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Virulence patterns of *Vibrio cholerae* non-O1 strains isolated from hospitalised patients with acute diarrhoea in Calcutta, India

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Summary. A collection of 28 strains of *Vibrio cholerae* non-O1 isolated during a 3-year period (1989–1991) from hospitalised patients with acute diarrhoea in Calcutta, India, were examined with regard to virulence-associated factors. Of the 28 isolates (each representing a case), 18 were isolated as the sole infecting agent; the remaining 10 were recovered as co-cultures from cases infected with *V. cholerae* O1. Of the strains isolated in this study, 82% could be serotyped, with serovars O5 (32·1%), O11 and O34 (14·3% each) predominant. Serovars O7, O14, O34, O39 and O97 were associated exclusively with sole infections. Two strains of *V. cholerae* non-O1 produced anti-cholera toxin IgG-absorbable cholera toxin (CT). Both CT-producing *V. cholerae* non-O1 strains hybridised with the DNA probe specific for the zonula occludens toxin (ZOT) but none of the remaining 26 strains hybridised with the ZOT probe. The majority of the strains were cytotoxic for CHO, HeLa and Vero cells, with end-point titres of 4–512. Fewer strains produced a cytotonic effect, with end-point titres of 2–16. Of the 28 strains of *V. cholerae* non-O1 examined, 75%, 75%, 25% and 14·3% produced haemolysin that was active against erythrocytes of rabbit, sheep (Eltor haemolysin), chicken and man, respectively. Strains that produced a haemolysin active against both rabbit and sheep erythrocytes were dominant (35·7%). Ten (35·7%) of the 28 strains examined showed cell-associated haemagglutinating activity on human blood. Of the 10 strains, nine were isolated as sole pathogen and only one strain was associated with mixed infection. Three distinct patterns of inhibition by sugars were detected; inhibition of haemagglutination by mannose 1% but not by fucose and galactose 1% was the dominant haemagglutination inhibition pattern. Six different virulence phenotypes were encountered among strains of *V. cholerae* non-O1 in this study. The prominent phenotype, which was associated commonly with isolates from patients solely infected by *V. cholerae* non-O1, was exhibited by strains that produced the Eltor haemolysin, a cytotoxin and a cell-associated haemagglutinin. The production of cell-associated haemagglutinin appeared to be the only distinctive phenotype that could distinguish between isolates from patients solely infected with *V. cholerae* non-O1 and those associated with mixed infections. From this study, it is apparent that the virulence of *V. cholerae* non-O1 is multifactorial and mediated by several traits functioning in an integrated fashion. The clinical significance of *V. cholerae* non-O1 must be assessed in its totality; the presence of a single factor should not be construed as the cause of enteropathogenicity.

Introduction

Whereas the aetiological role of *Vibrio cholerae* non-O1 (previously referred to as the non-agglutinating vibrios or NAG vibrios) as the causative agent of gastroenteritis is unequivocal, the virulence factor(s) responsible for eliciting the disease is obscure. Apart from gastrointestinal infections,[1] *V. cholerae* non-O1 has occasionally been implicated in various infections in man including cellulitis,[2] wound infections[3] and septicaemia.[4] This range of symptoms suggests a complex mechanism of pathogenesis and strains are likely to possess various virulence factors in

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different combinations. In recent years, several extracellular products have been documented as playing an important role in the disease process, including a heat-stable enterotoxin (NAG-ST) consisting of 17 amino acids, which bears close similarity to the heat-stable enterotoxins of enterotoxigenic *Escherichia coli*,[5–8] a heat-labile enterotoxin related to cholera toxin,[9–13] a heat-labile Eltor-like haemolysin[14,15] that induces fluid accumulation in the infant mouse,[16] a thermostable direct haemolysin similar to that of *V. parahaemolyticus*,[6,17] a Shiga-like toxin[18] and haemagglutinins.[19,20] However, attempts to correlate the production of the enterotoxin, cytotoxin, haemolysin and haemagglutinins with enteropathogenicity have not shown consistent results and no single virulence factor has been shown to predict whether a particular strain is pathogenic or not.

A recent volunteer study in man demonstrated that in the presence of adequate colonisation factor(s), a NAG-ST-producing strain of *V. cholerae* non-O1 caused diarrhoea of severity similar to that seen in cholera.[21] However, the frequency of occurrence of NAG-ST among strains of *V. cholerae* non-O1 is low[22] indicating that other, hitherto unknown enterotoxigenic factor(s) may be involved in the causation of acute secretory diarrhoea. This study was initiated to further our understanding of the virulence factor(s) associated with *V. cholerae* non-O1 strains isolated either as the sole infecting pathogen or as co-cultures in infections of polymicrobial aetiology from hospitalised patients with acute diarrhoea in Calcutta.

Materials and methods

Specimens

During the period July 1989–Nov. 1991, 591 patients hospitalised with acute diarrhoea and admitted to the Infectious Disease Hospital, Calcutta, were investigated. Stool specimens were collected immediately on admission, in sterile MacCartney bottles with sterile catheters. Soon after collection, stool specimens were transported to the laboratory and examined within 2 h for *V. cholerae* and for other common toxigenic enteropathogens such as enterotoxigenic *E. coli* (ETEC), *Shigella*, *Salmonella* and *Campylobacter* spp. by standard published techniques.[23]

Isolation and phenotypic characterisation

Plates of Thiosulphate-Citrate-Bile Salts-Sucrose Agar (TCBS; Eiken, Japan) were streaked with two or three loopfuls of stool specimens for selective isolation of *V. cholerae*. After incubation overnight at 37°C, 10–15 typical colonies from each stool sample showing growth on the selective agar were inoculated on to a multi-test medium,[24] which combines the principles of triple sugar-iron agar and Kligler's iron agar, to facilitate rapid presumptive identification of *V. cholerae*. In the multi-test medium, fermentation of glucose and inositol or rhamnose, arginine dehydrolation, indole production, H_2S production from sodium thiosulphate indicated by ferric ammonium citrate, and gas production can be recorded from a single tube. The rationale for examining 10–15 isolates/case was to explore whether *V. cholerae* non-O1 occurred as co-cultures with *V. cholerae* O1 from patients with cholera and, if so, how frequently. Strains that showed typical alkaline slant-acid butt reaction on the multi-test medium were examined for the oxidase reaction and slide agglutination was performed with polyvalent O1 and monospecific Ogawa-Inaba antisera prepared at the National Institute of Cholera and Enteric Diseases, Calcutta, India. Strains that did not agglutinate with the O1 antisera were characterised biochemically with the API 20E system (Analytab products, Plainview, NY, USA). Antibiotic susceptibility testing was performed by the disk diffusion technique.[23]

Serogrouping

The identities of the *V. cholerae* that did not agglutinate with the O1 antisera were re-confirmed and the serovars of strains were determined by the somatic O antigen serogrouping scheme[25] at the National Institute of Health, Tokyo, Japan.

Bacterial preparations

All the *V. cholerae* non-O1 strains were maintained in nutrient agar as stabs at room temperature until required. All media used in this study except TCBS were from Difco Laboratories, Michigan, USA. The broth media used for assessing production of cholera toxin-like enterotoxin was Casamino-Acid Yeast Extract (CAYE) medium supplemented with 90 μg/ml lincomycin (Sigma).[10] Trypticase soy broth containing yeast extract 0·6% was used for the tissue culture and haemolysin assays. The test strains were cultivated in the above media at 37°C for 24 h in a rotatory shaker (Firstek Scientific, USA) set at *c.* 200 rpm. After centrifugation (5000 rpm for 20 min at 4°C), the culture supernate was filtered (0·2-μm disposable filter; Sigma) and the cell-free culture filtrate was used for the various assays.

Assay for cholera toxin-like enterotoxin and thermostable direct haemolysin by bead-ELISA

All the culture filtrates of the *V. cholerae* non-O1 strains were examined for the presence of cholera toxin (CT)-like enterotoxin and for thermostable direct haemolysin in a highly sensitive bead-ELISA.[26–28] Various dilutions of purified CT (Sigma) or the culture filtrate of strain NICED 10 of Kanagawa phenomenon-positive *V. parahaemolyticus* (positive

controls) and uninoculated medium (negative control) were run concurrently whenever a batch of the culture filtrate of the test strains were assayed by the bead-ELISA.

Absorption assay with anti-CT IgG

An absorption assay by bead-ELISA was performed to determine whether CT present in culture supernates of strains yielding a positive result by the CT bead-ELISA could be absorbed with anti-CT IgG. In this assay, culture supernate of the CT-producing strain was pre-incubated at 37°C for 1 h with 10 μg of rabbit anti-CT IgG/ml. From earlier experiments it was determined that 10 μg of anti-CT IgG/ml can completely absorb 500 ng of pure CT/ml. Subsequently, the coated beads were introduced into 0·5 ml of the above-described incubation and the bead-ELISA was performed as described previously.[27]

Cytotoxin and cytotonic toxin assays

CHO and HeLa cells were grown as monolayers in Dulbecco's Minimum Essential Medium (Nissui Pharmaceutical Co. Ltd, Japan) supplemented with horse serum (Gibco Laboratories, USA) 10% v/v and Vero cells were grown as monolayers in Minimum Essential Medium (Gibco Laboratories) supplemented with horse serum 10%. Cultures were maintained in tissue-culture flasks (25 cm^2) at 37°C in a humidified CO_2 5% atmosphere. Samples (50 μl) of the culture filtrate of the test strains, serially diluted in Hanks's Balanced Salts Solution (Gibco Laboratories), were added to each well of 96-well tissue culture plates. A confluent monolayer of CHO, HeLa or Vero cells grown for 3–4 days was removed from the tissue culture flasks and 200 μl of the cell suspension (*c.* 4×10^3 cells) was added to each of the 96-well plates and incubated as described above. Cytotonic and cytotoxic changes were recorded at 24 and 48 h. For controls, wells received the uninoculated culture medium (negative control) and pure cholera toxin or the culture filtrate of reference strains of *E. coli* C984 producing Shiga-like toxin I and *E. coli* B1409 producing Shiga-like toxin II as positive controls (by courtesy of Nancy A. Strockbine, Centers for Disease Control, Atlanta, GA, USA).

Haemolysin assay

Haemolytic activity of the *V. cholerae* non-O1 strains with erythrocytes from rabbit, sheep, chicken and man was determined as described previously.[29] Briefly, washed erythrocytes were diluted to a final concentration of 1% in 10 mM phosphate-buffered saline (PBS; pH 7·0) containing NaCl 1·3% and mixed 1 to 1 with the culture filtrate of the test strains. The mixture was centrifuged at 1000 rpm for 5 min. The amount of released haemoglobin in the supernate was measured spectrophotometrically at 540 nm. An optical density of > 0·45 was considered to be a positive result.

DNA probes and colony hybridisation

A recombinant plasmid, pAO111, containing the coding sequence for NAG-ST[30] was used as the source of the DNA probe for the toxin. The digestion of pAO111 with restriction endonucleases, *Eco*RI and *Bam*HI released a 271-bp DNA fragment specific for the NAG-ST gene.[31] Similarly, *Eco*RI fragment (554 bp) of the plasmid, pKTN901 containing the A1 subunit of CT was used to screen all the *V. cholerae* non-O1 strains for the presence of a gene encoding CT.[31] Furthermore, the distribution of the *zot* gene that encodes the zonula occludens toxin (ZOT), was examined with the ZOT DNA probe constructed by Karasawa *et al.*[32] The procedure employed to purify the plasmid and to isolate and purify the DNA fragment has been described previously.[21,31,32] Purified DNA was labelled with ^{32}P by incorporating [α-^{32}P]dATP to a specific activity of 2×10^8–8×10^8 cpm/μg of DNA by nick translation. Radiolabelled probe DNA was purified by chromatography on NACS PREPAC as specified by the manufacturer (Bethesda Research Laboratories, USA). The colony blot was prepared on autoclaved, gridded nitrocellulose filter (Schleicher and Schuell Co.; BA 85/20) and hybridisation was performed under high stringency as described previously.[32]

Determination of cell-associated haemagglutinating activity

Pooled group O human blood obtained from the Nil Ratan Sarkar Hospital blood bank, Calcutta, was stored at 4°C for not more than 1 week before use. When required for assay, blood cells were washed three times in 10 mM PBS (pH 7·4) and a 3% suspension was prepared. Bacterial suspensions from test strains grown on CFA (Colonisation Factor Antigen) agar (casamino acid, Difco, 1%, yeast extract, Difco, 0·15%, magnesium sulphate 0·005%, manganese chloride 0·0005%, Bacto agar 2%, pH 7·4) were made to yield *c.* 10^9 cells/ml.[33] To determine the cell-associated haemagglutinating activity, 20 μl of bacterial suspension was mixed with the same volume of blood suspension on a glass slide at room temperature and rocked gently. Strains were recorded as 2+ if the reaction was immediate and complete, or as 1+ if the reaction was incomplete or not instantaneous but occurred within 5 min. A PBS-blood cell control was always included.

Inhibition of cell-associated haemagglutinating activity

Strains that agglutinated erythrocytes were tested for haemagglutination inhibition by L-fucose, D-ga-

Table I. Phenotypic characters of *V. cholerae* non-O1 isolates from hospitalised cases of acute diarrhoea

Characteric	Percentage positive (n = 28)
Oxidase	100
Ortho-nitro-phenyl-galactosidase	100
Arginine dihydrolase	0
Lysine decarboxylase	100
Ornithine decarboxylase	96·4
Simmons citrate	32·1
Hydrogen sulphide	0
Urease	0
Tryptophane deaminase	0
Indole	100
Acetoin production	89·3
Gelatin hydrolysis	100
Fermentation of:	
glucose	96·4
mannitol	100
inositol	0
sorbitol	0
rhamnose	0
sucrose	100
melibiose	3·6
amygdalin	3·6
arabinose	3·6

Table II. Isolation status of *V. cholerae* non-O1 in relation to serovar

Serovar	Isolation status		Total
	Sole isolate	Mixed infection	
O5	5	4	9
O7	1	0	1
O8	0	1	1
O11	2	2	4
O14	1	0	1
O26	0	1	1
O34	4	0	4
O39	1	0	1
O97	1	0	1
Untypable	3	2	5
Total	18	10	28

lactose or D-mannose. Bacterial suspensions were made in a 1% w/v solution of each sugar in PBS (pH 7·4). Haemagglutination was assessed by performing the test as described above. Inhibition was defined as $\geqslant 50\%$ reduction in agglutination compared to that of the test without the sugar.

Results

During the 3-year period of the study (1989–1991), *V. cholerae* non-O1 was isolated from 28 (4·7%) of the 591 patients hospitalised with acute diarrhoea. Of the 28 isolates (each strain representing a case), 18 were isolated as the sole infecting agent; the remaining 10 were isolated as co-cultures from cases infected with *V. cholerae* O1. The yearly isolation rate of *V. cholerae* non-O1 was 10% (5% each as sole pathogen and in association with mixed infections) in 1989, 9·5% (6·8% as sole and 2·7% as mixed infections) in 1990 and 2·6% (1·9% as sole and 0·72% as mixed infections) in 1991. The phenotypic characteristics of the 28 isolates of *V. cholerae* non-O1 in this study are shown in table I. API profile numbers 5147124 (57·1%) and 5347124 (25%) were predominant.

Of the 28 isolates of *V. cholerae* non-O1, 82% could be serotyped, with serovars O5 (32·1%), O11 and O34 (14·3% each) dominating (table II). Serovars O7, O14, O34, O39 and O97 were associated exclusively with sole infections. The culture supernates of two of the 28 strains of *V. cholerae* non-O1 (V254 and V315-1), both of which were untypable and were isolated as co-cultures with *V. cholerae* O1, yielded a positive result in the CT bead-ELISA. The CT produced by both strains could be absorbed completely by anti-CT IgG. The same two strains that produced CT hybridised with the CT and ZOT DNA probes; the remaining 26 strains isolated in this study did not. None of the 28 strains of *V. cholerae* non-O1 hybridised with the NAG-ST DNA probe.

The effect of culture filtrates of the 28 strains on CHO, HeLa and Vero cells is shown in table III. The majority of isolates produced a cytotoxic response in all the three cell lines, with end-point tires of 4–512. The cytotoxic response with disruption of the monolayer obtained with the *V. cholerae* non-O1 strains could be categorised into two types, one that was associated with complete membrane damage (figure, D) and the other was associated with cell rounding but without membrane damage (figure, E). Strains V59, V160 and V249 produced cytotoxic changes of the latter category in CHO cells; end-point titre of strain V59 was 512. In this category, no elongation of cells was observed just before the end-point titre. Fewer strains produced a cytotonic effect (figure, F); the end-point titres were 2–16. One of the two strains that produced CT (V315-1) showed a cytotonic effect on CHO and HeLa cells; in the other CT-producing strain (V254), the cytotonic effect appeared to be obliterated by a cytotoxic response on CHO and Vero cells without any changes being observed in HeLa cells. Apart from the two CT-producing isolates of *V. cholerae* non-O1, strain V157 showed a cytotonic response with HeLa cells and strains V160 and V249 showed cytotonic responses with HeLa and Vero cells, indicating the existence of cytotonic factors unrelated to CT.

None of the 28 strains produced a haemolysin that cross-reacted with the thermostable direct haemolysin of *V. parahaemolyticus* as determined by the bead-ELISA. However, 85·7% of the strains of *V. cholerae* non-O1 examined in this study haemolysed rabbit, sheep, chicken or human erythrocytes (table VI). Of the 28 strains of *V. cholerae* non-O1 examined, 75%, 75%, 25% and 14·3% produced haemolysin that was

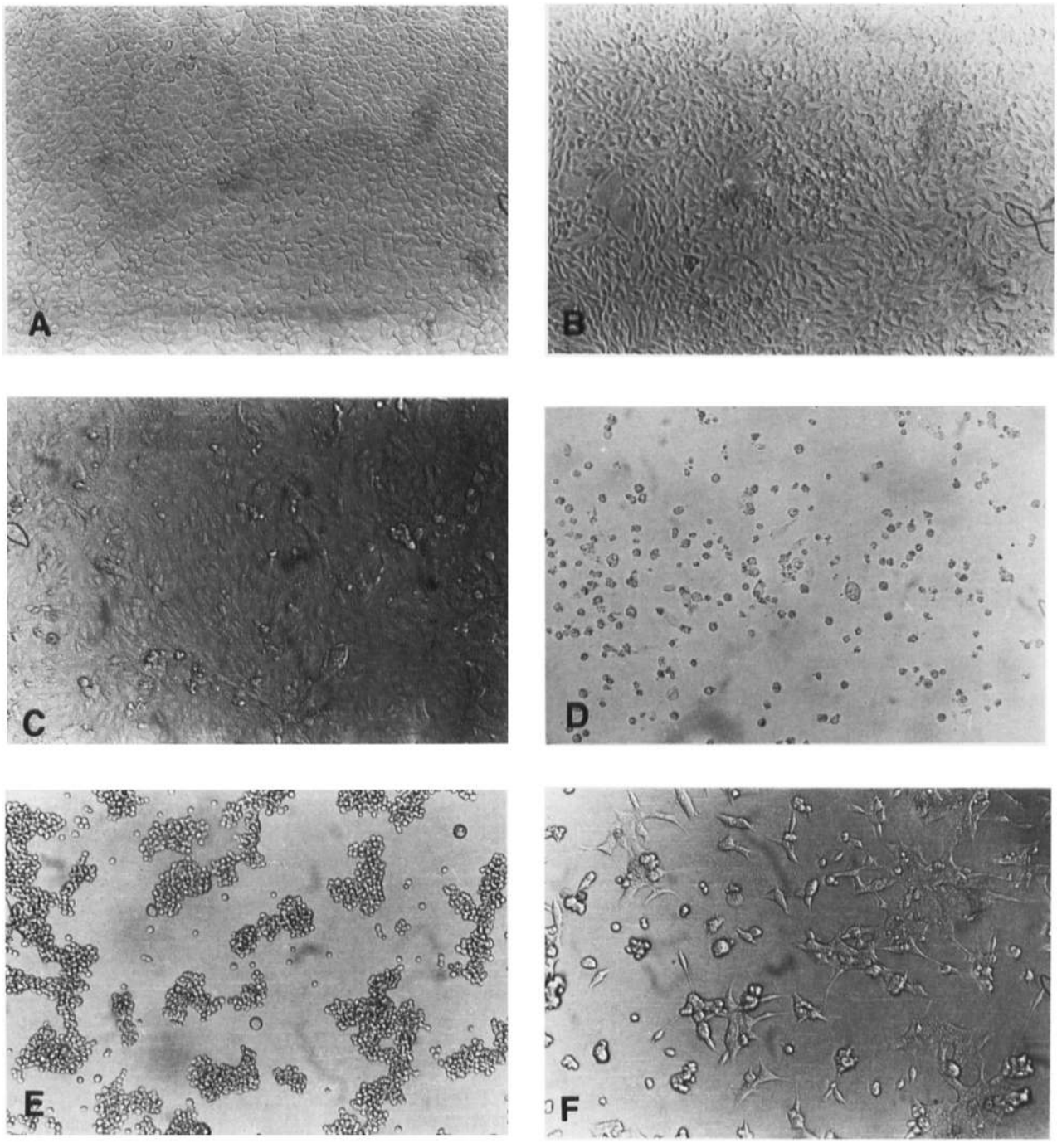

Figure 1. Effects of the culture filtrate of *V. cholerae* non-O1 strains isolated from clinical cases. Normal cells of HeLa (**A**), CHO (**B**) and Vero (**C**) cells with cytotoxic (**D** and **E**) and cytotonic (**F**) effects on the respective cells.

Table III. Response of CHO, HeLa and Vero cells to culture filtrates of *V. cholerae* non-O1

Morphological change	Titre range	Number of strains positive in		
		CHO cells	HeLa cells	Vero cells
Unaffected		0	1	2
Cytotoxic	4–8	5	10	7
	16–32	14	7	7
	64–128	4	6	9
	256–512	3	0	1
Cytotonic	2–4	1	2	0
	8–16	1	2	2

active against erythrocytes from rabbit, sheep (Eltor haemolysin), chicken and man, respectively. Strains that produced a haemolysin active against both rabbit and sheep erythrocytes were of the dominant type (35·7%). Two strains produced haemolysin(s) that was active against erythrocytes of rabbit, sheep, chicken and man (table IV). Ten (35·7%) of the 28 strains of *V. cholerae* non-O1 examined showed cell-associated haemagglutinating activity with human blood. Of the 10 strains, nine were isolated as sole pathogen and only one strain was associated with mixed infection (table V). Three distinct patterns of inhibition by sugars were detected; inhibition

Table IV. Haemolytic activity of the strains of *V. cholerae* non-O1 with rabbit, sheep, chicken and human erythrocytes

Haemolytis of erythrocyte	Number of strains
Rabbit, sheep, chicken, human	2
Rabbit, sheep, chicken	5
Rabbit, sheep, human	1
Rabbit, sheep	10
Rabbit, human	1
Rabbit	2
Sheep	3
None	4
Total	28

Table V. Patterns of inhibition of haemagglutination of *V. cholerae* non-O1 in relation to isolation status

Haemagglutination inhibition pattern*	Number of strains positive		Total
	Sole isolate	Mixed infection	
$M^+F^-G^+$	5	1	6
$M^+F^+G^-$	2	0	2
$M^+F^+G^+$	2	0	2
No haemagglutination	9	9	18
Total	18	10	28

*M, D-mannose; F, L-fucose; G, D-galactose; +, inhibition; −, no inhibition.

Table VI. Virulence phenotypes of the *V. cholerae* non-O1 isolates

Virulence phenotype*	Number of strains		Total
	Sole isolate	Mixed infection	
$Haem^+Cyt^+CT^-Hagg^+ZOT^-$	7	1	8
$Haem^+Cyt^+CT^-Hagg^-ZOT^-$	7	6	13
$Haem^-Cyt^+CT^-Hagg^-ZOT^-$	2	1	3
$Haem^-Cyt^+CT^-Hagg^+ZOT^-$	2	0	2
$Haem^-Cyt^+CT^+Hagg^-ZOT^+$	0	1	1
$Haem^-Cyt^-CT^+Hagg^-ZOT^+$	0	1	1
Total	18	10	28

*+, presence of the listed phenotype; − absence of the listed phenotype; Haem, ElTor haemolysin (active against sheep erythrocytes); Cyt, cytotoxin (CHO cells); CT, cholera toxin as determined by bead-ELISA; Hagg, haemagglutination (human O-group blood); ZOT, zonula occludens toxin determined by DNA probe.

of haemagglutination by mannose 1% but not by fucose and galactose 1% was the dominant haemagglutination inhibition pattern.

The composite virulence phenotypes of the 28 strains of *V. cholerae* non-O1 examined in this study are shown in table VI. A total of six different phenotypes was encountered. The prominent phenotype that was commonly associated with isolates from patients solely infected by *V. cholerae* non-O1 was exhibited by strains that produced the Eltor haemolysin, a hitherto unknown cytotoxin and a cell-associated haemagglutinin (table VI). The production of cell-associated haemagglutinin appeared to be the only distinctive phenotype that could distinguish between isolates from patients solely infected with *V. cholerae* non-O1 and those associated with mixed infections. Resistance of the 28 strains of *V. cholerae* non-O1 to antibiotics is shown in table VII. None of the isolates was resistant to tetracycline. The dominant antibiotic resistance pattern was ampicillin-streptomycin (17·8%) followed by neomycin-streptomycin (10·7%).

Discussion

Apart from serological differences, it is virtually impossible to differentiate between *V. cholerae* strains belonging to the serovar O1 and those belonging to serovars other than O1, collectively termed as the non-O1 group. However, these overtly similar organisms are very dissimilar in their pathogenic and epidemic potential, which has remained an enigma. Whereas CT is the major virulence factor in the pathogenesis of cholera, this study, like several others,[34] has clearly demonstrated that CT production is an exception among strains of *V. cholerae* non-O1. More interestingly, however, was the concurrent incidence of the gene for ZOT, a newly described enterotoxin of *V. cholerae*,[35] with the ability of the *V. cholerae* non-O1 strains to produce CT. The *V. cholerae* non-O1 strains that did not produce CT did not carry the ZOT gene, as reported previously,[32] which would indicate a possible synergic role for ZOT in the causation of the acute dehydrating diarrhoea mediated by CT-producing strains of *V. cholerae*. It has been suggested that ZOT may be responsible for the residual diarrhoea observed in human volunteers fed with recombinant strains of *V. cholerae* O1 attenuated by specific deletion of a major part of the gene encoding the A subunit of cholera toxin.[35,36]

The heat-stable enterotoxin (NAG-ST) was thought, at one time, to represent the principal virulence component of *V. cholerae* non-O1 but none of the isolates in this study hybridised with the NAG-ST gene probe. A recent study on the distribution of the NAG-ST gene among species of the genus *Vibrio* has shown conclusively that the incidence of the gene is very low among strains of *V. cholerae* non-O1.[22] However, when present in association with suitable colonisation ability, the NAG-ST gene of *V. cholerae* non-O1 is a formidable factor that can cause diarrhoea of severity similar to that of cholera, as demonstrated recently by study in human volunteers.[21]

From this study, it appears that most strains of *V. cholerae* non-O1 produce a membrane-damaging cytotoxin. Only one previous study has shown the production of a Shiga-like toxin by *V. cholerae* non-O1.[18] More than the Shiga-like toxin, the cytotoxic effect of *V. cholerae* non-O1 is probably related to the

Table VII. Antibiotic resistance of 28 *Vibrio cholerae* non-O1 isolates

Antibiotic	Resistant isolates (%)
Ampicillin	57·1
Chloramphenicol	7·1
Co-trimoxazole	10·7
Furazolidone	28·6
Gentamicin	25
Nalidixic acid	0
Neomycin	96·4
Streptomycin	46·4
Tetracycline	0

production of various haemolysins considering the range of activity with erythrocytes of different types seen in this study. Most of the strains were haemolytic and 75% of them produced a haemolysin active against sheep erythrocytes, also known as the Eltor haemolysin. Purified Eltor haemolysin evokes fluid accumulation in the rabbit ileal loop, the infant rabbit and the suckling mouse models, suggesting that the Eltor haemolysin is an enterotoxic factor.[16] The Eltor haemolysin of *V. cholerae* non-O1 is biologically, physicochemically and immunologically indistinguishable from the Eltor haemolysin elaborated by *V. cholerae* O1 belonging to the biotype Eltor.[14,15] Evidence against the Eltor haemolysin as an important enterotoxic factor are the findings emerging from the Center for Vaccine Development, USA, which indicated that recombinant strains carrying the Eltor haemolysin gene (JBK70 and CVD101) or in which the Eltor gene has been deleted (CVD104 and CVD105) did not seem to influence the efficacy of the recombinant vaccine strains, indicating that the Eltor haemolysin does not play a role in the causation of diarrhoea.[36,37]

When production of haemolysin or cytotoxin was analysed in conjunction with the haemagglutinating ability of the strain (a useful index of the attachment potential of pathogens),[38] it appeared that strains of *V. cholerae* non-O1 that produced haemolysin, that were cytotoxic and that produced haemagglutinins were isolated most often as the sole pathogen. The correlation of the ability of a strain to produce cell-associated haemagglutinin and its incidence as the sole pathogen in this study was remarkable; of the 18 strains of *V. cholerae* non-O1 isolated as the sole enteropathogen, 50% produced cell-associated haemagglutinin, as compared with only one strain among the 10 recovered from mixed infections. *V. cholerae* O1 is known to produce several haemagglutinins whose importance in the adherence process *in vivo* has been suggested.[19,20] The prominent haemagglutination-inhibition pattern exhibited by strains of *V. cholerae* non-O1 in this study was haemagglutination inhibited by mannose. Mannose-sensitive haemagglutination has been described as the predominant cell-bound haemagglutinin in *V. cholerae* O1 Eltor biotype.[20] However, the presence of haemagglutinins without an enterotoxic factor may not be significant, as > 62% of the environmental strains of *V. cholerae* non-O1 produce haemagglutinins or exohaemagglutinins[39] that are important for their survival in aquatic environments because they govern their ability to attach to different substrata.[40]

Considering the reported diversity of clinical symptoms elicited by *V. cholerae* non-O1[34] and considering its ubiquity in the environment,[41] a single microbial determinant could not be expected to discriminate a pathogenic strain from a non-pathogenic one. Virulence in such organisms is multifactorial and mediated by several traits functioning in an integrated fashion, as clearly demonstrated by the human volunteer study in which a strain of *V. cholerae* non-O1 that produced NAG-ST but did not colonise or a strain that could colonise but did not produce NAG-ST did not cause diarrhoea; only a strain that possessed both the attributes precipitated diarrhoea.[21] In conclusion, the clinical significance and virulence of *V. cholerae* non-O1 must be assessed in its totality and the presence of a single factor should not be construed as the cause of enteropathogenicity. It would be interesting in the future to trace the sequence of events which changes an innocuous isolate into an enteropathogenic one.

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