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Association of a disease approximating cholera caused by *Vibrio cholerae* of serogroups other than O1 and O139

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SUMMARY

One hundred and six patients suffering from severe dehydrating diarrhoea were studied of whom 36 patients were positive for *Vibrio cholerae*. Out of 36, 15 were positive for *V. cholerae* O1, 10 for *V. cholerae* O139 and 11 for *V. cholerae* non-O1 non-O139. O1 and O139 were positive for the 301-bp *ctxA* amplicon and 471-bp *tcpA* amplicon indicating that the strains possessed toxigenic capability whereas no non-O1 non-O139 strain possessed *ctxA* or *tcpA* genes. Post-admission severity of purging and amount of ORS required were less in the *V. cholerae* non-O1 non-O139 group ($P < 0{\cdot}05$) compared to the *V. cholerae* O1 and O139 groups. It appears from this study that a cholera-like clinical condition can be caused in the absence of CT as exemplified by strains of non-O1 non-O139.

INTRODUCTION

The unprecedented appearance of a novel epidemic strain of *V. cholerae* non-O1 classified as *V. cholerae* O139 Bengal causing major epidemics of cholera in the Indian subcontinent is of considerable scientific and public health interest [1, 2]. It was a long-held premise that only *V. cholerae* belonging to O1 serogroup is capable of causing epidemic cholera. The outbreaks of severe clinical cholera due to *V. cholerae* O139 [3] alerted us to look for an association of typically severe clinical cholera with *V. cholerae* serogroups other than O1 and O139. This type of monitoring should help us to detect promptly the emergence of newer strains of cholera. In this report, we provide clinical and bacteriological evidence for the occurrence of severe cholera-like disease in association with *V. cholerae* strains not belonging to O1 or O139 serogroups in Calcutta, an area which is endemic for cholera.

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MATERIALS AND METHODS

Clinical procedures

Male patients (for easy collection of stool and urine separately) aged 18–55 years, with a history of acute watery diarrhoea of less than 24 h duration and severe degree of dehydration and a systolic blood pressure of less than 50 mmHg admitted to the Infectious Diseases Hospital between September 1995 and March 1996, were included in the study. A complete history (including pre-admission duration of diarrhoea, fre-

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quency of stool, presence of abdominal cramp, vomiting) was recorded on a predesigned form. All patients were placed on a cholera cot and were rehydrated with intravenous Ringer's lactate solution followed by administration of oral rehydration solution as recommended by WHO. The amount of intravenous and oral rehydration fluids administered, volume of stool, duration of diarrhoea and body weights were monitored till discharge and were recorded. Cessation of diarrhoea was defined as the passage of last liquid stool. Blood samples were obtained from patients for estimation of electrolytes on admission and recovery. Verbal consent was obtained from the close relatives of the patients. The study was ethically cleared by the Institutional Scientific Advisory Committee. All the patients received doxycycline 300 mg capsule as a single dose, after correction of initial dehydration and when vomiting had stopped. Food was provided when the patients asked for it.

Bacteriological procedures

On admission a fresh stool sample was collected from each patient using a sterile rectal catheter and was processed within 1 h of arrival at the laboratory for the isolation of *V. cholerae* using standard techniques [4]. A multitest medium was used for presumptive identification of *V. cholerae* [5]. All isolates were subsequently examined for the oxidase reaction and the identity of *V. cholerae* 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]. *V. cholerae* 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 Infectious Diseases, Tokyo, Japan [7]. Antimicrobial susceptibility testing of the isolated strains were done using standard techniques [8].

Detection of virulence genes by multiplex PCR

The *V. cholerae* strains isolated in this study were examined by multiplex PCR to determine the presence of *ctxA* (encoding the enzymatic subunit of cholera toxin) and *tcpA* (encoding the major structural subunit of the toxin co-regulated pilus) genes using published methods [9]. Three pairs of primers as described previously [9] were used to detect the following: a 301-bp sequence of *ctxA* [10], a 471-bp fragment of the E1Tor variant of the *tcpA* sequence and a 671-bp sequence of the classical variant of *tcpA* [11].

The following were added to each 100 μl of PCR mixture: 10 μl of Mg-free 10 $\times$ 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 the 2 mM of 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 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, NSW, 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 E1Tor Ogawa) and 569B (*V. cholerae* O1 classical Inaba) were run as controls. Amplified products from PCR were electrophoresed on 2·5% agarose gels and were stained with ethidium bromide. A 1-kb molecular size ladder (Gibco BRL, Gaithersburg, MD) was run with each gel.

Tissue culture assay

Culture supernatant of strains of *V. cholerae* non-O1, non-O139 were examined by tissue culture assay using CHO cells for CT or CT-like toxins identified by cell elongation. The strains were grown in tryptic soy broth (Difco, Detroit, MI) 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) [12] with shaking for 18 h. The culture supernatant obtained by centrifugation at 4 °C was made cell free by passing through a 0·22 μm-pore-size filter unit (Millex-GS; Millipore, Bedford, MA) and collected in sterile test-tubes which were

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kept at 4 °C until used. CHO cells were grown as monolayers in Dulbecco's minimum essential medium (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan), supplemented with 10 % (vol/vol) horse serum (Gibco Laboratories, Grand Island, NY). 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 cells grown for 3–4 days was removed from the tissue culture flasks, and 200 μl of the cell suspension (*c.* $6{\cdot}4 \times 10^3$ 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 cells were recorded at 24 h. For negative control, wells received the uninoculated culture medium, and pure cholera toxin (Sigma, St Louis, MO) was used as the positive control.

RESULTS

A total of 106 patients admitted to the I.D. Hospital were examined of whom 36 patients were positive for *V. cholerae* and these patients formed the basis of this report. Of these 36 patients, 15 were positive for *V. cholerae* O1, 10 for *V. cholerae* O139 and 11 stool cultures grew *V. cholerae* not belonging to serogroups O1 or O139 and therefore classified as non-O1 non-O139.

All the 11 strains which belonged to the non-O1 non-O139 category could be further serogrouped, with 3 strains belonging to the O39 serogroup, 2 each to O144 and O6 serogroups and 1 each to O8, O9, O19 and O58 serogroup. All the O1 and O139 strains isolated in this study were positive for the 301-bp *ctxA* amplicon and 471-bp *tcpA* amplicon, indicating that the strains possessed toxigenic capability and the surface organelle required for intestinal colonization was intact. However, none of the non-O1 non-O139 strains isolated in this study possessed the *ctxA* or *tcpA* genes, indicating that the strains did not possess the genetic potential to produce CT. Nine non-O1 non-O139 strains were examined by tissue culture assay using CHO cells to determine if any of the strains were capable of increasing intracellular cyclic AMP levels, which causes elongation of CHO cells. None of the nine non-O1 non-O139 strains produced a factor which was capable of cell elongation, indicating that none of the strains produced CT (confirming the PCR results) or a CT-like toxin.

Admission characteristics were similar in the three groups (Table 1). They all developed severe dehydration with hypovolaemic shock after a short period of diarrhoea and vomiting (mean preadmission duration of less than 12 h in all three groups). There was a trend towards higher rate of abdominal cramp in the group with *V. cholerae* O139 (relative risk = 1·73, 95 % C.I. 0·83 and 3·60, $P = 0{\cdot}17$) compared to the other two groups. All three groups required large and similar amounts of intravenous fluids for correction of initial dehydration and hypovolaemic shock. However, post-admission severity of purging was less in the *V. cholerae* non O1, non O139 group ($P < 0{\cdot}05$) compared to the *V. cholerae* O1 and O139 groups. Consequently, they also needed smaller volumes of oral rehydration solution ($P < 0{\cdot}05$). Both the purging rate and consumption were closely similar in the groups with *V. cholerae* O1 and O139.

Antimicrobial susceptibility pattern showed that all isolated strains of O1 and O139 were resistant to ampicillin, chloramphenicol, cotrimoxazole and furazolidone, and were susceptible to neomycin, nalidixic acid, tetracycline and norfloxacin, while non-O1 non-O139 strains were uniformly susceptible to all the above-mentioned drugs. All the patients were successfully rehydrated with intravenous and oral fluids. No patient required any unscheduled i.v. fluid. There was no mortality in the study patients. The serum electrolyte profiles in the three groups were similar on recovery.

DISCUSSION

V. cholerae is currently classified into 155 'O' serogroups [7, 13]. Until recently the disease cholera was caused by *V. cholerae* O1 serogroup. The non-O1 serogroups were recognized as causes of sporadic and localized outbreaks of diarrhoeal illness [14]. The dramatic and unprecedented emergence of a new epidemic strain of *V. cholerae* non-O1 now designated as *V. cholerae* O139 and recent genetic studies indicating the horizontal transfer of polysaccharide genes between O1 and non-O1 strains [15] have led to renewed interest in other *V. cholerae* non-O1 serogroups. Further, recent studies with nucleotide sequence of *asd* genes of 45 strains of *V. cholerae* suggest that both classical and EITor biotypes of *V. cholerae* O1 independently evolved from non-toxigenic environmental strains of *V. cholerae* non-O1 [16], indicating the feasibility of similar natural events occurring in future. In both *V. cholerae* O139 and *V. cholerae* O1, the virulence genes encoding cholera

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Table 1. *Admission features, course of illness and serum electrolyte values of patients**

	V. cholerae			
	O139 ($n = 10$)	O1 ($n = 15$)	non-O1 non-O139 ($n = 11$)	P†
Admission				
Age (years)	32·5 ± 11·5	30·3 ± 8·3	28·6 ± 9·1	
Pre-admission duration (h)	11·8 ± 5·4	11·7 ± 6·2	8·4 ± 4·8	
Number of purges	10·3 ± 5·1	10·9 ± 4·8	8·7 ± 4·8	
Proportion vomited [% (n)]	100 (10)	93·3 (14)	100 (11)	
Abdominal cramp [% (n)]	60 (6)	33·3 (5)	36·4 (4)	
Clinical course				
Total iv required (L)	7·2 ± 1·2	7·7 ± 0·9	7·3 ± 0·9	N.S.
Total stool (L)	5·9 ± 1·8	6·4 ± 2·1	3·9 ± 1·5	0·005
Total ORS consumed (L)	9·7 ± 3·4	10·1 ± 3·3	5·7 ± 0·9	0·003
Diarrhoea duration (h)	50·9 ± 9·5	49·0 ± 10·9	35·9 ± 10·1	0·003
Serum electrolytes (mmol/l)				
Admission				
Na^+	140·1 ± 5·2	140·4 ± 2·9	140·9 ± 4·6	
K^+	5·1 ± 0·7	4·7 ± 0·5	5·0 ± 0·5	
Cl^-	106·3 ± 2·1	106·7 ± 2·1	109·4 ± 1·1	
Total CO_2	12·1 ± 1·8	11·9 ± 1·7	12·5 ± 1·6	
Recovery				
Na^+	137·3 ± 4·5	134·8 ± 2·0	134·6 ± 3·5	
K^+	4·3 ± 0·4	4·1 ± 0·5	4·4 ± 0·5	
Cl^-	105·6 ± 4·2	103·6 ± 3·9	103·2 ± 2·3	
Total CO_2	20·1 ± 1·9	19·9 ± 2·6	21·1 ± 2·9	

* All figures are expressed as mean ± S.D. or [% (no.)].

† Clinical course is closely similar in the groups with *V. cholerae* O1 and *V. cholerae* O139; they were pooled for comparison with *V. cholerae* non-O1, non-O139.

toxin, zona occludens toxin, accessory cholera enterotoxin, and core encoded pilin are present in a 4·5 kb 'virulence cassette' region of the chromosome [17].

The hallmark of clinical cholera is the development of severe dehydration with signs of hypovolaemic shock in adults and other children after a short duration of watery diarrhoea and vomiting usually requiring resuscitation with i.v. fluids. The patients associated with *V. cholerae* non-O1 non-O139 in this study were indistinguishable in their presentation on admission from those associated with *V. cholerae* O1 and *V. cholerae* O139, although the subsequent course of illness was milder as evidenced by less severe purging and shorter duration of diarrhoea.

Resistance to a broad spectrum of antimicrobial agents has recently been identified with increasing frequency in *V. cholerae* strains around the world. This trend seems to be more pronounced after the genesis of the O139 serogroup of *V. cholerae* which was resistant to trimethoprim-sulphamethoxazole, streptomycin, furazolidone and the vibrio static agent O/129 [18, 19]. In the present study, all strains of *V. cholerae* O1 and O139 were resistant to cotrimoxazole and furazolidone, both of which are used in antimicrobial therapy against cholera when indicated specially among children. Tetracycline is generally contraindicated in children less than 8 years old because it causes staining of permanent teeth [20]. Therefore, resistance to furazolidone of the strains of *V. cholerae* O1 and O139 isolated in this study has complicated antimicrobial therapy in cholera and this is an alarming trend.

This study documents the ability of serogroups of *V. cholerae* other than O1 and O139 to cause a clinical condition which closely approximates cholera and which necessitates the hospitalization of the patient. How exactly these strains potentiate the cholera-like clinical condition is enigmatic since the PCR studies reveal that none of the non-O1 non-O139 strains isolated in this study has the virulence genes encoding for CT production and for surface organelle that are required for intestinal colonization. The clinical state of cholera is principally attributed to CT which activates adenylate cyclase resulting in increased levels

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of cyclic AMP leading to hypersecretion of salt and water [21]. However, this is a situation not without parallel. *V. cholerae* strains belonging to the O1 serogroup which do not produce CT and which lack the toxin structural genes also known as non-toxigenic (NT) strains have been associated with a cholera-like disease. In fact, we have recently documented a cluster of cases associated with an outbreak of cholera due to the NT *V. cholerae* O1 serogroup Inaba biotype E1Tor [22]. Therefore, it appears that there are instances when a cholera-like clinical condition can be caused in the absence of CT as exemplified by NT *V. cholerae* and by strains of non-O1 non-O139 isolated in this study.

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