Article

Toxin(s), Other Than Cholera Toxin, Produced by Environmental Non O1 Non O139 *Vibrio cholerae*

Kohinur Begum[1, 3], Chowdhury R. Ahsan[1, 4], Mohammad Ansaruzzaman[2], Dilip K. Dutta[2], Qazi S. Ahmad[2] and Kaisar A. Talukder[2]

A total of 39 *Vibrio cholerae* non O1 non O139 strains were isolated from surface waters of different parts of Dhaka City, Bangladesh. All these strains showed lack of *ctx* or *zot* gene, as demonstrated by the PCR analysis. Eighteen representative strains were tested for enterotoxin production using a rabbit ileal loop model, of which live cells of 8 strains and culture filtrates of 6 strains produced fluid accumulation in ileal loops. However, none of them produced heat stable toxin (ST), as detected by suckling mouse assay. On the other hand, 15% of isolates produced cytotoxin as detected by the Chinese Hamster Ovary (CHO) cell assay. Fifty times concentrated culture filtrates of the representative strains did not give any precipitin band against the anti-cholera toxin, suggesting the strains produced an enterotoxin, which is antigenically different from known cholera toxin (CT). Eighty percent of the total isolates were found to be positive for heat labile haemolysin detected by tube method, whereas, 39% were found positive by the Christie-Atkins-Munch-Petersen (CAMP) method. However, 87% of the isolates were positive for haemagglutinin/protease and all of the strains were positive for mannose-sensitive-haemagglutinin assay. *Cellular & Molecular Immunology*. 2006;3(2):115-121.

Key Words: non O1 non O139 *Vibrio cholerae*, enterotoxin, cytotoxin, haemolysin, haemaglutinin/protease

Introduction

Vibrio cholerae is a natural inhabitant of the aquatic environment where water plays an important role in the transmission and epidemiology of cholera (1). Strains of *V. cholerae* belonging to serogroup O1, biotype El Tor and serogroup O139 have been described as causative agents of diarrhoea (2). In 1992, *V. cholerae* O1 in Bangladesh belonging to both biotypes caused regular epidemics of cholera and since then, both *V. cholerae* O1 and O139 have been significant causes of infection, although the frequency of infection varied from year to year in different regions of the country (3). On the other hand, the non O1 non O139 serogroup of *V. cholerae,* comprising a heterogeneous group of organisms caused cholera like diseases in patients (4). Clinically apart from the O1 and O139 serogroups, the non O1 non O139 serogroups continue to be of importance since these strains are associated with illness in patients hospitalized due to acute secretory diarrhoea (3). In most instances, the clinical profile of patients infected with *V. cholerae* non O1 non O139 is virtually indistinguishable from that of patients with cholera (4). Thus, it became evident that the non O1 non O139 serogroups of *V. cholerae* are capable of causing a disease mimicking cholera in absence of cholera toxin. At least three localized outbreaks of diarrhoea caused by non O1 non O139 serogroups have been described in the literatures. These include an outbreak caused by *V. cholerae* O10 and O12 in February 1994 in Lima, Peru (5). Another caused by O10 in East Delhi, India (6) and an epidemic caused by non O1 *V. cholerae* that produced ST among Khmers in a camp in Thailand (7).

The epidemiological impact of environmental non O1 non O139 *V. cholerae* strains is not clearly understood, since majority of these strains isolated from the environment do

[1]Department of Microbiology, University of Dhaka, Dhaka 1000, Bangladesh;

[2]ICDDR, B: International Centre for Health and Population Research, Bangladesh, GPO Box 128, Dhaka 1000, Bangladesh;

[3]Department of Pharmacy, University of Asia Pacific, House 73, Road 5A, Dhanmondi R/A, Dhaka 1209, Bangladesh;

[4]Corresponding to: Dr. CR Ahsan, Tel: +880-2-966-1900 (ext-6156), Fax: +880-2-861-5583, E-mail: crahsan@yahoo.com.

Received Sep 8, 2005. Accepted Mar 20, 2006.

Copyright © 2006 by The Chinese Society of Immunology

Abbreviations: ST, heat stable toxin; CHO, Chinese hamster ovary; CAMP, Christie-Atkins-Munch-Petersen; CT, cholera toxin; BHI, brain heart infusion; TCBS, thiosulphate-citrate-bile salt-sucrose; KRT, Krebs-Ringer solution; HA/P, haemagglutinin/protease; MSHA, mannose-sensitive-haemagglutinin.

not produce cholera toxin and lack not only the virulence gene cassette for cholera toxin but also zonula occuludens toxin and accessory cholera enterotoxin (8, 9). However, production of cholera like infection or acute secretory diarrhoae by these non O1 non O139 *V. cholerae* organisms made us in looking into the factor(s) contributing to the pathogenicity of these organisms. In this study, an attempt has been made to investigate the toxin(s) or other secretogenic factor(s) produced by the non O1 non O139 *V. cholerae*, which may contribute to the pathogenicity of these organisms.

Materials and Methods

Bacterial strains

One hundred and ten environmental water samples were collected from various surface water reservoirs and ponds of Dhaka City. Samples were collected at every 15 days for six months from September 1998 to March 1999. Samples were enriched in alkaline peptone water at 37°C for overnight and incubated on TCBS agar plate at 37°C for 18-24 h. One hundred and forty-four distinct yellow colonies were subcultured on gelatin-agar plates. All suspected single colonies were identified by a series of biochemical tests (10) and serotyping was performed by slide agglutination with specific antiserum (11). Thirty-nine strains were confirmed as non O1 non O139 *V. cholerae* which were stored in T_1N_1 (1% trypticase, 1% NaCl) soft agar at room temperature until studied.

Preparation of culture filtrates

Culture filtrates of all the 39 non O1 non O139 *V. cholerae* were prepared following the method of Sanyal et al. (12). Ten milliliters of brain heart infusion (BHI) broth contained in a 50 ml Ehlenmeyer flask was inoculated with 0.1 ml of 6 h growth of the organisms in T_1N_1 broth in a shaking water bath (120 oscillations per min) for 18 h at 37°C. The cultures were centrifuged at 12,000 g for 20 min and the supernatants were filtered through millipore membranes of 0.45 µm average pore diameter and preserved at -20°C. Similar preparation of culture filtrate was made from the known toxigenic *V. cholerae* 569B strain.

PCR assay for ctx and zot genes

Presence of *ctx* and *zot* genes in all the non O1 non O139 isolates was checked by amplifying primers specific for *ctx* and *zot* genes. The sequences of primers used for the detection of *ctx* 1 and *ctx* 2 were 5'-CTC AGA CGG GAT TTG TTA GGC ACG-3' and 5'-TCT ATC TCT GTA GCC CCT ATT ACG-3', respectively (13) and *zot* 1 and *zot* 2 were 5'-GAA CGC ATA GCT AAG TAC-3' and 5'-CCT GTC GCC CAT AGA CCA-3', respectively (29). All these primers were synthesized using Oligo 1000 DNA Synthesizer (Beckman) and were available from the Laboratory Science Division of ICDDR, B.

Double diffusion assay

Double diffusion assay in agar gel was performed with approximately 50 times concentrated culture filtrates of 18 representative non O1 non O139 *V. cholerae* strains and *V. cholerae* strain 569B (14). Approximately 2 mm thick agar slabs were made by solidifying 1% agar (Difco) in veronal buffer on microscopic glass slides. Small wells were punched out of the agar and two fold dilutions of the concentrated culture filtrates were applied in the wells surrounding the well that contained the anti-cholera toxin (anti-CT, Sigma, USA). The antigen-antibody reactions were allowed to react in a moist chamber at room temperature for 24-36 h. The agar slab containing antigen-antibody complexes was washed with normal saline for 24-36 h with periodic changes of the saline. The slab was dried and stained with 0.1% Coomassie blue for 30 min and the de-staining solution (7% acetic acid) was used to remove any unbound dye. The slide was then dried and looked for any precipitin band showing reaction of identity, partial identity or non-identity.

Rabbit ileal loop assay

Live cells and culture filtrates of 18 representative strains of non O1 non O139 *V. cholerae* were tested for enterotoxin production in adult New Zealand rabbits (15). For live cells, 1 ml preparation of 4 to 6 h bacterial culture (~10^6 cfu/ml) in peptone water was inoculated into rabbit ileal loops. The toxigenic *V. cholerae* 569B and sterile peptone water served as positive and negative control, respectively. Culture filtrates of all strains prepared in brain heart infusion (BHI) broth were tested by the same method (15) using 1 ml inocula. Each test was done in two rabbits and all rabbits were sacrificed after 18 h. The ratios of volume (ml) of fluid accumulation to the length (cm) of the loop in the two rabbits were averaged to get the results.

Suckling mice assay

Culture filtrates of the representative strains were also tested in suckling mouse assay (16). Swiss albino suckling mice (2-4 days old) were separated from their mothers immediately before use. Each mouse was inoculated intragastrically with 0.1 ml of culture filtrate containing 2 drops of 2% pontamine sky blue dye solution per ml. Two mice were used for each culture filtrate, which were then incubated at 25°C for 4 h. After incubation, the mice were killed by excess of chloroform and the abdomen was opened by a forceps. The ratio of gut weight to remaining body weight of the two mice was calculated and results averaged. The ratio of less than 0.07 was considered negative, 0.07-0.085 was considered borderline and the ratio over 0.085 was considered positive. The culture filtrate of a heat stable toxin producer *E. coli* strain 36004 and BHI broth with dye served as positive and negative controls, respectively.

Activity against Chinese Hamster Ovary (CHO) cells

Culture filtrates of all the 39 strains were tested in CHO cell monolayer following the method of Guerrant et al. (17). Monolayer of CHO cells were grown in F12 medium with 10% fetal calf serum and 100 µl of CHO cell suspension was delivered into each well of a 96-well microtitre plate (flat bottom) and incubated at 37°C for 1-2 h followed by the

addition of 100 μl culture filtrate of each strain to all well containing the cell suspensions in duplicate. The cells were incubated at 37°C for overnight and examined under an inverted microscope for necrosis, elongation or rounding.

Haemolysin assay

The isolation of all the non O1 non O139 *V. cholerae*s was tested for the production of haemolysin by the tube (18) and Christie-Atkins–Munch-Petersen (CAMP) method (19). In the tube method, 0.5 ml supernatant of the original bacterial culture in BHI broth was mixed with an equal volume of 1% suspension of sheep RBC. The mixture was incubated for 2 h at 37°C and held overnight at 4°C to check the haemolysis of the RBC. The whole process was repeated with culture supernatants, which were heated at 56°C for 30 min. An El Tor O1 strain and classical O1 strain were used as positive and negative controls, respectively.

In the CAMP test, the adjacent stab inoculation of *Staphylococcus aureus* and *V. cholerae* on a blood agar plate was checked for synergistic haemolysis. A CAMP haemolysin positive El Tor O1 strain and a CAMP haemolysin-negative classical O1 strain were used as controls.

Haemagglutinin/protease (HA/P) assay

Haemagglutinin/protease activity of the isolates was detected on milk-agar plates (20), where bacteria were inoculated and incubated at 37°C for 24 h. A clear zone around the bacterial growth indicated HA/P activity. A known *V. cholerae* O1 El Tor strain was included as a positive control.

Mannose-sensitive-haemagglutinin (MSHA) assay

Mannose-sensitive-haemagglutinin activity of the isolations was checked by mixing 15 μl of bacterial suspension in Krebs-Ringer solution (KRT, ~10^{10} organisms/ml) with an equal volume of a 3% (v/v) chicken erythrocyte suspension in KRT. The slide was slowly rotated for 3 min to detect any agglutination reaction of the erythrocytes (21). Both positive and negative O139 *V. cholerae* MSHA were included in the test as controls.

Results

Bacterial strains

All the 110 environmental water samples were enriched in alkaline peptone water at 37°C overnight and then incubated on TCBS agar plates. From here 144 distinct yellow colonies were sub-cultured on gelatin agar plates and suspected colonies were taken for biochemical and serological tests. A total of 39 strains were identified as non O1 non O139 *V. cholerae* isolated from 110 environmental surface water samples collected between September, 1998 and March, 1999. All these strains were confirmed following different biochemical and immunological methods.

PCR analysis for ctx and zot genes

Polymerase chain reaction is a highly specific technique for the detection of any specific gene. In this study, PCR was carried out by amplifying the primers specific for *ctx* or *zot* genes. However, all the 39 strains were negative in the PCR assay for *ctx* and *zot* genes indicating that the strains did not produce cholera (CT) or zonula occludens (zot) toxins.

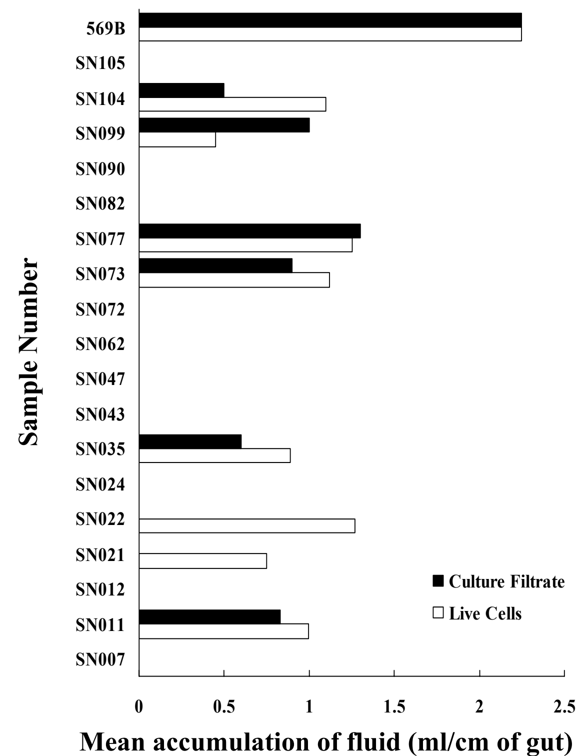

Figure 1. Volume of fluid accumulated in rabbit ileal loops after inoculation of live cells and culture filtrates. Eighteen representative non O1 non O139 *V. cholerae* strains were tested in rabbit ileal loops and the results were determined as the volume of fluid accumulation (ml) per unit length (cm) of gut.

Double diffusion assay with culture filtrates

Double diffusion assay in agar gel was performed with approximately 50 times concentrated culture filtrates so that we can detect the precipitin bands (if there is any) after Coomassie blue staining. However, none of the 50 times concentrated culture filtrates prepared from the 18 representative strains of non O1 non O139 *V. cholerae* produced any precipitation band in double diffusion technique when tested against the anti-CT. On the other hand, the positive control *V. cholerae* 569B culture filtrate gave a prominent precipitin band.

Rabbit ileal loop test with live cells and culture filtrates

Fluid outpouring in ileal loops can be attributed due to liberation of an entertoxin(s) by the organisms during multiplication in the intestine. In this study, 8 out of the 18 representative strains tested in rabbit ileal loop caused fluid accumulation in different ranges (Figure 1). The range of fluid accumulation per cm of gut varied from strain to strain (from 0.45 to 1.27 ml/cm of the gut). The *V. cholerae* 569B

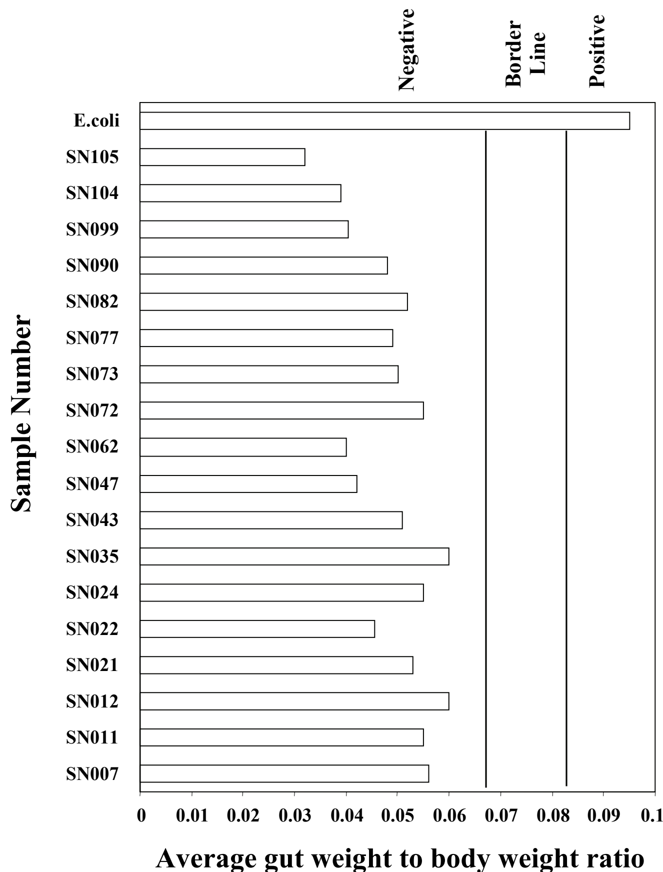

Figure 2. Heat stable (ST) enterotoxin activity of the 18 representative non O1 non O139 *V. cholerae* were tested in sucking mice assay. All the representative culture filtrates were found to be negative showing gut to remaining body weight ratios less than 0.064. However, the culture filtrate of a heat stable toxin producer *E. coli* strain gave positive results.

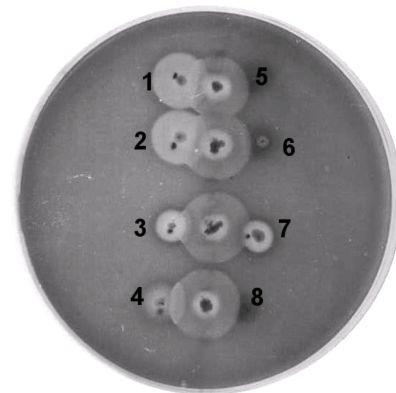

Figure 3. Christie-Atkins-Munch-Petersen (CAMP) haemolysin assay showed synergistic haemolysis from *V. cholerae* O1 El Tor positive control strains (zones 1, 2, 3, and 4). However, the negative control of *V. cholerae* O1 classical strain did not show any haemolysis (zone 8). Zone 7 is the positive reaction and zones 5 and 6 are the negative reactions shown by the non O1 non O139 *V. cholerae* strains.

(Classical Inaba), which was included as a positive control, produced more than 2.5 ml/cm of loop. On the other hand, culture filtrates of 6 strains out of the above 18 representative strains produced fluid accumulation in rabbit ileal loop assay (Figure 1) and some of them were comparable to that of *V. cholerae* 569B culture filtrate.

Suckling mice assay

Suckling mice assay is considered to be specific for heat stable entertoxin and gut to body weight ratio over 0.085 is considered positive. In this study, all 18 culture filtrates were negative in this assay showing gut to remaining body weight ratios of less than 0.064 (Figure 2). However, the *E. coli* strain 36004 culture filtrate showed a gut to body weight ratio of 0.094 ± 0.003, indicating a positive reaction and the BHI broth showed a negative reaction.

Activity against CHO cells

The assay in principle is that a monolayer of Chinese hamster Ovary cell is carried in a tissue culture flask. When measured quantity of the prepared toxin is added to the cell suspension in a micro-titre plate and incubated overnight, a toxin positive preparation will change the morphology of the cells. Here culture filtrates prepared from the 39 strains of non O1 non O139 of *V. cholerae* were tested for cytotoxin (Table 1). Among them, only six strains were found to be positive, changing the morphology of CHO cells into round after overnight incubation at 37°C.

Haemolysin assay

A total of 31 strains were found to be positive in the haemolysin assay (tube method), which showed haemolysis of the sheep erythrocytes and reddish color of the suspension (Table 1). In the negative results, the sheep erythrocytes only precipitated at the bottom of the tube and no haemolysis occurred. In addition, culture supernatant of all these 31 strains when heated at 56°C for 30 min and tested in the same way, could not produce RBC haemolysis and it indicated heat labile nature of the haemolysin.

On the other hand, in the CAMP method, only 15 out of the 39 strains had positive reactions (Table 1). The CAMP positive reactions of *V. cholerae* El Tor strains appeared as large sausage, bean, or football-shaped zones of complete haemolysis, significantly overlapping the circular zone of non-CAMP haemolysis and extending well beyond its periphery (Figure 3). The positive non O1 non O139 *V. cholerae* also gave El Tor type synergistic haemolysis in the CAMP plate.

Haemagglutinin/protease (HA/P) and mannose-sensitive haemagglutinin assay (MSHA)

In the HA/P assay, 87% non O1 non O139 *V. cholerae* produced protease (Table 1), which was demonstrated by a clear zone around the bacterial growth (Figure 4). However, in the MSHA assay, all strains were found to be positive (Table 1).

Table 1. Cytotoxin, haemagglutinin/protease, mannose-sensitive haemagglutinin assay (MSHA), and haemolysin activity of the non O1 non O139 *V. cholerae* organisms

Sample No	Cytotoxic response CHO cell	Haemagglutinin/ protease activity	MSHA	Haemolysin (CAMP)	Haemolysin (tube)	
					Haemolysin	Haemolysin*
SN007	-	-	+	+	+	-
SN008	+	+	+	-	+	-
SN010	-	+	+	-	+	-
SN011	-	+	+	-	+	-
SN012	+	+	+	+	+	-
SN015	-	+	+	-	+	-
SN019	+	+	+	+	+	-
SN021	-	+	+	+	+	-
SN022	-	+	+	-	-	-
SN024	-	+	+	-	-	-
SN025	+	+	+	-	+	-
SN029	-	+	+	-	+	-
SN035	-	+	+	+	+	-
SN043	-	+	+	-	+	-
SN047	-	+	+	-	+	-
SN051	-	+	+	+	-	-
SN053	-	+	+	+	+	-
SN054	-	+	+	-	-	-
SN056	-	+	+	-	-	-
SN060	-	+	+	-	-	-
SN062	-	-	+	+	+	-
SN063	-	+	+	-	+	-
SN072	-	+	+	-	+	-
SN073	-	-	+	+	+	-
SN077	-	+	+	+	+	-
SN079	-	-	+	+	+	-
SN082	-	-	+	+	-	-
SN084	-	+	+	-	-	-
SN090	+	+	+	+	+	-
SN099	-	+	+	+	+	-
SN102	+	+	+	-	+	-
SN103	-	+	+	+	+	-
SN104	-	+	+	-	+	-
SN105	-	+	+	-	+	-
SN130	-	+	+	-	+	-
SN132	-	+	+	-	+	-
SN136	-	+	+	-	+	-
SN140	-	+	+	-	+	-
SN144	-	+	+	-	+	-
ATCC 36004	+	NA	NA	NA	NA	NA
ATCC 3600	-	NA	NA	NA	NA	NA
O1 El Tor *V. cholerae*	NA	+	NA	NA	NA	NA
O139 *V. cholerae* (*positive MSHA)*	NA	NA	+	NA	NA	NA
O139 *V. cholerae (negative MSHA)*	NA	NA	-	NA	NA	NA
O1 El Tor *V. cholerae*	NA	NA	NA	+	NA	NA
O1 classical *V. cholerae*	NA	NA	NA	-	NA	NA
O1 El Tor *V. cholerae*	NA	NA	NA	NA	+	NA
O1 classical *V. cholerae*	NA	NA	NA	NA	-	NA

+, Positive; -, Negative; NA, Not applicable; *Culture filtrate heated at 56°C for 30 min.

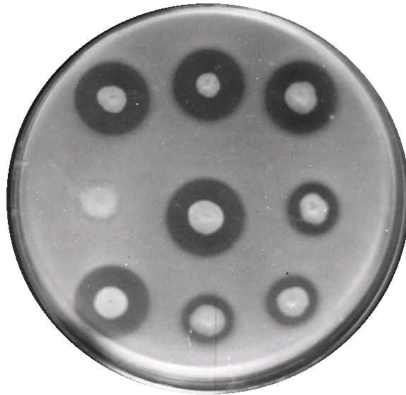

Figure 4. Haemagglutinin/protease activity of non O1 non O139 *V. cholerae.* Clear zones around the bacterial growth indicated protease activity. A *V. cholerae* El Tor isolate was included as a positive control (shown in the middle).

Discussion

Thirty-nine non O1 non O139 *V. cholerae* strains were isolated from 110 surface water samples collected from different parts of Dhaka City, Bangladesh. In the PCR assay, we failed to detect the presence of *ctx* or *zot* gene in all the 39 strains. This was confirmed again by the double diffusion assay, and none of the concentrated culture filtrates of the representative strains produced any precipitin band against anti-CT. On the other hand, culture filtrate of the toxigenic *V. cholerae* 569B strain gave a prominent precipitin band. These results suggested that the cholera toxin gene was negative in non O1 non O139 *V. cholerae* strains, so did not produce any toxin antigenically similar to cholera toxin.

In the rabbit ileal loop assay, 8 of 18 live cells representative strains caused fluid accumulation in different ranges (Figure 1). The range of fluid accumulation per cm of gut varied from 0.45 to 1.27 ml/cm of the gut. Also culture filtrates prepared from 6 of 18 representative strains produced fluid accumulation in rabbit gut, the level of them can be comparable to that of toxigenic *V. cholerae* 569B culture filtrate (Figure 1). Fluid outpouring in ileal loops can be attributed due to liberation of an enterotoxic substance(s) by the organisms during multiplication in the intestine or *in vitro* multiplication in the medium. This has been shown with many other organisms such as, *V. cholerae* (22), enterotoxigenic *E. coli* (23), *Cl. perfringens* (24), *A. hydrophila* (25), NAG vibrios (15). The present study indicates that sufficient amount enterotoxic substance(s) of the organisms accumulated in rabbit loop during multiplication to induce fluid outpouring. The differences in fluid accumulation between the strains may have been due to variation in the release of toxin. Therefore, it is clear from this study, that in the absence of *ctx* or *zot* genes, these non O1 non O139 *V. cholerae* strains produced a toxin, which caused fluid accumulation in the gut by a mechanism entirely different from that of the toxigenic *V. cholerae* O1 or O139, which contain *ctx* or z*ot* genes.

In the suckling mice assay, all 18 representative culture filtrates were found to be negative showing gut to remaining body weight ratios of less than 0.064 (Figure 2). However, the culture filtrate of *E. coli* strain 36004 showed a gut to body weight ratio of 0.094, indicating a positive reaction. This assay which is considered to be specific for heat stable (ST) enterotoxin (16) indicated that none of the non O1 non O139 *V. cholerae* strains produced heat stable toxin. On the other hand, 6 of the 39 strains were found to be positive in the CHO cell assay, indicating that these strains produced a cytotoxin (Table 1). As the cytotoxin activity was demonstrated by the strains, which were both positive and negative in ileal loop assay, therefore this study also suggested that the cytotoxin production in non O1 non O139 *vibrio* strains was independent of toxin production responsible for fluid accumulation.

Haemolysin tests of all the isolates were performed in this study using both tube and CAMP methods and it was found that 80% and 39% of the strains were haemolysin positive in tube and CAMP methods, respectively (Table 1). However, these strains did not produce haemolysis in the tube method, when tested with the culture supernatant exposed to heat at 56°C for 30 min, which indicated the nature of the haemolysin to be heat-labile (Table 1). In the HA/P assay, 87% of the strains were HA/P positive and in the MSHA assay, all strains were found to be MSHA positive (Table 1). All these characteristics of non O1 non O139 *vibrio* are similar to those of El Tor *vibrios* (21). Previously the El Tor haemolysin has been implicated as a virulence factor for toxigenic *V. cholerae* (26). The haemolysin has also been described as a potent toxin with both enterotoxin and cytotoxin activities (7, 27, 28). Similarly, the *V. cholerae* O10 and O12 strains associated with the outbreak in Peru were all positive for El Tor haemolysin, cytotoxin, invasiveness, and mannose-sensitive-haemagglutinin, which led the investigators to conclude that a combination of all these factors and perhaps some other unknown factors may have caused the diarrhoea in Peru (5).

In the present study, the *ctx* and *zot* genes negative non O1 non O139 *V. cholerae* strains were found to produce a toxin(s), other than cholera toxin, which is responsible for causing fluid accumulation in rabbit ileal loops. The ST negative strains also showed cytotoxic activity in CHO cell assay. The organisms also produced heat-labile haemolysin, haemagglutinin/protease and mannose-sensitive-haemagglutinin. All these results suggest that the cholera like symptom produced by non O1 non O139 *V. cholerae* (4, 5) could be due to different factor(s) responsible for the toxigenic effects of these organisms.

References

1. Islam MS, Drassar BS, Sack RB. The aquatic environment as a reservoir of *Vibrio cholerae*: a review. J Diarrhoeal Dis Res. 1993;11:197-206.
2. Mukhpadhyay AK, Garg S, Mitra R, et al. Temporal shifts in traits of *Vibrio cholerae* strains isolated from hospitalized

patients in Calcutta: a 3-year (1993 to 1995) analysis. J Clin Microbiol. 1996;34:2537-2547.

3. Baqui AH, Yunus MD, Zaman K, Mitra AK, Hossain KMB. Surveillance of patients attending a rural diarrhoea treatment centre in Bangladesh. Top Geogr Med. 1991;43:17-22.
4. Mukhpadhyay AK, Saha PK, Garg S, et al. Distribution and virulence of *Vibro cholerae* belonging to serogroups other than O1 and O139: a nation-wide survey. Epidemiol Infect. 1995; 114:65-70.
5. Dalsgaard AM, Albert MJ, Taylor DN, et al. Characterization of *Vibrio cholerae* non O1 serogroup obtained from an outbreak of diarrhoea in Lima, Peru. J Clin Microbiol. 1995;33:2715-2722.
6. Rudra S, Mahajan R, Mathur M, Kathuria K, Talwar V. Cluster of cases of clinical cholera due to *Vibrio cholerae* O10 in East Delhi Indian. J Med Res. 1996;103:71-73.
7. Bagchi K, Echeverria P, Arthur JD, Sethabutr O, Serichantalergs O, Hoge CW. Epidemic diarrhoea caused by *Vibrio cholerae* non O1 that produced heat-stable toxin among Khmers in a camp in Thailand. J Clin Microbiol. 1993;31:1315-1317.
8. Kurazono H, Pal A, Bag PK, et al. Distribution of genes encoding cholera toxin, zonula occludens toxin, accessory cholera toxin, and El tor hemolysin in *Vibrio cholerae* of diverse origins. Microb Pathog. 1995;18:231-235.
9. Sharma C, Thungapathra M, Ghosh A, et al. Molecular analysis of non-O1, non-O139 *Vibro cholerae* associated with an unusual Upsurge in the incidence of cholera-like disease in Calcutta, India. J Clin Microbiol. 1998;36:756-763.
10. WHO (1974). World Health Organization Guidelines for the Laboratory Diagnosis of cholera. Bacterial Disease Unit. World Health Organization, Geneva, Switzerland.
11. Shimada T, Arakawa E, Itoh K, et al. Extended serotyping scheme for *Vibrio cholerae*. Curr Microbiol. 1994;28:175-178.
12. Sanyal SC, Saraswathi B, Sharma P. Enteropathogenicity of Plesiomonas shigelloides. J Med Microbiol. 1980;13:401-409.
13. Shirai H, Nishibuchi M, Ramamurthy T, Bhattacharya SK, Pal SC, Takeda Y. Polymerase chain reaction for detection of the cholera enterotoxin operon of *Vibrio cholerae*. J Clin Microbiol. 1991;29:2517-2521.
14. Ouchterlony O. Antigen-antibody reactions in gels. Acta Pathol Microbiol Scand. 1969;26:507-515.
15. Singh SJ, Sanyal SC. Enterotoxicity of the so-called NAG vibrios. Ann Soc Belg Med Trop. 1978;58:133-140.
16. Dean AG, Chig YC, Williams RG, Harden LB. Test for *Escherichia coli* enterotoxin using infant mice: application in a study of diarrhoea in children in Honolulu. J Infect Dis. 1972;125:407-411.
17. Guerrant RL, Brunton LL, Schnaitman TC, Rebhun LI, Gilman AG. Cyclic adenosine monophosphate and alteration of Chinese Hamster Ovary cell morphology: a rapid, sensitive *'in vitro'* assay for the enterotoxins of *Vibro cholerae* and *Escherichia coli*. Infect Immun. 1974;10:320-327.
18. Barrett TJ, Blake PA. Epidemiological usefulness of changes in hemolytic activity of *Vibrio cholerae* biotype El Tor during the seventh pandemic. J Clin Microbiol. 198;13:126-129.
19. Lesmana M, Subekti D, Tjaniadi P, Pazzaglia G. Modified CAMP test for biogrouping *Vibrio cholerae* O1 strains and distinguishing them from strains of *V. cholerae* non-O1. J Clin Microbiol. 1994;320:235-237.
20. Honda T, Booth BA, Boesman-Finkelstein M, Finkelstein RA. Comparative study of *Vibrio cholerae* non-O1 protease and soluble haemagglutinin with those of *Vibrio cholerae* O1. Infect Immun. 1987;55:451-454.
21. Johnson G, Sanchez J, Svennerholm AM. Expression and detection of different biotype-associated cell-bound haemagglutinins of *Vibrio cholerae* O1. J Gen Microbiol. 1989;135:111-120.
22. De SN. Enterotoxicity of bacteria-free culture filtrate of *Vibrio cholerae.* Nature. 1959;183:1533-1534.
23. De SN, Bhattacherium K, Sarkar JK. A study of pathogenicity of strains *Bacterium coli* from acute and chronic enteritis. J Path Bacteriol. 1956;71:201-209.
24. Duncan Cl, Strong DH. Ileal loop fluid accumulation and production of diarrhoea in rabbits by cell-free products of *Clostridium perfringens*. J Bacteriol. 1969;100:86-94.
25. Annapurna E, Sanyal SC. Enterotixicity of *Aeromonas hydrophila*. J Med Microbiol. 1977;10:317-323.
26. Nagamune K, Yamamoto K, Honda T. Cloning and sequencing of a novel hemolysis gene of *Vibro cholerae*. FEMS Microbiol Lett. 1995;128:265-269.
27. Honda T, Finkelstein RA. Purification and characterization of a hemolysin produced by *Vibrio cholerae* biotype El Tor: another toxic substance produced by cholera vibrios. Infect Immun. 1979;26:1020-1027.
28. Ichinose Y, Yamamoto K, Nakasone N. Enterotoxicity of El Tor-like hemolysin of non-O1 *Vibrio cholerae*. Infect Immun. 1987;55:1090-1093.
29. Baudry B, Fasano A, Ketley J, Kaper JB. Cloning of a gene (zot) encoding a new toxin produced by *Vibrio cholarae*. Infect Immun. 1992;60:428-434.