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Biotype traits and antibiotic susceptibility of *Vibrio cholerae* serogroup O1 before, during and after the emergence of the O139 serogroup

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SUMMARY

Sixty-nine strains of *Vibrio cholerae* O1 isolated at different times were analysed to investigate if there were any differences among the O1 strains isolated before, during and after the advent of the O139 serogroup. Of the 69 O1 strains examined, 68 belonged to the Ogawa serotype while one belonged to the Inaba serotype. With the exception of one strain all other strains of *V. cholerae* O1 belonged to the eltor biotype. A single O1 strain isolated before the emergence of the O139 serogroup could not be classified as either eltor or classical biotype because it was resistant to both classical and eltor specific bacteriophages. Marked variations in the susceptibility to antibiotics of *V. cholerae* O1 isolated during the different periods were observed. In addition, strains of *V. cholerae* isolated after the epidemic of serogroup O139 in Calcutta showed an expanding R-type with resistance to a variety of drugs as compared to the O1 strains isolated before the advent of the O139 serogroup. From this study, it is clear that there is a substantial mobility in genetic elements of *V. cholerae* O1 which necessitates a continuous monitoring to keep abreast of the changing traits of the etiologic agent of cholera.

INTRODUCTION

Vibrio cholerae belonging to the serogroup O1, until recently the only causative agent of epidemic and pandemic cholera, is classified into two biotypes namely classical and eltor. Each biotype is further sub-classified into two major serotypes

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namely Ogawa and Inaba. From October 1992, a novel strain of *V. cholerae* which did not agglutinate with the O1 antiserum was responsible for explosive epidemics of cholera-like infection in several parts of India [1, 2] and in several countries in the Asian continent [3]. These strains were assigned to a new serogroup O139 synonym Bengal because they did not agglutinate with any of the existing 138 'O' antisera of *V. cholerae* [4]. Detailed serological studies have now shown that the O139 serogroup possess antigenic components which are shared with serogroups O22 and O155 [5] indicating somatic antigenic sharing between the O139 serogroup and the non-O1 serogroups of *V. cholerae*.

A continuous surveillance on the incidence of *V. cholerae* among patients hospitalized in the Infectious Diseases Hospital (IDH), Calcutta has been ongoing for several years at the National Institute of Cholera and Enteric Diseases (NICED), the National Reference Centre for Cholera in India. The objective of the surveillance is to monitor the variations in biotypes, serotypes, serogroups and phage types of *V. cholerae*. This surveillance enabled us to promptly detect the emergence of the O139 serogroup [1] with the first strain being isolated in Calcutta around 20 November 1992 [2]. The present study was performed to understand whether the emergence of *V. cholerae* O139 had any impact on strains of *V. cholerae* O1 which was temporarily displaced by the O139 serogroup for a period of 6 months in Calcutta [2].

MATERIALS AND METHODS

Sources of strains

Stool samples from patients with acute watery diarrhoea admitted to the IDH, Calcutta were collected and processed as described previously [6]. Isolation and identification of *V. cholerae* was done following previously published procedures [7]. Slide agglutination was done with polyvalent O1 and monospecific Ogawa and Inaba antisera and with O139 antiserum prepared at NICED [8]. Strains that did not agglutinate with either O1 or O139 antisera were grouped by the somatic 'O' antigen serogrouping scheme of *V. cholerae* developed at the National Institute of Health, Tokyo [5].

Categorization of strains

The strains of *V. cholerae* O1 characterized in this study were classified into three groups, based on the time of isolation in relation to the genesis of the O139 serogroup. Group 1 was comprised of strains isolated before the emergence of O139 in Calcutta (April to Noember 1992), Group II was comprised of strains isolated during the O139 epidemic in Calcutta (July to December 1993) while group III represented strains isolated after the O139 epidemic in Calcutta (February to April 1994). A total of 69 strains including 27, 20 and 22 strains from Groups I, II and III, respectively, were characterized in detail. Seventeen strains of *V. cholerae* O139 isolated from February to May 1994 which represent the current isolates of this serogroup were also included in this study for purposes of comparison. Selection of strains from each time-period was done in a random fashion by an individual blind to the date of isolation of the strains.

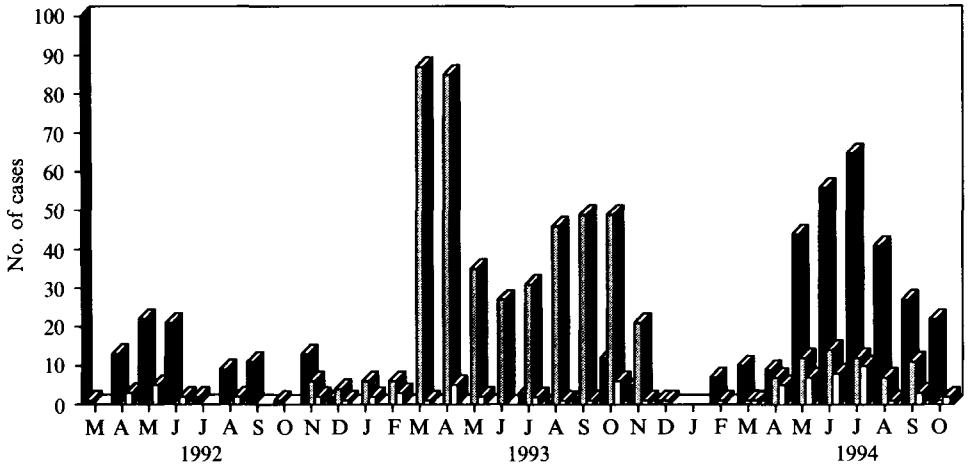

Fig. 1. Distribution of various serogroups of *Vibrio cholerae* isolated from patients with acute secretory diarrhoea admitted to the Infectious Diseases Hospital, Calcutta, India, ■, O1; ▨, O139; □, non-O1, non-O139.

Biotyping of V. cholerae *O1*

Strains of *V. cholerae* O1 examined in this study were biotyped by determining the following reactions: chicken cell agglutination, Voges-Proskauer reaction and sensitivity to polymyxin B and bacteriophages ϕ149 (classical phage IV) and e5 (eltor phage 5). The above tests were performed as described [9].

Detection of cholera toxin gene

The presence of the CT gene in all strains of *V. cholerae* O1 characterized in this study was determined. Colony blots were prepared and hybridization was performed under stringent conditions as described previously [10].

Susceptibility to antibiotics

Susceptibility of the strains to a variety of antimicrobial agents was performed by the dry disc diffusion technique [7] with commercially available disks listed in Table 2. Strains showing intermediate zone of inhibition were interpreted as resistant to that drug.

RESULTS

Variations in the incidence of serogroups of *V. cholerae* among patients admitted to the IDH in Calcutta between March 1992 and October 1994 is shown in Figure 1. For a span of 6 months starting from January 1993, serogroup O139 completely replaced O1 serogroup in Calcutta, an area hyperendemic for cholera. Serogroup O1, however, reappeared in July 1993 and currently both serogroups cause cholera in Calcutta with the O1 serogroup predominating. This pattern of incidence afforded us an opportunity to examine the various traits of *V. cholerae* O1 isolated before, during and after the O139 epidemic in Calcutta. Of the total of 69 *V. cholerae* O1 stains examined, 68 belonged to the Ogawa serotype and one to the Inaba serotype.

All the 69 representative strains of *V. cholerae* O1 and all the 17 strains of *V. cholerae* O139 examined were resistant to polymyxin B (Table 1). With the

Table 1. *Characteristics of* V. cholerae *O1 and O139 examined in this study*

Categ strain (no.)*	f	Presence of CT gene (%)	Resistance to O/129 (150 μg) (%)	Biotyping traits (%)				
				Agglutination of chicken erythrocytes	Positive Voges-Proskauer reaction	Resistance to polymixin B (50 U)	Lysis by	
							Classical phage IV	El Tor phage 5
Grou (*n* =		26 (96·3)	17 (63)	27 (100)†	21 (77·8)	27 (100)	0 (0)	26 (96·3)
Grou (*n* =		20 (100)	5 (25)	20 (100)	20 (100)	20 (100)	0 (0)	20 (100)
Grou (*n* =		22 (100)	0 (0)	22 (100)	22 (100)‡	22 (100)	0 (0)	22 (100)
V. ch O13	: 17)	17 (100)	17 (100)	ND§	17 (100)	17 (100)	0 (0)	0 (0)

* Group I incl strains isolated between April and November, 1992; Group II includes strains isolated between July and December, 1993 and Group III includ ains isolated between February and April, 1994.

† One of the 2 itive strains showed weak agglutination.

‡ Two of the : itive strains showed weak reactions.

§ ND, not don

Table 2. *Drug resistance of* Vibrio cholerae *O1 and O139*

Category of strains (no.)	Antibiotic (% resistant)*										
	A	C	Co	Cf	Fz	J	N	Na	Nf	S	T
Group I ($n = 27$)	33·3	14·8	37·0	0	96·3	0	25·9	7·4	0	100	11·1
Group II ($n = 20$)	25	30	80	0	85	0	10	5	0	100	10
Group III ($n = 22$)	81·8	63·6	100	0	100	0	54·5	95·5	0	100	9
V. cholerae O139 ($n = 17$)	70·6	29·4	100	0	100	0	58·8	0	0	100	0

* A, ampicillin (10 mcg); C, chloramphenicol (30 mcg); Co, co-trimoxazole (25 mcg); Cf, ciprofloxacin (5 mcg); Fz, furazolidone (100 mcg); J, gentamicin (10 mcg); N, neomycin (30 mcg); Na, nalidixic acid (30 mcg); Nf, norfloxacin (10 mcg); S, streptomycin (10 mcg); T, tetracycline (30 mcg).

exception of one strain (VC100) which was isolated before the advent of serogroup O139, all other strains were sensitive to eltor phage 5 but resistant to classical phage IV placing them in the eltor biotype. VC100 showed a positive Voges-Proskauer reaction, agglutinated chicken red cells and was resistant to polymixin B, classical phage IV and eltor phage 5. Again apart from one strain VC14 belonging to the eltor biotype, Ogawa serotype, all other O1 strains examined in this study hybridized with the DNA probe specific for the CT gene (Table 1).

Susceptibility of *V. cholerae* O1 isolated before, during and after the O139 epidemic in Calcutta showed interesting variations (Table 2). Resistance of *V. cholerae* O1 to co-trimoxazole (Co), nalidixic acid (Na) and chloramphenicol (C) showed an increasing trend when strains isolated before the O139 epidemic were compared to strains currently being isolated. Interestingly, strains of *V. cholerae* O1 resistant to tetracycline (T) were also encountered, although low in numbers. Strains of O1 and O139 currently being isolated in Calcutta showed a similar pattern of resistance with the exception of Na with most (91·3%) of the O1 strains being resistant to this drug while all the O139 strains were sensitive to Na.

Comparison of resistance types (R-types) of *V. cholerae* O1 isolated before, during and after the O139 epidemic in Calcutta also showed a shifting pattern (Table 3). Strains of *V. cholerae* isolated after the O139 epidemic showed an expanding R-type with resistance to a variety of drugs as compared to strains of *V. cholerae* isolated before the emergence of O139 serogroup. Multi-drug resistance was more common among *V. cholerae* O1 strains currently being isolated as compared to the O139 serogroup.

DISCUSSION

Since for a span of 6 months, the O1 serogroup of *V. cholerae* was completely replaced by the O139 serogroup in Calcutta, we became interested to determine if there were any differences among the O1 strains which reappeared in July 1993 as compared to those which prevailed before January 1993. The differentiation of *V. cholerae* O1 into classical and eltor biotypes is accomplished by four tests which include haemagglutination tests, haemolysis and susceptibility to polymyxin B (50 U) and bacteriophages [11, 12]. Among the tests for biotyping of *V. cholerae*

Table 3. *R-types of multiply drug resistant strains of* V. cholerae *O1 and O139 encountered in this study*

Category of strains (no.)	R-type								No.
Group 1 ($n = 27$)	C	Co	Fz	N	Na	S	T		1
	C	Co	Fz	Na	S	T			1
	C	Co	Fz	S	T				1
	A	Fz	N	S					4
	C	Co	Fz	S					1
	Co	Fz	N	S					1
	A	Fz	S						5
	Co	Fz	S						4
	Fz	N	S						1
	Co	S							1
	Fz	S							7
Group II ($n = 20$)	A	C	Co	Fz	S				3
	C	Co	Fz	S					3
	Co	Fz	N	S					1
	A	Co	S						2
	Fz	Co	S						6
	Fz	N	S						1
	Co	S							1
	Fz	S							3
Group III ($n = 22$)	A	C	Co	Fz	N	Na	S	T	1
	A	C	Co	Fz	N	Na	S		4
	A	C	Co	Fz	Na	S			5
	A	Co	Fz	N	Na	S			5
	A	Co	Fz	Na	S	T			1
	C	Co	Fz	N	Na	S			1
	A	Co	N	Na	S				1
	A	Co	Fz	Na	S				1
	C	Co	Fz	Na	S				3
V. cholerae ($n = 17$)	A	C	Co	Fz	N	S			2
	A	C	Co	Fz	S				1
	A	Co	Fz	N	S				6
	C	Co	Fz	N	S				2
	A	Co	Fz	S					3
	Co	Fz	S						3

Abbreviation of antibiotics same as in Table 2.

O1, bacteriophage susceptibility is considered one of the most important and least variable trait. Classical biotype of *V. cholerae* O1 are usually sensitive to bacteriophage classical phage IV while the eltor isolates are usually resistant [13] and eltor isolates are susceptible to bacteriophage eltor 5 while classical strains are resistant to the same [14]. *V. cholerae* O139 has been shown to be resistant to both the classical and eltor bacteriophages [15].

Intriguingly, one strain of *V. cholerae* O1 which was isolated before the emergence of the O139 serogroup was resistant to the classical and eltor specific bacteriophages, a pattern similar to that shown by the O139 serogroup, and could not be classified as either eltor or classical biotype. All the strains of *V. cholerae* O1 examined in this study were resistant to polymyxin B, a trait similar to that exhibited by eltor strains. From this data, it appears that before the appearance

of O139 serogroup, changes in bacteriophage susceptibility in some strains of *V. cholerae* O1 took place which was followed by the emergence of the O139 serogroup. This could indicate that bacteriophages may have had a contributing role in the genesis of O139 serogroup and this needs to be examined. With the above exception, apparently, *V. cholerae* O1 strains being currently isolated are in effect, similar to the O1 strains which existed prior to the emergence of the O139 serogroup.

Susceptibility to antibiotics also showed marked variations with strains of O1 isolated after the O139 epidemic exhibiting higher percentage of multiply antibiotic resistant strains as compared to before and during the O139 epidemic. Co-trimoxazole and furazolidone, both considered the second line of treatment in adults and the drug of choice among children when antimicrobial therapy is indicated in cholera patients, are clearly ineffective for the current O1 and O139 mediated infections. The appearance of nalidixic acid resistance among *V. cholerae* O1 strains currently isolated is also a disturbing event. This is probably related to the widespread use of nalidixic acid in the treatment of multiply resistant *Shigella dysenteriae* type 1 cases in Calcutta [16]. The transmission of resistant plasmids from *Shigella* sp. to *V. cholerae* has been established although resistance was not stable under drug-free conditions [17, 18]. Susceptibility of vibrios to the vibriostatic agent 2,4-diamino-6,7-diisopropylpteridine (O/129) has been used as an important taxonomical trait to differentiate between vibrios and other Gram-negative bacilli. However, all the *V. cholerae* O139 and several of the *V. cholerae* O1 strains examined in this study were resistant to the vibriostatic agent and the taxonomical usefulness of this test no longer remains valid. In an earlier study, we observed that resistance of *V. cholerae* to O/129 was invariably linked with resistance to co-trimoxazole [19].

The past 2 years have witnessed incredible changes in *V. cholerae*, the causative agent of cholera indicating an enhanced mobility in genetic elements encoding various phenotypic traits. These rapid changes which are currently taking place in a highly cholera endemic area like Calcutta underscores the need to maintain a continuous vigilance on the changing trend in the etiologic agent of cholera. Among others, environmental factors and possibly the widespread use of antibiotics may be playing an important role in the shifting trends of *V. cholerae* that is being currently witnessed.

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