Cholera toxin, zonula occludens toxin and accessary cholera enterotoxin gene-negative *Vibrio cholerae* non-O1 strains produce the new cholera toxin

Vibrio cholerae non-O1 have been reported to produce cholera toxin (CT), although certain strains did not do so[1]. In recent years, several other extracellular products such as a heat stable toxin (NAG-ST), a thermostable direct haemolysin, El Tor-like haemolysin, a shiga-like toxin, haemagglutinin and zot[2] produced by *V. cholerae* non-O1, have been reported to play some role in causing disease. However, none of these virulence factors alone was considered

as the cause of enteropathogenicity. Moreover, the recent observation of Kurazono *et al.*[3] that the majority strains of *V. cholerae* non-O1 of clinical and environmental origin lack the virulence genes cassette comprising CT, zonula occludens toxin (zot) and accessary cholera enterotoxin (ace), indicates that these strains may possess another unidentified virulence gene encoding an yet unrecognized secretogen.

Earlier we demonstrated that CT^- or CT^+ strains of *V. cholerae* O1, biotype El Tor or classical, serotype Ogawa or Inaba of clinical or environmental origin or genetically engineered in the laboratory produce a new cholera toxin (NCT)[4–6], and the disease cholera may be caused either by CT or NCT or both. However, the production of NCT by *V. cholerae* non-O1 has not yet been reported, although these organisms cause diarrhoea in humans and a secretory response in experimental animals. In this study, an attempt was therefore, made to examine the production of NCT by *V. cholerae* non-O1, isolated from the River Ganga in Varanasi, India. These strains of *V. cholerae* non-O1 were *ctx*, *zot* and *ace* negative as tested using specific DNA probes[3] and were, therefore, devoid of the core dynamic region of *V. cholerae* O1[7] (G.B. Nair, pers. commun.).

Live cells and one of the five isolates of *V. cholerae* non-O1 tested in ligated ileal loops of adult albino rabbits (Belgian strain), following the method of De and Chatterje[8], caused accumulation of fluid in the initial test (0.7–1.2 ml/cm of RIL). The other four isolates did so after one to four consecutive passage(s) through rabbit gut in the range of 0.5–1.1 ml/cm of RIL, and thereafter outpouring of fluid by every strain increased with each passage. Culture filtrates (CFs) of these strains prepared in AKI medium[9], only after their live cells caused fluid accumulation in RILs, when tested in the same assay showed a similar secretory response (0.5–1.2 ml/cm of RIL), although slightly less than that of toxigenic *V. cholerae* strain 569B (1.1–1.4 ml/cm of RIL) but did not cause lysis of sheep erythrocytes when tested by conventional method.

These observations indicate that the non-O1 *V. cholerae* strains that lack gene for all known toxin factors such as CT, zot and ace, except NCT, produce a secretogen. Enhancement of secretory response upon passage through the gut of a susceptible host suggests that if such strains circulate in the community, because aquatic life is the reservoir of non-O1 *V. cholerae* strains, its virulence may increase further.

In RIL assays, the enterotoxic activity of non-O1 *V. cholerae* strains was completely neutralized by the antiserum against purified NCT diluted in 1:32 (Figures 1 and 2) prepared with CT^- strain of *V. cholerae* X-392, which was shiga or shiga-like toxin gene-negative as tested using specific DNA probes (P. Echeverria, pers. commun.) and was non-haemolytic when tested by conventional method.

The observation that CF of one isolate showed complete identity and neutralization by anti NCT indicates that this strain produces a secretogen antigenically similar to NCT. However, the other four isolates that showed partial identity with NCT and complete neutralization of enterotoxic activity in RIL by its antiserum in the same dilution may suggest that these strains also produce NCT but differ in some weaker epitopes[10], as has been observed in CT-B subunits produced by classical and El Tor biotype strains[11]. Tamplin *et al.*[12] 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 with some of the anti-classical monoclinal antibodies. Although the subunit structure of NCT could yet be determined, its

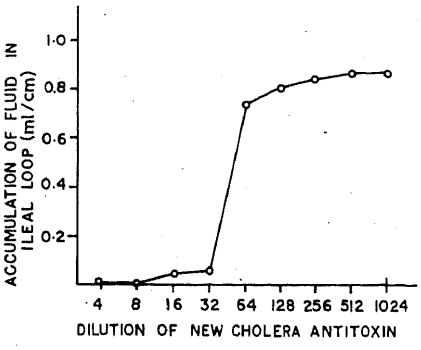

Figure 1. Neutralization of non-O1 *V. cholerae* enterotoxin by new cholera antitoxin. The results are means of filtrates of five cultures.

Figure 2. Neutralization of non-O1 *V. cholerae* enterotoxin by new cholera antitoxin. Loop 1: Positive control, 0.15 ml of culture filtrate (CF, optimal loop-reacting dose) mixed with 0.85 ml of phosphate buffered saline (PBS, 0.02 M, pH 7.4); Loop 2–9: 0.15 ml of CFs in 0.35 ml of PBS with 0.5 ml of 1 in 512, 1 in 256, 1 in 128, 1 in 64, 1 in 32, 1 in 16, 1 in 8 and 1 in 4 dilutions of new cholera antitoxin. Loop 10: negative control, 1.0 ml of PBS.

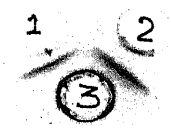

Figure 3. Immunological identity of the NCT with non-O1 *V. cholerae* (strain no. 88 SH) enterotoxin. Ouchterlony immunodiffusion analysis of concentrated CF of CT^- *V. cholerae* strain X-392 (well 1) and *V. cholerae* non-O1 strain 88 SH (well 2) against antiserum of X-392 NCT (well 3).

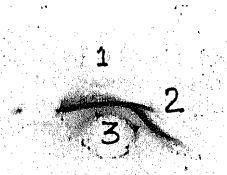

Figure 4. Immunodiffusion comparison of the NCT and *V. cholerae* non-O1 strain 86-TG2 enterotoxin. Concentrated CF of the strain X-392 (well 1) was compared with concentrated CF of the strain 86 TG2 (well 2) against antiserum of X-392 NCT (well 3).

molecular weight being as large as 61,000 Da (unpublished data) there is every likelihood that this toxin possesses some subunits, the epitopes of which may differ slightly from strain to strain. This difference, however, is minor and does not affect the neutralizing capability of the antitoxin against X-392.

In gel-diffusion test, 10 times concentrated CF of CT⁻ *V. cholerae* X-392 that produces NCT[4] and *V. cholerae* non-O1 strains gave a precipitation band against anti-NCT. Only one isolate showed reaction of identity (Figure 3) and the other four showed reaction of partial identity (Figure 4).

The results of this study suggest that the strains of *V. cholerae* non-O1 can produce NCT in the absence of *ctx*, *zot* and *ace* or when these genes are deleted. They, thus possess the potential to cause diarrhoea. These observations are of importance in understanding the pathogenesis of diarrhoea caused by *V. cholerae* non-O1 strains as this toxin seems to play an important role in the causation of diarrhoea[4]. However, further study with a large number of isolates is needed to strengthen this conclusion.

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D. V. SINGH
ANJALI TIKOO
A. PAL*
S. C. SANYAL

Department of Microbiology,
Institute of Medical Sciences,
Banaras Hindu University,
Varanasi 221 005, India
**National Institute of Cholera and Enteric Diseases,*
P-33, CIT Road, Scheme XM,
Post Box 177, Beliaghata,
Calcutta 700 010, India