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Temporal Shifts in Traits of *Vibrio cholerae* Strains Isolated from Hospitalized Patients in Calcutta: a 3-Year (1993 to 1995) Analysis

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This study presents results of a surveillance on cholera conducted with hospitalized patients admitted to the Infectious Diseases Hospital, Calcutta, India, from January 1993 to December 1995. The O139 serogroup of *Vibrio cholerae* dominated in 1993 but was replaced by O1 as the dominant serogroup in 1994 and 1995. The isolation rate of *V. cholerae* non-O1 non-O139 did not exceed 4.9% throughout the study period, while the isolation rate of the O139 serogroup in 1994 and 1995 was below 9%. No temporal clustering of any non-O1 non-O139 serogroup was observed. With the exception of 1 strain, none of the 64 strains belonging to the non-O1 non-O139 serogroup hybridized with *ctx*, *zot*, and *ace* gene probes, while 97.3 and 97.7% of the O139 and O1 strains, respectively, hybridized with all the three probes. Multiplex PCR studies revealed that all the O1 strains belonged to the ElTor biotype. There was a progressive increase in the cytotoxic response on CHO and HeLa cells evoked by culture supernatants of strains of *V. cholerae* non-O1 non-O139 isolated during 1994 and 1995 compared with the response evoked by those isolated in 1993. Dramatic shifts in patterns of resistance to antibiotics between strains of *V. cholerae* belonging to different serogroups and within strains of a serogroup isolated during different time periods were observed. There was a discernible increase in the incidence of multidrug-resistant strains of *V. cholerae* O1 isolated in 1994 and 1995 compared with that in 1993. On the basis of the results of this study, we predict the possibility of newer variants of *V. cholerae* emerging in the future.

The past few years have witnessed a remarkable resurgence in the global incidence of cholera. With the spread of the seventh pandemic of cholera into Latin America, the number of countries reporting cholera cases has dramatically increased from 35 in 1984 to 54 in 1991 (37). The epidemiology of cholera continues to be bewildering, and several events which have occurred in recent years defy explanation. Included among these enigmatic events are the sudden and explosive entry of the *Vibrio cholerae* O1 ElTor biotype in Latin America after a 100-year absence in that continent (16, 27), the exclusive distribution of the classical biotype of *V. cholerae* O1 in southern Bangladesh (30, 34), the explosive emergence of *V. cholerae* O139 (4, 25), and the reappearance in the Indian subcontinent of the O1 serogroup of *V. cholerae*, which apparently is a new clone different from those which existed earlier (18, 41).

Considering the alarming increase in the incidence of cholera and being the National Reference Centre for Cholera in India, we have fortified our surveillance at the national level in general and at Calcutta, an area where cholera is hyperendemic, in particular. Among others, we have adopted a holistic approach and are monitoring *V. cholerae* as a whole with emphasis on no particular serogroups, as was previously done. Our rationale to adopt this strategy was to understand changes at all levels and to be prepared for newer emerging variants of *V. cholerae*. In this study, we report the findings of a systematic holistic surveillance which has enabled us to precisely detect subtle changes in traits of *V. cholerae* which would have gone unnoticed in a more superficial surveillance.

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MATERIALS AND METHODS

Hospital surveillance. The present study is a part of the continuing surveillance program of the National Institute of Cholera and Enteric Diseases on cholera. Analysis for this study was made for a period of 36 months encompassing the 3 years from January 1993 to December 1995. Stool specimens were obtained from patients admitted to the Infectious Diseases Hospital, the only hospital which admits cholera patients from the metropolitan city and suburban areas, with sterile catheters in sterile McCartney bottles. Rectal swabs were taken with sterile cotton-tipped swabs from patients from whom stool samples could not be obtained. Stool specimens and rectal swabs in Cary-Blair medium were transported within 1 h of collection and were bacteriologically examined within 1 h of arrival at the laboratory.

Bacteriology and serogrouping. Thiosulfate-citrate-bile salts-sucrose agar (Eiken, Tokyo, Japan) was used as the selective medium for the isolation of *V. cholerae*. Stool samples were inoculated on thiosulfate-citrate-bile salts-sucrose agar plates with sterile cotton-tipped swab sticks and then streaked for colony isolation. About 250 μl of the neat stool sample or the rectal swab was also introduced into 2 ml of alkaline peptone water (Bacto Peptone, 1%; NaCl, 1%; pH 8.5) used as an enrichment medium and incubated at 37°C for 18 h. The inoculated plates (directly from stool samples or from enrichment medium) were incubated at 37°C for 18 to 24 h and subsequently examined for the growth of typical *V. cholerae*-like colonies appearing as round, yellow, sucrose-fermenting colonies with elevated centers. A multitest medium was used for presumptive identification of *V. cholerae* (9, 21). All isolates were subsequently examined for the oxidase reaction, and the identity of *V. cholerae* O1 was thereafter confirmed by serogrouping, using growth from the multitest medium, with polyvalent O1 and monospecific Inaba and Ogawa antisera raised at this institute. *V. cholerae* strains which did not agglutinate with the O1 antiserum were checked with monoclonal O139 antiserum developed at our institute (6). The quality of the antisera was periodically checked with appropriate reference strains (MAK 757 [*V. cholerae* O1 ElTor biotype], 569B [*V. cholerae* O1 classical biotype], and ATCC 51394 [*V. cholerae* O139 Bengal]). *V. cholerae* strains which did not

agglutinate with either O1 or O139 antisera were assumed to belong to the non-O1 non-O139 serogroups, and these strains were further serogrouped by the somatic O-antigen serogrouping scheme of *V. cholerae* developed at the National Institute of Health, Tokyo, Japan (32).

Tissue culture assay. A representative 77 strains of *V. cholerae* non-O1 non-O139 isolated during the study period were examined by tissue culture assay using CHO and HeLa cells. The strains were grown in tryptic soy broth (Difco, Detroit, Mich.) supplemented with 0.6% yeast extract (Difco) and in AKI medium (Bacto Peptone, 1.5%; yeast extract, 0.4%; NaCl, 0.5%; $NaHCO_3$, 0.3%; pH 7.4 [8]) with shaking for 18 h. The culture supernatant obtained by centrifugation at 4°C was made cell free by passing it through a 0.22-μm-pore-size filter unit (Millex-GS; Millipore, Bedford, Mass.) and collected in sterile test tubes which were kept at 4°C until used.

CHO and HeLa cells were grown as monolayers in Dubbecco's minimum essential medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% (vol/vol) horse serum (Gibco Laboratories, Grand Island, N.Y.). Cell lines were maintained in 25-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) at 37°C in a humidified 5% CO_2 atmosphere. A confluent monolayer of CHO and HeLa cells grown for 3 to 4 days was removed from the tissue culture flasks, and 200 μl of the cell suspension (ca. 6.4×10^3 cells) was added to each of the 96-well plates along with 50 μl of the cell-free culture filtrate and incubated as described above. Morphological changes in CHO or HeLa cells were recorded at 24 h. For negative controls, wells received the uninoculated culture medium, and pure cholera toxin (Sigma, St. Louis, Mo.) was used as the positive control.

Antimicrobial susceptibility. Representative strains of *V. cholerae* O1, O139, and non-O1 non-O139 from each of 3 years under analysis were examined for resistance to ampicillin (10 μg), chloramphenicol (30 μg); cotrimoxazole (25 μg), ciprofloxacin (5 μg), furazolidone (100 μg), gentamicin (10 μg), neomycin (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), streptomycin (10 μg), and tetracycline (30 μg) with commercial discs (Hi Media, Bombay, India). A 3-h culture of *V. cholerae* O139 or a 4-h culture of *V. cholerae* O1 and non-O1 non-O139 in tryptic soy broth (Difco) was spread plated on well-dried Mueller-Hinton agar (Difco). Plates were incubated for 24 h at 37°C. Characterization of strains as susceptible, intermediately resistant, or resistant was based on the size of the inhibition zones around each disc according to the manufacturer's instructions, which matched the interpretive criteria recommended by World Health Organization (38). Strains showing intermediate zones of inhibition were interpreted as resistant to that drug on the basis of previous MIC studies conducted with *V. cholerae* (39).

DNA probes. A 540-bp *Xba*I-*Cla*I fragment of the *ctxA* gene (10), an *Eco*RI-*Pst*I fragment of pMZP11 containing a 790-bp *Sca*I-*Acc*I fragment of the *zot* gene (12), and an *Eco*RI-*Hin*dIII fragment of pKK2 which contains a 355-bp *Nru*I-*Acc*II fragment of the *ace* gene (15) were used as the specific DNA probes for the *ctx*, *zot*, and *ace* genes, respectively. The procedure employed to purify plasmid DNA and to isolate and purify the DNA fragment has been described previously (12, 36). Purified DNA was labelled by incorporating [α-^{32}P]dATP to a specific activity of 2×10^8 to 8×10^8 cpm/μg of DNA by nick translation. Radiolabelled probe DNA was purified by chromatography on NACSPREPAC as specified by the manufacturer (Bethesda Research Laboratories, Gaithersburg, Md.). The colony blot was prepared on autoclaved nitrocellulose filters (BA 85/20; Schleicher and Schuell Co., Keene, Germany), and hybridization was performed under high-stringency conditions as described previously (12).

Multiplex PCR. A multiplex PCR-based assay was used to biotype strains of *V. cholerae* by determining the presence of *ctxA* (encoding the enzymatic subunit of cholera toxin) and *tcpA* (encoding the major structural subunit of the toxin-coregulated pilus), as described elsewhere (13). Thirty, 30, and 40 representative strains of *V. cholerae* belonging to the O1, O139, and non-O1 non-O139 serogroups, respectively, were examined. Three pairs of primers as described previously (13) were used to detect the following: a 301-bp sequence of *ctxA* (33), a 470-bp fragment of the ElTor variant of the *tcpA* sequence, and a 671-bp sequence of the classical variant of *tcpA* (35).

The following were added to each 100 μl of PCR mixture: 10 μl of Mg-free 10× amplification buffer (500 mM KCl, 100 mM Tris HCl [pH 9.0], 0.1% Triton X-100); 8 μl of 25 mM $MgCl_2$; 2 μl each of 2 mM dATP, dTTP, dGTP, and dCTP; 50 pmol each of the primers; and 2.5 U of *Taq* DNA polymerase (Takarashuzo, Otsu, Japan). PCR was carried out in 0.5-ml microcentrifuge tubes, with 43.5 μl of the PCR mixture described above and 6.5 μl of Luria broth (Difco) culture of the test strains heated at 94°C for 5 min. The solution was overlaid with a drop of sterile mineral oil (Sigma), and PCR was performed in an automated thermocycler (FTS 320; Corbett Research, N.S.W., Australia). PCR amplification was performed for 30 cycles, and the cycling conditions were as follows: denaturation at 94°C for 1.5 min, annealing at 60°C for 1.5 min, and extension at 72°C for 1.5 min. A reagent blank (containing all the components of the reaction mixture and water instead of broth containing template DNA), VC20 (*V. cholerae* O1 ElTor Ogawa), and 569B (*V. cholerae* O1 classical Inaba) were run as controls. Amplified products from PCR were electrophoresed on 1% agarose gels and were stained with ethidium bromide. A 1-kb molecular size ladder (Gibco BRL, Gaithersburg, Md.) was run with each gel.

Statistical analysis. The Mantel-Haenszel chi-square, Yates correction, and Fisher exact chi-square tests (14) were employed to compare and assess yearwise significance in the increase or decrease of drug resistance of *V. cholerae* O1, O139, and non-O1 non-O139.

TABLE 1. Isolation rates of *V. cholerae* O1, O139, and non-O1 non-O139 from patients with acute secretory diarrhea admitted to the Infectious Diseases Hospital, Calcutta

Yr	No. of samples screened	No. (%) of samples				
		Positive for *V. cholerae*	Of serogroup:			
			O1		O139	Non-O1 non-O139
			Inaba	Ogawa		
1993	890	491 (55.2)	1 (0.1)	23 (2.6)	443 (49.8)	24 (2.7)
1994	748	400 (53.5)	0	296 (39.6)	67 (8.9)	37 (4.9)
1995	873	399 (45.7)	1 (0.1)	304 (34.8)	62 (7.1)	32 (3.7)

RESULTS

A total of 2,511 patients hospitalized with acute secretory diarrhea were examined during the 3-year period under analysis. The rate of isolation of the various serogroups of *V. cholerae* is shown in Table 1. The O139 serogroup dominated in 1993 but was replaced by O1 as the dominant serogroup in 1994 and 1995. Monthly isolation of various serogroups of *V. cholerae* is shown in Fig. 1 along with prominent events which occurred in relation to subtle changes in the traits of *V. cholerae* at that point. The O1 serogroup of *V. cholerae*, after being displaced for a period of 6 months, reappeared sporadically in July 1993 and became the dominant serogroup by February 1994. In 1994, the peak incidence of *V. cholerae* O1 occurred in July, while in 1995, the peak incidence occurred in May. The pattern of monthly isolation of the O139 and the non-O1 non-O139 serogroups followed trends more or less similar to that of the O1 serogroup in 1994 and 1995 with the exception of September to November 1995, when the incidence of the O139 serogroup was anticoincidental to that of the O1 serogroup. All the O1 strains of *V. cholerae*, with the exception of two, isolated in the 3 years belonged to the Ogawa serotype. Two strains of the Inaba serotype were isolated, one in December 1993 and one in May 1995.

The isolation rate of non-O1 non-O139 *V. cholerae* did not exceed 4.9% throughout the study period, while the isolation rate of the O139 serogroup in 1994 and 1995 was below 9%. Table 2 shows the different non-O1 non-O139 serogroups isolated from patients with acute secretory diarrhea during the 3 years. No temporal clustering of any non-O1 non-O139 serogroup was observed. Of the possible 155 currently recognized O serogroups (31), 16 (10%) were encountered in 1993, 22 (14.2%) were encountered in 1994, and 18 (11.6%) were encountered in 1995. During the later half of 1995, three strains of *V. cholerae* belonging to newly designated serogroups O174, O178, and O183 were isolated.

Representative strains of *V. cholerae* O1, O139, and non-O1 non-O139 isolated during the period of study were examined with DNA probes specific for *ctxA*, *zot*, and *ace*. With the exception of 1 strain, none of the 64 strains of *V. cholerae* non-O1 non-O139 hybridized with the three probes (Table 2), while 73 of the 75 O139 strains and 130 of the 133 O1 strains hybridized with all the three probes. One strain each, among the O139 and O1 serogroups, was nontoxigenic, while the remaining strains (one in O139 and two in O1) possessed an incomplete virulence cassette, with either *zot* or *ace* missing. The single non-O1 non-O139 strain (SG36) which hybridized with the *ctx*, *zot*, and *ace* gene probes belonged to serogroup O74 and was isolated in February 1993. Multiplex PCR studies revealed that all the representative strains of *V. cholerae* O1 and O139 examined were positive for the 301-bp *ctxA* and the 470-bp *tcpA* (of the ElTor biotype) amplicons, indicating that the O1

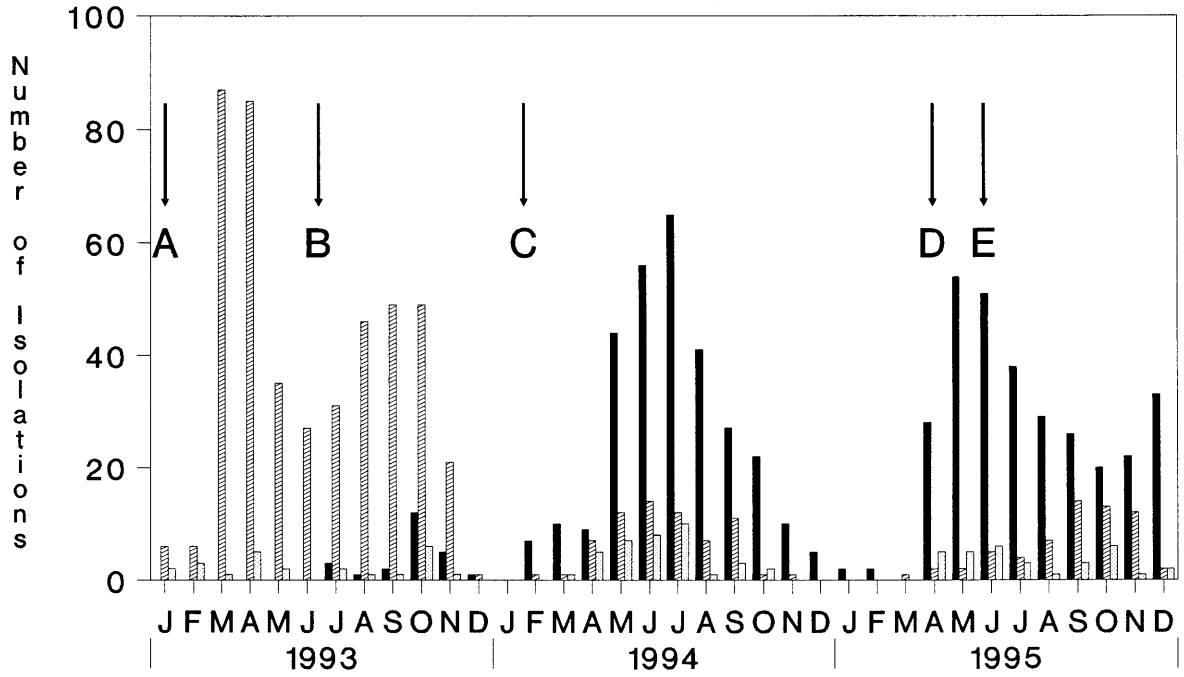

FIG. 1. Monthly isolation of various serogroups of *V. cholerae* from patients hospitalized because of acute secretory diarrhea at the Infectious Diseases Hospital, Calcutta, from January 1993 to December 1995 (J to D, respectively). Arrows denote the precise month when an unusual event in relation to the traits of *V. cholerae* occurred: A, replacement of *V. cholerae* O1 by *V. cholerae* O139 in January 1993; B, reappearance of *V. cholerae* O1 in July 1993; C, domination of *V. cholerae* O1 and the abrupt appearance of nalidixic acid-resistant strains of *V. cholerae* O1 in February 1994; D, first nontoxigenic strain of *V. cholerae* O139 isolated in April 1995; E, appearance for the first time of cotrimoxazole-susceptible strains of *V. cholerae* O139. ■, O1; ▨, O139; ▧, non-O1 non-O139.

strains belonged to the ElTor biotype. Only 1 of the 40 non-O1 non-O139 strains was positive for the 301-bp *ctxA* amplicon, but the same strain was negative for the *tcpA* amplicon.

To further understand the probable virulence factor associated with infection caused by *V. cholerae* non-O1 non-O139, we examined 77 representative strains of *V. cholerae* non-O1 non-O139 isolated during the study period by tissue culture assay using CHO and HeLa cell lines. There was a progressive increase in the cytotoxic response evoked by culture supernatant of strains of *V. cholerae* non-O1 non-O139 isolated during 1994 and 1995 compared with that by those isolated in 1993 (Table 2). This pattern was consistent in both cell lines used as well as in both the media used. Fewer non-O1 non-O139 strains, as shown in Table 2, caused morphological changes of CHO and HeLa cells consistent with cell elongation.

The patterns of resistance of representative strains of *V. cholerae* O1, O139, and non-O1 non-O139 isolated during the 3 years against commonly used antibiotics are shown in Fig. 2a, b, and c, respectively. Interesting shifts in patterns of resistance between strains of *V. cholerae* belonging to different serogroups and within strains of a serogroup isolated during different time periods were observed. For instance, there was striking increase in resistance of *V. cholerae* O1 strains isolated in 1994 and 1995 to nalidixic acid, ampicillin, and neomycin compared with that of those isolated in 1993. Resistance of *V. cholerae* O1 to chloramphenicol showed an increase in 1994 compared with the level in 1993 and subsequently declined in 1995. A statistical analysis comparing the differences in resistance to each drug of *V. cholerae* O1, O139, and non-O1 non-O139 for each year is shown in Table 3. The remarkable difference in antimicrobial resistance patterns between O1 and O139 serogroups was with nalidixic acid, with the O139 serogroup being mostly susceptible to nalidixic acid. The other interesting event was the appearance, for the first time since its genesis in Calcutta, of cotrimoxazole-susceptible *V. cholerae* O139 strains in June 1995 (Fig. 1).

The resistance pattern of the *V. cholerae* non-O1 non-O139 serogroups (Fig. 2c) differed from those of the O1 and O139 serogroups (Fig. 2a and b), with few strains being resistant to tetracycline, norfloxacin, and ciprofloxacin. The dominant drug resistance patterns of *V. cholerae* O1 in 1993, 1994, and 1995 were CoFzS (25.0%), ACCoFzNNaS (43.5%), and ACoFzNNaS (53.6%), respectively (see Table 2, footnote *a*, for definitions of resistance abbreviations), while the dominant drug resistance patterns for *V. cholerae* O139 in 1993, 1994, and 1995 were ACCoFzS (30%), ACCoFzNS (48.1%), and ACoFzS (49.0%), respectively. A discernible increase in the incidence of multidrug-resistant O1 strains isolated in 1994 and 1995 compared with that of 1993 was observed. In the case of O139 strains, there was a decline in the occurrence of multidrug-resistant strains compared with that of 1993 and 1994, and a similar pattern was found for the non-O1 non-O139 strains.

DISCUSSION

The genesis of *V. cholerae* O139 as the new serogroup associated with cholera (20) and its probable evolution as a result of horizontal gene transfer between non-O1 and O1 strains (3) has led to a heightened interest in the *V. cholerae* non-O1 serogroups. Clinically, apart from the O139 serogroup, the non-O1 serogroups continue to be of negligible significance since these strains are associated with only a low percentage of patients hospitalized because of acute secretory diarrhea, as evident from this and from previous studies conducted in Calcutta and elsewhere (17, 19, 24). However, recent trends indicate that the non-O1 non-O139 strains evidently play an im-

TABLE 2. Serogroups, tissue culture activities, and antibiograms of *V. cholerae* non-O1 non-O139 strains isolated from hospitalized patients with acute secretory diarrhea during the study period[a]

Strain no.	Date of isolation (day.mo.yr)	Sero-group[b]	Tissue culture activity in:				Antibiogram
			CHO cells		HeLa cells		
			TSB-YE	AKI	TSB-YE	AKI	
SG33	2.2.93	O39	Ce	Nc	Nc	Nc	ND
SG34	10.2.93	O56	Nc	Cr	Nc	Cr	ND
SG36[c]	19.2.93	**O74**	Ce	Nc	Ce	Ce	ND
SG38	23.2.93	O74	Ce	Nc	Ce	Ce	ND
SG43	1.3.93	O32	Nc	Cr	Nc	Nc	ND
SG89	15.3.93	O7	Ce	Nc	Nc	Nc	ND
CO47	1.4.93	OUT	Nc	Nc	Nc	Nc	ACoFzNaS
CO81	7.4.93	O145	Nc	Nc	Ce	Ce	ACoFzNST
CO95	13.4.93	O5	Nc	Nc	Nc	Ce	AFz
CO98	13.4.93	O36	Nc	Nc	Nc	Nc	ACoFzNS
CO130	16.4.93	O37	ND	ND	ND	ND	ACoFzNS
CO170	24.5.93	OUT	Ce	Nc	Nc	Nc	AFzNS
CO216	23.6.93	**O54**	Nc	Nc	Nc	Nc	AFz
CO219	19.7.93	ND	Nc	Cr	Nc	Cr	AFzNS
CO236	28.7.93	**O43**	Nc	Nc	Nc	Nc	AFzNS
CO248	5.8.93	**O53**	Ce	Nc	Nc	Nc	ACoFzNS
CO325	27.9.93	**O5**	Ce	Nc	Ce	Nc	AFzNS
CO351	4.10.93	**O10**	Ce	Nc	Nc	Nc	AFzNST
CO375	11.10.93	**O6**	Nc	Nc	Nc	Nc	AFzNS
NT638/1	14.10.93	**O34**	Nc	Cr	Nc	Cr	AFzN
CO395	15.10.93	**OUT**	Ce	Nc	Nc	Nc	AFzNNx
CO399B	18.10.93	**O134**	Nc	Cr	Nc	Cr	ACoCfFzNNaS
CO400	18.10.93	**O149**	Nc	Nc	Nc	Nc	AFzN
CO421	3.11.93	**O54**	Nc	Nc	Nc	Nc	AFzN
CO456	28.3.94	**O27**	ND	ND	ND	ND	ND
CO464	4.4.94	**O26**	ND	ND	ND	ND	ND
CO469	7.4.94	**O5**	ND	ND	ND	ND	ND
CO470	11.4.94	**O23**	ND	ND	ND	ND	ND
CO476	19.4.94	**O37**	ND	ND	ND	ND	ND
CO484	28.4.94	**O5**	ND	ND	ND	ND	ND
CO491	6.5.94	**O65**	ND	ND	ND	ND	ND
CO498	10.5.94	**O26**	ND	ND	ND	ND	ND
CO507	13.5.94	**O9**	ND	ND	ND	ND	ND
CO520	18.5.94	**O11**	ND	ND	ND	ND	ND
CO536	26.5.94	**O18**	ND	ND	ND	ND	ND
CO545	31.5.94	**O5**	Ct	Ct	Ct	Ct	ACCoFzNS
CO546	31.5.94	**O10**	Ct	Cr	Nc	Ce	ACCoFzNS
CO560	8.6.94	**O27**	Ct	Ct	Ct	Cr	AFzN
CO567	13.6.94	**O7**	Ct	Ct	Ct	Cr	AfzNS
CO568	13.6.94	**O56**	Ct	Ct	Ct	Ct	AFzT
CO599	27.6.94	**O2**	Ct	Nc	Ct	Ct	ACoFzNaST
CO603B	27.6.94	O108	Ct	Nc	Ct	Ct	ACCoFzNNaS
CO604	27.6.94	**O10**	Ct	Ct	Ct	Ct	AFzNST
CO611B	28.6.94	**O2**	Ct	Nc	Ct	Nc	CoFzNaT
CO621B	30.6.94	**O44**	Ct	Cr	Ct	Cr	ACCoFzNS
CO625	1.7.94	**O110**	Ct	Ct	Ct	Cr	ACCoFzNS
CO627B	1.7.94	**O8**	Ct	Cr	Ce	Cr	ACCoFzNS
CO638	5.7.94	**O6**	Ct	Ct	Ct	Cr	AFzNS
CO639	5.7.94	**O11**	Ct	Ct	Ct	Cr	AFzN
CO651	11.7.94	**O5**	Ct	Ct	Ct	Ct	FzNa
CO668	13.7.94	**OUT**	Ce	Ct	Ce	Ct	ACCoFzNS
CO672	14.7.94	**OUT**	Ct	Cr	Ct	Cr	ACCoFzNS
CO679	19.7.94	**O5**	Ct	Nc	Ct	Ce	FzN
CO684	21.7.94	**O2**	Ct	Nc	Ct	Nc	CCoN
CO702	28.7.94	**O54**	Ct	Ct	Ct	Ct	AFzT
CO742	26.8.94	**O41**	Ct	Cr	Ct	Cr	AFzNS
CO775	13.9.94	**O37**	Ce	Nc	Ce	Cr	AFzNNa
CO794	27.9.94	**O14**	Nc	Nc	Nc	Nc	AFzNS
CO795	27.9.94	**O9**	Ct	Ct	Ce	Ct	ACCoFzNS
CO809	18.10.94	**O7**	Ct	Ct	Ct	Cr	AFzNS
CO818	25.10.94	**O43**	Ct	Ct	Ct	Ct	AFzNS
CO844	3.4.95	**O5**	Ct	Ct	Ct	Ct	AFzNS
CO845	4.4.95	**O8**	Ct	Ct	Ct	Ct	AFzNS
CO848B	5.4.95	**O26**	Ct	Nc	Ct	Ct	ACoFzNa
CO861	25.4.95	**O54**	Ct	Ct	Ct	Ct	AFzNaS
CO872	27.4.95	**O13**	Ct	Ct	Ct	Ct	AFzNS
CO883	3.5.95	**O8**	Ct	Cr	Ct	Cr	AFzNS
CO890	5.5.95	**O110**	Ct	Ct	Ct	Ct	ACCoFzNS
CO923	24.5.95	**O8**	Ct	Ct	Ct	Ct	AFzNS
CO926	26.5.95	**O10**	Ce	Nc	Ce	Nc	ACCoFzNS
CO932	30.5.95	**O14**	Ct	Ct	Ct	Ct	AFzNS
CO942	5.6.95	**O71**	Ct	Ct	Ce	Ct	AFzNT
CO944	5.6.95	**O45**	Ct	Ct	Ce	Nc	AFz
CO963	14.6.95	**O183**	Ct	Ct	Ct	Ct	ACFzT
CO984	27.6.95	**O12**	Ct	Ct	Ct	Cr	AFz
CO987	27.6.95	**O9**	Cr	Cr	Cr	Ct	ANa
CO990	28.6.95	**O174**	Nc	Nc	Nc	Ce	FzNaS
CO1007	6.7.95	O178	Nc	Nc	Ct	Ct	ND
CO1035	25.7.95	O128	Ct	Ct	Ct	Nc	ACoCfFzNT
CO1039	26.7.95	ND	ND	ND	ND	ND	ND
CO1070	25.8.95	O39	Ct	Ct	Ct	Ct	AFzNS
CO1098	14.9.95	O4	ND	ND	ND	ND	ND
AM2	15.9.95	O9	Nc	Nc	Ct	Nc	ACfCoFzNxT
AM25	28.9.95	O39	Nc	Nc	Ct	Nc	AFzNS
AM29	9.10.95	O6	ND	ND	ND	ND	ND
AM33	10.10.95	O39	Ct	Ct	Nc	Ct	AFz
AM37	11.10.95	O43	Ct	Ct	Ct	Ct	ND
AS10	20.10.95	O5	Ct	Ct	Ct	Ct	ACoFz
AS12/1	21.10.95	O10	Ct	Ct	Ct	Ct	AFzNST
AS15	23.10.95	O9	ND	ND	ND	ND	ACoFzGNNaS
AS16	24.10.95	O144	Ct	Ct	Ct	Ct	ACoFzNST
AS50	7.12.95	ND	Ct	Ct	Ct	Nc	ACoFzNxS
AS58	21.12.95	ND	Nc	Ct	Ct	Nc	FzNS

[a] Abbreviations: Nc, no change; Ct, cytotoxic; Ce, cell elongation; Cr, cell rounding; ND, not done; OUT, O untypeable; TSB-YE, tryptic soy broth with yeast extract; A, ampicillin; C, chloramphenicol; Co, cotrimoxazole; Cf, ciprofloxacin; Fz, furazolidone; G, gentamicin; N, neomycin; Na, nalidixic acid; Nf, norfloxacin; S, streptomycin; T, tetracycline.

[b] Serogroups in boldface were examined by DNA probes specific for *ctxA*, *zot*, and *ace*. Underlined serogroups were examined by multiplex PCR specific for *ctxA*, *tcpA* (ElTor), and *tcpA* (classical).

[c] Strain SG36 was the only non-O1 non-O139 serogroup strain in this study which hybridized with the DNA probes specific for *ctxA*, *zot*, and *ace* and was positive for the 301-bp *ctxA* amplicon but negative for the *tcpA* amplicon in the multiplex PCR.

portant role in the shifting dynamics of *V. cholerae* and in the evolution of new strains. This was the rationale to include the non-O1 non-O139 serogroups in our surveillance. In previous studies (22, 26), we monitored the incidence of non-O1 non-O139 serogroups among hospitalized patients and also examined the potential of these strains to produce cholera toxin, which fortuitously led to the discovery of the O139 serogroup (25). In this study, we performed a more comprehensive surveillance and also examined the non-O1 non-O139 strains for a variety of other virulence-associated genes.

After a brief period of displacement, *V. cholerae* belonging to the O1 serogroup reappeared rapidly and became the dominant serogroup causing cholera in Calcutta from 1994 onwards. Although phenotypically very similar (18), the O1 strains of *V. cholerae* which reappeared after being temporarily displaced by the O139 serogroup in Calcutta were quite dissimilar to the O1 strains which prevailed prior to the genesis of O139 when examined by pulsed-field gel electrophoresis (41). In this study, we compared the O1 and O139 strains isolated in the past 3 years but did not see any remarkable variations in

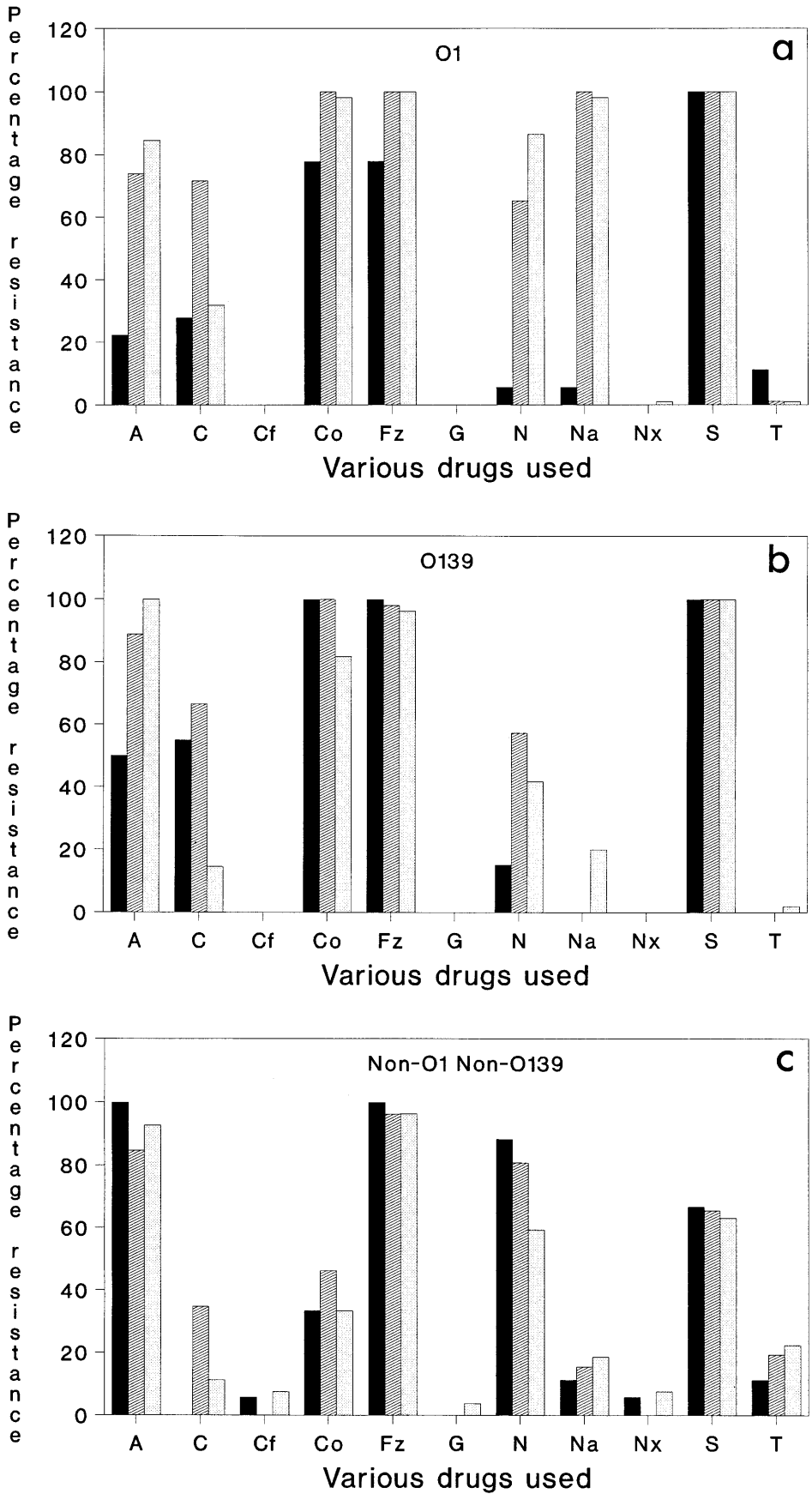

FIG. 2. Antibiotic resistance patterns of *V. cholerae* O1 (a), O139 (b), and non-O1 non-O139 (c) to commonly used antibiotics. Abbreviations are explained in Table 2, footnote *a*. ■, 1993; ▨, 1994; ▤, 1995.

TABLE 3. Statistical analysis showing significant changes in drug resistance (either increase or decrease) between years among *V. cholerae* O1, O139, and non-O1 non-O139

Antibiotic	Serogroup	*P* value[a]		
		1993 vs 1994	1993 vs 1995	1994 vs 1995
Ampicillin	O1	0.0003*	<0.0001*	0.0619
	O139	<0.0001*	<0.0001*	0.0127*
	Non-O1 non-O139	0.1101	0.3545	0.5828
Chloramphenicol	O1	0.0004*	0.7327	<0.0001**
	O139	0.1785	<0.0001**	<0.0001**
	Non-O1 non-O139	0.0044*	0.2061	<0.0429**
Cotrimoxazole	O1	0.0005*	0.0035*	0.2953
	O139	NC	<0.0001**	0.0007**
	Non-O1 non-O139	0.4005	1.000	0.3447
Furazolidone	O1	0.0005*	0.0003*	NC
	O139	0.4029	0.1642	0.5069
	Non-O1 non-O139	0.5909	0.6000	0.7453
Neomycin	O1	<0.0001*	<0.0001*	0.0004*
	O139	<0.0001*	0.0005*	0.1055
	Non-O1 non-O139	0.3879	0.0698	0.0912
Nalidixic acid	O1	<0.0001*	<0.0001*	0.2953
	O139	NC	<0.0001*	0.0016*
	Non-O1 non-O139	0.5244	0.4092	0.5252
Tetracycline	O1	0.0690	0.0516**	0.7050
	O139	NC	0.4074	0.5046
	Non-O1 non-O139	0.3879	0.2424	0.7903

[a] *, statistically significant increase in resistance to the antibiotic in question between the years of comparison; **, statistically significant decrease in resistance to the antibiotic in question between years of comparison; NC, not comparable data.

phenotypic traits and in virulence-associated genes with the exception of rapid changes in antibiotic resistance patterns.

Comparison of the O1 strains isolated between the 3 years revealed interesting fluctuations in the patterns of resistance to various antibiotics. For instance, there was a striking and abrupt emergence of nalidixic acid-, ampicillin-, and neomycin-resistant strains of *V. cholerae* O1 from 1994 onwards. The pattern of resistance of *V. cholerae* O1 to cotrimoxazole, on the other hand, varied between years. These rapid shifts in antimicrobial resistance are in contrast to earlier decades, when antibiotic resistance among *V. cholerae* O1 strains was not a usual phenomenon and multiple-antibiotic-resistant strains were few and far between (23). However, this pattern of quick shifts is consistent with reports in recent years which indicate an enhanced mobility in genetic elements which confers resistance to antibiotics (18). A multiple-antibiotic resistance plasmid belonging to incompatibility group C has been associated with drug resistance plasmids of *V. cholerae* O1 (7) and O139 (40). It has been predicted that since the multiple-drug resistance plasmid is self-transmissible by conjugation, the incidence of plasmid-carrying strains may increase (40). There were differences in the antibiotic resistance patterns of *V. cholerae* O1 and O139 isolated during the 3 years, with the O139 strains being susceptible, by and large, to nalidixic acid and neomycin compared with the O1 strains. Cotrimoxazole-susceptible strains of *V. cholerae* O139 began appearing from June 1995, representing another shift because since the serogroup's genesis, O139 strains isolated in Calcutta and elsewhere were completely resistant to cotrimoxazole and furazolidone (1, 25). Yamamoto et al. (39) revealed differences in antimicrobial susceptibility patterns between clones of *V. cholerae* isolated from different geographic areas, with the Indian O1 ElTor strains (97%) and Bangladeshi ElTor strains (50%) being highly resistant to streptomycin, sulfamethoxazole, and trimethoprim and moderately resistant to chloramphenicol and furazolidone, which sharply contrasted with the resistance of the O1 Peruvian ElTor and Bangladeshi O1 classical strains.

The most interesting observation in this study was the progressive increase in the production of a factor which was cytotoxic to CHO and HeLa cells by strains belonging to the non-O1 non-O139 serogroups. Among the 1993 strains of *V. cholerae* non-O1 non-O139, cytotoxin production was not observed and most of the strains did not affect the cell lines used. However, among the 1994 and 1995 strains, there was a dramatic increase in the number of non-O1 non-O139 strains producing a cytotoxic effect. We are currently examining these strains to understand the nature of the cytotoxin. By analysis of the sequence variation of the *asd* gene (encodes aspartate semialdehyde dehydrogenase; a housekeeping gene) within *V. cholerae*, Karaolis et al. (11) concluded that the sixth and seventh cholera pandemics were due to independent clones separately derived from environmental, nontoxigenic, non-O1 *V. cholerae* and that among *V. cholerae* strains there is a high level of genetic exchange and a relatively low level of clonality. If this be the case, the incidence of cytotoxic *V. cholerae* non-O1 non-O139 may be an ominous sign and might be a prelude to the genesis of newer strains of *V. cholerae* having hitherto unrecognized toxigenic traits.

Indications to this effect are already coming to light with the recent report on outbreaks of cholera-like disease caused by unusual strains of *V. cholerae*, including nontoxigenic strains of *V. cholerae* O1 which produce a factor that evokes a rapid cell rounding effect when introduced on confluent layer of CHO and HeLa cells (29) and *V. cholerae* non-O1 strains producing the heat-stable enterotoxin (2), and two recent reports in which serogroups such as O10 and O12 have been causes of concern since they were associated with outbreaks of cholera-like diarrhea (5, 28). The precise reasons for such rapid shifts and emergence of newer variants of *V. cholerae* is not certain but might relate to the same factors responsible for the overall resurgence of infectious diseases. Effective surveillance, especially in high-risk areas, could abort some of the damages caused by the emerging variants.

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