Epidemiol. Infect. (2000), **124**, 393–399. Printed in the United Kingdom

Expanding multiple antibiotic resistance among clinical strains of *Vibrio cholerae* isolated from 1992–7 in Calcutta, India

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(*Accepted 20 December 1999*)

SUMMARY

Antimicrobial susceptibilities of *Vibrio cholerae* strains isolated from cholera patients admitted to the Infectious Diseases Hospital, Calcutta, India for 6 years were analysed to determine the changing trends; 840 *V. cholerae* strains isolated in 1992–1997 were included in this study. Among *V. cholerae* serogoup O1 and O139, ampicillin resistance increased from 1992 (35 and 70%, respectively) to 1997 (both serogroups 100%). Resistance to furazolidone and streptomycin was constantly high among *V. cholerae* O1 strains with gradual increase in resistance to other drugs such as ciprofloxacin, co-trimoxazole, neomycin and nalidixic acid. *V. cholerae* O139 strains exhibited susceptibilities to furazolidone and streptomycin comparable with those of O1 strains. However, after initial increase in resistance to chloramphenicol and co-trimoxazole, all the *V. cholerae* O139 strains became susceptible to these two drugs from 1995 onwards. Both *V. cholerae* O1 and O139 remained largely susceptible to gentamicin and tetracycline. *V. cholerae* non-O1, non-O139 strains, in contrast, exhibited high levels of resistance to virtually every class of antimicrobial agents tested in this study especially from 1995. Kruskal–Wallis one-way analysis showed that *V. cholerae* O1 Ogawa serogroup exhibited significant yearly increase in resistance to nine antibiotics followed by non-O1 non-O139 and O139 strains to six antibiotics and two antibiotics respectively. Interesting observation encountered in this study was the dissipation of some of the resistant patterns commonly found among *V. cholerae* non-O1 non-O139 or O1 serogroups to the O139 serogroup and *vice versa* during the succeeding years.

INTRODUCTION

The definition of emerging infectious diseases in the Institution of Medicine report includes drug-resistance infections, which have been on the upsurge for the past several years [1–3]. Recent examples include multi drug resistance in *Mycobacterium tuberculosis* in USA [4], *Shigella dysenteriae* type I infection in Africa [5], *Salmonella typhi* in India [6], and *Vibrio cholerae* in Ecuador [7]. *Vibrio cholerae* O1 and O139 serogroups are the well-known aetiologic agents of epidemic cholera. Less is reported about *V. cholerae* belonging to the non-O1 non-O139 serogroups, but their participation in causing cholera-like diarrhoea should not be under-estimated particularly after the

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emergence of O139 Bengal. Clinical laboratories do not always test for non-O1 non-O139 serogroups on a routine basis, both with respect to serotype and with respect to susceptibility to different classes of antimicrobial agents. It is important to ascertain the variations in resistance and to relate these variations to mechanisms of resistance. We have been monitoring different serogroups of *V. cholerae* among hospitalized cholera patients for the past several years in Calcutta, India [8]. The major objective of this study was to analyse the trends in multiple antibiotic resistance among clinical strains of *V. cholerae* isolated in 1992–7 in Calcutta.

METHODS

Vibrio cholerae strains

Eight hundred and forty strains of *V. cholerae* isolated between 1992 and 1997 from cholera and cholera-like patients admitted in the Infectious Diseases Hospital, Calcutta were included in this study. All the *V. cholerae* strains were isolated and identified by standard laboratory methods [9], and further confirmed by serology using antisera prepared in our Institute. The 840 strains of *V. cholerae* strains included 326 *V. cholerae* O1 Ogawa, 314 *V. cholerae* O139 and 200 *V. cholerae* non-O1 non-O139 strains. All the strains were stored in nutrient agar stabs at room temperature (20–30 °C) and antibiotic susceptibility testing was performed at monthly intervals.

Antimicrobial susceptibility testing

Antimicrobial susceptibility analysis of *V. cholerae* was performed by the disk diffusion technique [10], with commercial discs (Hi Media, Bombay, India). The following antibiotics were used, ampicillin (A, 10 mcg), chloramphenicol (C, 30 mcg), ciprofloxacin (Cf, 5 mcg), co-trimoxazole (Co, 25 mcg), furazolidone (Fz, 50 mcg), gentamicin (G, 10 mcg), nalidixic acid (Na, 30 mcg), neomycin (N, 30 mcg), norfloxacin (Nf, 10 mcg), streptomycin (S, 10 mcg) and tetracycline (T, 30 mcg). Characterisation of strains as susceptible, intermediately resistant, or resistant was based on the size of the inhibition zones according to the manufacturer's instructions, which matched the interpretive criteria recommended by the World Health Organization [11]. In this study, strains showing intermediate zones of growth inhibition were interpreted as resistant on the basis of previous MIC studies with *V. cholerae* [12].

Statistical analysis

The data were entered into a data base software package KEDIT 3.5 in a personal computer separately by two individuals and converted into EPI-ENFO 6.1 as rec. file for matching data to derive the consistency and validity. The validated data were random checked and then compiled and analysed using the SPSS 4.0 version software package. For comparing the mean rank differences in resistance to each drug for each year, the duration of 6 years from 1992 to 1997 were divided into six groups each for *V. cholerae* O1, O139 and non-O1 non-O139 for all drugs. For non-parametric tests, Kruskal–Wallis one-way analysis of variance was employed to compare the mean rank differences in resistance to each drug for each year, 1992–7. A '*P*' value of $< 0{\cdot}05$ was considered statistically significant.

RESULTS

Results on the drug resistance of *V. cholerae* O1 strains are furnished in Table 1. Increase in resistant to ampicillin, co-trimoxazole, nalidixic acid and neomycin was constantly recorded from 1994. Almost all the *V. cholerae* O1 strains were uniformly resistant to furazolidone and streptomycin throughout the study period. From low levels of resistance to chromphenicol in 1992 and 1993, 73% were resistant to this drug in 1994 and in subsequent years showed a wavering trend (Table 1). *V. cholerae* O1 strains were mostly susceptible to gentamicin and tetracycline. A perceptible increase in the isolation of ciprofloxacin and norfloxacin resistant strains was noticed from 1995.

Like *V. cholerae* O1, O139 strains were resistant to ampicillin, furazolidone and streptomycin and mostly susceptible to nalidixic acid, norfloxacin and tetracycline (Table 2). Ciprofloxacin resistant *V. cholerae* O139 strains first appeared in 1995, but the isolation frequency was low. Frequency in the isolation of chloramphenicol and co-trimoxazole resistant strains of *V. cholerae* O139 was highest during 1994–5 and thereafter declined sharply in the succeeding years (Table 2). Ampicillin, co-trimoxazole, furazolidone, neomycin and streptomycin resistant strains of *V.*

https://doi.org/10.1017/S0950268899003957 Published online by Cambridge University Press

Table 1. *Resistance of* Vibrio cholerae *O1 strains to different antibiotics*

	Year*					
Drug	1992 ($n = 26$)	1993 ($n = 20$)	1994 ($n = 74$)	1995 ($n = 84$)	1996 ($n = 69$)	1997 ($n = 53$)
Ampicillin	9 (34·6)	5 (25·0)	54 (73·0)	74 (88·1)	69 (100)	53 (100)
Chloramphenicol	4 (15·4)	6 (30·0)	54 (73·0)	22 (26·2)	54 (78·3)	10 (18·9)
Ciprofloxacin				2 (2·4)	4 (5·8)	10 (18·9)
Co-trimoxazole	6 (23·1)	16 (80·0)	74 (100)	83 (99·0)	69 (100)	52 (98·1)
Furazolidone	25 (96·1)	17 (85·0)	74 (100)	82 (97·6)	69 (100)	53 (100)
Gentamicin					6 (8·7)	
Neomycin	7 (27·0)	2 (10·0)	35 (47·3)	56 (66·7)	64 (92·7)	23 (43·4)
Nalidixic acid	2 (7·7)	1 (5·0)	73 (98·6)	82 (97·6)	68 (98·5)	50 (94·3)
Norfloxacin				3 (3·6)		4 (7·5)
Streptomycin	26 (100)	20 (100)	74 (100)	83 (99·0)	69 (100)	59 (94·3)
Tetracycline	3 (11·5)	2 (10·0)	1 (1·3)	1 (1·2)	1 (1·4)	

* n, total number of strains. The numbers in parentheses indicate percentage.

Table 2. *Resistance of* Vibrio cholerae *O139 strains to different antibiotics*

	Year*					
Drug	1992 ($n = 10$)	1993 ($n = 87$)	1994 ($n = 40$)	1995 ($n = 42$)	1996 ($n = 64$)	1997 ($n = 71$)
Ampicillin	7 (70·0)	60 (69·0)	35 (87·5)	42 (100)	64 (100)	71 (100)
Chloramphenicol		49 (56·3)	26 (65·0)	6 (14·3)	9 (14·1)	2 (2·8)
Ciprofloxacin				1 (2·4)	3 (4·7)	1 (1·4)
Co-trimoxazole	10 (100)	87 (100)	40 (100)	36 (85·7)	1 (1·6)	
Furazolidone	10 (100)	86 (98·8)	39 (97·5)	41 (97·6)	64 (100)	71 (100)
Gentamicin					2 (3·1)	
Neomycin	1 (10·0)	28 (32·2)	14 (35·0)	15 (35·7)	61 (95·3)	42 (59·1)
Nalidixic acid		2 (2·3)		9 (21·4)	5 (7·8)	5 (7·0)
Norfloxacin				1 (2·4)		
Streptomycin	7 (70·0)	87 (100)	40 (100)	41 (98·0)	61 (95·3)	25 (35·2)
Tetracycline		1 (1·1)		1 (2·4)	5 (7·8)	1 (1·4)

* n, total number of strains. The numbers in parentheses indicate percentage.

cholerae non-O1 non-O139 strains were generally high between 1992 and 1997 as shown in Table 3. In contrast to *V. cholerae* O1 and O139, the non-O1, non-O139 strains were more frequently resistant to ciprofloxacin, norfloxacin and tetracycline (Table 3).

To determine statistically the yearly increase in antibiotic resistance among *V. cholerae* strains isolated between 1992 and 1997, we used Kruskal–Wallis one-way analysis of variance and the results are depicted in Table 4. Except for gentamicin and tetracycline, *V. cholerae* O1 strains were increasingly resistant to all the tested antibiotics. Among *V. cholerae* O139 strains, significant yearly increase in resistance was recorded only for ampicillin and neomycin. However, significant decrease in resistance was recorded among *V. cholerae* O139 to chloramphenicol, co-trimoxazole, nalidixic acid, and streptomycin. *V. cholerae* non-O1, non-O139 strains showed significant increase in resistance to most of the tested antibiotics such as ampicillin, chloramphenicol, ciprofloxacin, neomycin, nalidixic acid and norfloxacin.

One hundred and nineteen different multidrug resistance profiles were encountered in this study (data not shown). Two processes (emergence and dissemination) account for strains of antibiotic resistance in *V. cholerae*. It seems that antibiotic resistance characters confined to one serogroup for a particular period of time was presumably dissipated to the other *V. cholerae* serogroup(s), in which it became stable and then became dominant in the

https://doi.org/10.1017/S0950268899003957 Published online by Cambridge University Press

Table 3. *Resistance of* Vibrio cholerae *non-O1 non-O139 strains to different antibiotics*

	Year*					
Drug	1992 ($n = 13$)	1993 ($n = 20$)	1994 ($n = 14$)	1995 ($n = 27$)	1996 ($n = 53$)	1997 ($n = 73$)
Ampicillin	13 (100)	20 (100)	11 (78·6)	25 (92·6)	53 (100)	73 (100)
Chloramphenicol	1 (7·7)		4 (28·6)	3 (11·1)	14 (26·4)	24 (32·9)
Ciprofloxacin	3 (23·1)	1 (5·0)		2 (7·4)	19 (35·8)	24 (32·9)
Co-trimoxazole	2 (15·4)	6 (30·0)	7 (50·0)	9 (33·3)	25 (47·1)	39 (53·4)
Furazolidone	13 (100)	19 (95·0)	14 (100)	26 (96·3)	53 (100)	72 (98·6)
Gentamicin				1 (3·7)	6 (11·3)	10 (13·7)
Neomycin	11 (84·6)	18 (90·0)	10 (71·4)	16 (59·2)	52 (98·1)	55 (75·3)
Nalidixic acid		2 (10·0)	3 (21·4)	5 (18·5)	19 (35·8)	30 (41·1)
Norfloxacin		1 (5·0)		2 (7·4)	11 (20·7)	15 (20·5)
Streptomycin	5 (38·5)	14 (70·0)	10 (71·4)	17 (63·0)	36 (67·9)	31 (42·5)
Tetracycline	1 (7·7)	2 (10·0)	4 (28·6)	6 (22·2)	14 (26·4)	28 (38·3)

* n, total number of strains. The numbers in parentheses indicate percentage.

Table 4. *Kruskal–Wallis one-way analysis of variance exploring significant changes in drug resistance* (*either mean rank increase or mean rank decrease*) *among years and* V. cholerae *O1, O139 and non-O1 non-O139*

	Ties significance		
Antibiotics	O1	O139	Non-O1 non-O139
Ampicillin	< 0·00001*	< 0·00001*	< 0·00001*
Chloramphenicol	< 0·00001*	< 0·00001†	0·0148*
Ciprofloxacin	< 0·00001*	0·2789	0·0018*
Co-trimoxazole	< 0·00001*	< 0·0001†	0·0808
Furazolidone	0·0003*	0·6488	0·5823
Gentamicin	0·0004†	0·1654	0·1511
Neomycin	< 0·00001*	< 0·0001*	0·0006*
Nalidixic acid	< 0·00001*	0·0008†	0·0057*
Norfloxacin	0·0312*	0·2626	0·0559*
Streptomycin	0·0532*	< 0·0000†	0·0151†
Tetracycline	0·0147†	0·0913	0·0754

* Statistically significant increases mean rank in resistance to the antibiotic in question among the years of comparison.
† Significantly decreasing.

subsequent years. As shown in Table 5, the profiles AFz and AFzN are recorded among *V. cholerae* non-O1 and non-O139 strains from 1992 and these profiles are respectively recorded among 17% (12/71 strains) and 39·4% (28/71 strains) of *V. cholerae* O139 in 1997. Similarly, the profile AFzNS was first recorded among *V. cholerae* O1 and non-O1 non-O139 strains from 1992 and most likely was transferred to 67·7% of O139 serogroup (42/64 strains), which was one of the dominant profiles in 1996. The profile ACoFzNaS was first recorded during 1993 among *V. cholerae* O139 and non-O1 and non-O139 strains followed by *V. cholerae* O1 strains from 1994 (4/74 strains), in which preponderance of this profile reached to 30% (16/53 strains). AFzS profile was recorded first among *V. cholerae* O1 in 1992 followed by non-O1 and non-O139 strains until 1996. In 1997, this profile was dominantly found among 18·3% of *V. cholerae* O139 strains (13/71 strains).

DISCUSSION

Even though the therapy for cholera is principally supportive, antimicrobial therapy can be useful in decreasing the volume of stools and length of illness [13, 14]. While tetracycline has been the mainstay of

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Table 5. *Emergence and dissemination of multiply antibiotic resistance among* V. cholerae *in Calcutta*

Dominant profile	Year/serogroup																	
	1992			1993			1994			1995			1996			1997		
	O1	O139	Non-O1 non-O139	O1	O139	Non-O1 non-O139	O1	O139	Non-O1 non-O139	O1	O139	Non-O1 non-O139	O1	O139	Non-O1 non-O139	O1	O139	Non-O1 non-O139
AFz	—	—	1	—	—	1	—	—	—	—	—	3	—	—	1	—	12	8
AFzN	—	—	6	—	—	4	—	—	2	—	—	—	—	—	8	—	28	14
AFzNS	4	—	2	—	—	6	—	—	—	—	—	8	—	42	10	—	9	—
ACoFzNaS	—	—	—	—	1	1	4	—	—	11	4	—	2	—	—	16	—	—
AFzS	5	—	—	—	—	—	—	—	2	—	1	—	—	1	—	—	13	—

therapy, chloramphenicol, furazolidone and co-trimoxazole are the other reported alternatives [15, 16]. Multidrug resistant classical *V. cholerae* strains and simultaneous epidemic outbreaks of both classical and ElTor biotypes of *V. cholerae* has been reported in Bangladesh [17]. Majority of the ElTor strains in this study was resistant to ampicillin and furazolidone and a similar trend is seen in Calcutta. Since cholera is a non-invasive disease, drugs such as co-trimoxazole, which is not absorbed from the gastrointestinal tract, was widely used for the treatment [18, 19]. Resistance of an ElTor strain of *V. cholerae* to trimethoprim, streptomycin and the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine) is due to a transposon inserted into the chromosome [20], whose transfer is being enhanced by pretreatment with these drugs for which the markers encode resistance. This phenomenon may, in large part, be responsible for the rapid dissemination and high incidence of co-trimoxazole and streptomycin resistance among *V. cholerae* isolated from 1989 in Calcutta [21]. Almost all the *V. cholerae* O1 strains were resistant to co-trimoxazole versus none of *V. cholerae* O139 strains isolated during 1996–7 [22]. The higher incidence of *V. cholerae* non-O1, non-O139 strains resistant to tetracycline compared to O1 and O139 strains in this study could be a prelude to the possible emergence of tetracycline resistant strains of *V. cholerae* O1 and O139.

Reservation about promotion of ciprofloxacin as a first line drug for the treatment of cholera in developing countries has been expressed [23], since it is an important substitute drug for treatment of multidrug resistant enteric and other pathogens. Extensive use of this drug and empirical therapy for treating diarrhoeal infection might have promoted incidence of ciprofloxacin resistant *V. cholerae*, which has emerged for the first time in Calcutta during 1992 among *V. cholerae* non-O1 non-O139 and during 1995 among *V. cholerae* O1 and O139 strains [24].

Since tetracycline resistant *V. cholerae* O1 strains have been responsible for major epidemics of cholera in Latin America, Tanzania, Bangladesh and Zaire [7, 25–27], norfloxacin is widely used as an alternative to tetracycline for the treatment. Even though the incidence level of norfloxacin resistant strains among *V. cholerae* O1 and O139 is less in the present study, the non-O1 non-O139 strains exhibited a higher level of incidence, especially during 1996–7.

Based on the chronological evidence gleaned from this study it appears that some of the drug resistance

https://doi.org/10.1017/S0950268899003957 Published online by Cambridge University Press

expression might have transferred from one serogroup of *V. cholerae* to the other. However, the possibility of acquiring antibiotic resistance by inter/intra generic transfer cannot be ignored. The major patterns of multiple antibiotic resistance determined in Calcutta are comparable with those from other endemic locals in India and Bangladesh. The antibiotic resistance profiles AFz; AFzN and AFzNS encountered in this study were also reported common among *V. cholerae* O139 strains isolated from Madras, Nagpur [28]; Dhaka, Bangladesh [29]; Nagpur, Midnapur, Madras, Amravati [30] respectively. The profile ACoFzNaS was common among *V. cholerae* O1 strains isolated from Bhillai, Ahmadabad, Allephey, Madras, Vellore and Dibrugarh [31].

Early studies conducted in India showed that the prevalence of multidrug resistant strains of *V. cholerae* non-O1 was a rare event [32, 33]. Sundaram & Murthy [34] reported that only 2·7% non-O1 isolates were multi drug resistant in Madras; an area endemic for cholera in south India, but none of the strains was resistant to nalidixic acid or furazolidone. In the current study, we have observed that like O1, non-O1 and non-O139 isolates exhibited resistance to furazolidone and nalidixic acid.

It is amply clear that long-term surveillance programmes are essential to identify changes in the spectrum of microbial pathogens causing serious infection and to monitor trends in antimicrobial resistance patterns [35–37]. The information gleaned from the surveillance efforts is integral to the designing approaches to the therapy of serious infection and also to defining appropriate control measures for antimicrobial-resistance pathogens.

ACKNOWLEDGEMENTS

This work was supported, in part, by the Japan International Co-operation Agency (JICA/NICED Project 054-1061-E-O), Tokyo, Japan. We thank A. M. Mukhopadhyay, A. Basu and R. Mitra for their input in this study.

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