noteworthy that several of *V. cholerae*-O1 strains isolated in this region display multiple drug resistance which includes G (unpublished).

The results emphasize the importance of antimicrobic susceptibility determination of *V. cholerae* isolates regardless of their serotypes before commencing chemotherapy.

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