**Production of the new cholera toxin by environmental isolates of Vibrio cholerae non-O1**

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One of five strains of Vibrio cholerae non-O1 isolated from environmental sources caused fluid accumulation in an initial rabbit ileal loop (RIL) test. The four strains that caused little or no accumulation of fluid gave a positive response after one-to-three consecutive passages through RILs. The amount of fluid produced increased after each passage. Filtrates of cultures of all five environmental isolates caused fluid accumulation similar to that produced by live cells. The enterotoxin showed a precipitin band with new cholera antitoxin and was neutralised completely by new cholera antitoxin diluted 1 in 32, indicating its close immunobiological relationship to the new cholera toxin. The present study indicates that V. cholerae non-O1 strains produce an enterotoxin that is similar to the new cholera toxin.

**Introduction**

Vibrio cholerae non-O1 has been recognised as a causative agent of sporadic and localised outbreaks of cholera-like diarrhoea [1]. This organism has also been implicated as a cause of extra-intestinal infections such as wound infection, septicemia and cellulitis in man [2-4]. A large number of V. cholerae non-O1 strains of clinical and environmental origins produce an enterotoxin that is similar immunologically and biologically to cholera toxin (CT) [5-10]. Recently, several other extracellular products, all of which may play an important role in the pathogenesis of the disease, have been documented. These include heat-stable enterotoxin (NAG-ST) similar to the heat-stable enterotoxin of Escherichia coli that caused fluid accumulation in an infant mouse model [11-13], a thermostable direct haemolysin similar to that of V. parahaemolyticus [12, 14], a heat-labile El Tor-like haemolysin [15-17], a shiga-like toxin [18], haemagglutinins [19, 20] and Zonula Occludens Toxin (Zot) [7]. It seems that the virulence of V. cholerae non-O1 is multifactorial and is mediated by several traits in an integrated fashion [7]. However, there is no published information about the production of new cholera toxin (NCT) by V. cholerae non-O1. NCT can be produced by cholera toxin gene-negative and -positive strains of V. cholerae O1, both classical and El Tor biotypes from both clinical and environmental origins [21-26]. Therefore, the present study of environmental isolates of V. cholerae non-O1 was undertaken to see whether fluid accumulation in RILs following inoculation with culture filtrates could be neutralised by an antitoxin against NCT produced by CT gene-negative V. cholerae O1. The study also investigated whether there is any antigenic similarity with NCT produced by a CT gene-negative V. cholerae O1 strain and the toxin(s) produced by non-O1 environmental isolates.

**Materials and methods**

**Bacterial strains**

Five strains of V. cholerae non-O1 isolated from the environment by this laboratory were identified by standard techniques [27]. A CT gene-negative V. cholerae O1 (strain X-392) which produced NCT was obtained from J. B. Kaper, Center for Vaccine Development, MD, USA, and included for preparation of antiserum against purified NCT.

**Medium**

Preliminary tests for toxin production were made in AKI medium. The AKI medium (Bacto-peptone 1.5% v/v, yeast extract 0.4%, NaCl 0.5% and NaHCO3 0.3%) was prepared as described by Iwanaga et al. [28]. The sodium bicarbonate was filter sterilised separately and mixed with the other autoclaved ingredients. Freshly prepared medium was always used; the pH of this medium was c. 7.4 without adjustment.

**Preparation of culture filtrates**

Culture filtrates (CFs) of the five isolates of V. cholerae non-O1, once their live cells were known to cause fluid...
accumulation in ileal loops, were prepared by the method of Annapurna and Sanyal [29]. Briefly, 10 ml of AKI medium in a 50-ml conical flask were inoculated with five or six colonies from an overnight nutrient agar plate culture. The flasks were incubated at 37°C in a water-bath with shaking for 16–18 h with 80–120 oscillations/min. The cultures were centrifuged at 22 000 g for 20 min at 4°C and supernates were filtered through membrane filters (Millipore, 0.22 μm) and stored in small volumes at 4°C.

Ileal loop test

Live cells of the five V. cholerae non-O1 isolates were tested for enterotoxin production in adult New Zealand and albino rabbits by the method of De and Chatterje [30]. The strains were grown in peptone water for 3–4 h, diluted 10-fold in the same medium and inoculated in RILs in doses of 1 ml (10^7–6 cfu). The known toxigenic V. cholerae strain 956B grown in peptone water and unseeded peptone water served as positive and negative controls, respectively. CFs of all these strains prepared in AKI medium were tested in RILs as described earlier [6,22]. Briefly, such strains were inoculated in RILs in doses of 1 ml (ch). The cultures were centrifuged at 37°C for 34 h and 1 ml of diluted culture was inoculated again into RIL. This process was continued until good positive responses were obtained.

Passage through RILs

Consecutive passages of the V. cholerae non-O1 strains that caused little or no accumulation of fluid in the initial test were made in RILs as described earlier [6,22]. Briefly, such strains were recovered from the ileal loops on nutrient agar plates and, following overnight incubation, five or six colonies were inoculated into peptone water, incubated at 37°C for 3–4 h and 1 ml of diluted culture was inoculated again into RIL. This process was continued until good positive responses were obtained.

Determination of the optimum loop reacting dose

Cell-free CFs of five strains of V. cholerae non-O1 were injected in volumes ranging between 0.05 and 1.0 ml into RILs to find the dose causing a maximal reaction [29].

Neutralisation of V. cholerae non-O1 enterotoxin by NCT in RIL

CFs of five strains in 0.15-ml amounts contained in 0.5 ml of phosphate-buffered saline (PBS, pH 7.4) were mixed with equal volumes of anti-NCT serum in serial double dilutions as described previously [6,22,31]. The mixtures were incubated at 37°C in a water-bath for 30 min and then tested in RILs to find the highest dilution of anti-NCT serum that would completely neutralise the enterotoxigenic activity in CFs. CF-PBS mixture and PBS alone served as positive and negative controls, respectively.

Immuno diffusion test

Ouchterlony immunodiffusion tests were performed with 10-fold concentrated CFs (with polyethylene glycol 4000, Ranbaxy, India) of the test strains against anti-NCT serum. CF of strain X-392 along with those of V. cholerae non-O1 strains were tested individually against anti-NCT serum.

Results

In the initial experiments, live cells of only one of the five isolates tested caused fluid accumulation in RILs, comparable to that of V. cholerae O1 toxigenic strain 569B. Of the four isolates that caused little or no accumulation of fluid in the initial tests, two each became positive after two and three passages through rabbit gut, respectively (Table 1). These strains showed marked increase in fluid accumulation on each consecutive passage through the rabbit gut (data not shown). CFs of all these strains also caused fluid accumulation in the range similar to those produced by live cells.

Filtrates in doses of 0.15 ml caused fluid accumulations in the range 0.74–1.2 ml/cm of RIL. The amount of fluid accumulation was no greater when larger volumes of CFs were injected (Fig. 1). The 0.15-ml dose of CF was used in neutralisation tests with the antiserum against NCT.

The highest dilution of anti-NCT serum that completely neutralised the enterotoxigenic activity in these CFs was 1 in 32. There was proportionately less neutralisation, i.e., more fluid accumulation, with higher dilutions of anti-NCT serum.

When CFs of the CT gene-negative V. cholerae O1 (strain X-392) and V. cholerae non-O1 strains were tested in gel diffusion, one (88-SH) gave a precipitin band against the NCT antiserum showing reaction of identity, whereas the remaining four showed a reaction of partial identity.

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\text{Table 1. Enhancement of fluid accumulation of } V. \text{ cholerae non-O1 strains after passage through RILs} \\
\begin{array}{|c|c|c|}
\hline
\text{Strain no.} & \text{Before passage} & \text{After passage} \\
\text{(number of passages)} & \text{(ml/cm of rabbit ileal loop)} & \\
\hline
86-HG & 0 & 0.5–0.9 (3) \\
86-TG-2 & 0 & 0.7–1.1 (3) \\
88-AS & 0 & 0.6–0.7 (2) \\
88-SH & 0 & 0.5–0.7 (2) \\
86-DSM & 0.5–0.9 & 0.7–1.2 (0) \\
86-TG-3 & 0.8–1.1 & 1.0–1.4 \\
\hline
\text{Positive control}^* & 0.8–1.1 & 1.0–1.4 \\
\text{Negative control}^† & 0 & 0 \\
\hline
\end{array}
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*Peptone water culture of V. cholerae strain 569B.
†Peptone water.
Discussion

The present study suggests that all strains of *V. cholerae* non-O1 produce enterotoxin as judged by fluid accumulation in RILs, the classical model for detection of enterotoxin activity of bacteria. Moreover, strain-to-strain and loop-to-loop variations in fluid accumulation as observed might have been due to variations in the amounts of toxin released by them and to biological variations [32].

Four of the five isolates of *V. cholerae* non-O1 that caused little or no accumulation of fluid in the initial test showed enhancement of enterotoxic activity after one-to-three consecutive passages through the gut of a susceptible host, i.e., rabbit ileum. Fluid outpouring increased after each passage. Similar effects of passage on enterotoxicity have been observed earlier with *V. cholerae* non-O1 [32, 33], and with other organisms such as *V. cholerae* O1 [21, 22], *Aeromonas* spp. [34–37], *Plesiomonas shigelloides* [38], *V. fluvialis* [39], *V. mimicus* [40] and *Klebsiella pneumoniae* [41]. Furthermore, it is well known that the so-called hypotoxicogenic strain 569 B of *V. cholerae* O1 also requires passage through rabbit gut for enhancement of toxin production. A mechanism of repression of a toxin gene that becomes expressed on passage in a susceptible host may account for this phenomenon [32]. There is also a possibility that toxigenicity of the isolates might have decreased during subculture or storage in the laboratory before the ileal loop tests. It appears that repression/derepression of a virulence factor in a bacterial strain is a means for its adaptation to a particular microenvironment. The present study thus suggests that all the *V. cholerae* non-O1 strains were potentially enterotoxigenic.

The observation that the toxin produced by one *V. cholerae* non-O1 strain (no. 88-SH) gave a precipitin band against anti-NCT serum showing reaction of identity suggests that this strain produced an enterotoxin antigeniically similar to NCT. This conclusion is further reinforced by the fact that the enterotoxic activity of this strain was completely neutralised by anti-NCT serum at a dilution of 1 in 32.

The CFs of the four other strains showed only partial identity with NCT and complete neutralisation of enterotoxic activity in ileal loops, suggesting that these strains also produced NCT but possibly with some weaker epitopes, as has been observed in CT-B subunits produced by classical and El Tor biotype strains [42]. Tamplin *et al.* [43] also observed five shared and one unshared epitope between classical and El Tor CFs as well as some variation in the extent of cross-reactivity between different El Tor CT-B preparations. Although the subunit structure of NCT has not yet been determined, its large mol. wt (61 000, unpublished data) suggests that this toxin possesses some subunits, the epitopes of which may differ slightly from strain to strain. However, these differences are minor and do not affect the neutralising capability of the antitoxin prepared against strain X-392.

This study thus suggests that the strains of *V. cholerae* non-O1 isolated from environmental sources produce an enterotoxin that is similar to the new cholera toxin.

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References

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