

## Reassessment of the Prevalence of Heat-Stable Enterotoxin (NAG-ST) among Environmental *Vibrio cholerae* Non-O1 Strains Isolated from Calcutta, India, by Using a NAG-ST DNA Probe

AMIT PAL,<sup>1</sup> T. RAMAMURTHY,<sup>1</sup> R. K. BHADRA,<sup>1</sup> TAE TAKEDA,<sup>2</sup> TOSHIO SHIMADA,<sup>3</sup> YOSHIFUMI TAKEDA,<sup>4</sup> G. BALAKRISH NAIR,<sup>1\*</sup> S. C. PAL,<sup>1</sup> AND S. CHAKRABARTI<sup>1</sup>

National Institute of Cholera and Enteric Diseases, Beliaghata, Calcutta 700010, India,<sup>1</sup> and Department of Infectious Diseases Research, National Children's Medical Research Center, Setagaya-ku, Tokyo 154,<sup>2</sup> Laboratory of Bacteriology I, National Institute of Health, Shinagawa-ku, Tokyo 141,<sup>3</sup> and Department of Microbiology, Faculty of Medicine, Kyoto University, Kyoto 606,<sup>4</sup> Japan

Received 27 January 1992/Accepted 6 June 1992

**A collection of 521 environmental isolates of *Vibrio cholerae* which were previously examined by the suckling mouse assay and found to be negative for the heat-stable enterotoxin NAG-ST were reassessed by a recently developed DNA probe for NAG-ST. A total of 12 (2.3%) of the isolates hybridized with the NAG-ST probe. By using a cholera toxin (CT) DNA probe, the CT gene was detected in six of the strains in the collection, although none of the isolates of *V. cholerae* non-O1 hybridized with both of the toxin probes. All of the NAG-ST and CT probe-positive strains were hemolysin positive. Thirty-fold-concentrated supernatants of the three representative NAG-ST DNA probe-positive *V. cholerae* non-O1 strains gave positive fluid accumulation ratios in the suckling mouse assay even after heating (100°C for 5 min) and also inhibited the binding of a NAG-ST monoclonal antibody to the bound NAG-ST in a competitive enzyme-linked immunosorbent assay (ELISA). Likewise, all six CT probe-positive *V. cholerae* non-O1 strains produced in vitro CT when examined by the CT bead ELISA. *Hind*III digest patterns of chromosomal DNA from the representative NAG-ST gene-positive strains were visually indistinguishable. Between the groups of NAG-ST probe-positive strains examined, there was a variation in the hybridizable fragments, with one group of strains exhibiting a hybridizable fragment similar to that of the NRT 36 reference strain; a smaller *Hind*III fragment hybridized with the NAG-ST probe in the other group of strains. The significance or role of these toxigenic *V. cholerae* non-O1 strains in the environment needs to be studied in greater detail.**

*Vibrio cholerae* non-O1, a ubiquitous inhabitant of natural aquatic environments (13, 20), has been implicated as the etiologic agent of human gastroenteritis (1, 7, 12, 15, 35) and occasionally associated with wound infections (3, 9) and primary septicemia (24). The mechanism of pathogenesis of non-O1 gastroenteritis is incompletely understood, although in recent years a variety of virulence factors such as cholera toxin-like enterotoxin (5, 34), Kanagawa hemolysin (10, 36), Shiga-like toxin (21), and a heat stable-like enterotoxin (NAG-ST) (2, 10) have been proposed to explain the clinical manifestations of non-O1 gastroenteritis. The NAG-ST, a 17-amino-acid peptide (2) which exhibits remarkable similarity, especially in the carboxyl-terminal toxic domain, to the heat-stable enterotoxins (STs) produced by enterotoxigenic *Escherichia coli* (ETEC) (28, 37), is the best studied virulence factor of *V. cholerae* non-O1. A recent human volunteer study has clearly demonstrated that in the presence of adequate colonization factor(s), a NAG-ST-producing strain of *V. cholerae* non-O1 caused diarrhea of severity comparable to that seen in cholera (16).

The epidemiology of NAG-ST-producing *V. cholerae* non-O1 and the extent of the occurrence of these strains in the environment, in foods, and among clinical cases is still obscure. This hiatus in information stems largely from the lack of a suitable assay system for detection of NAG-ST. The routinely used test for detection of NAG-ST is the

suckling mouse assay, which is riddled with inaccuracies because of the presence of an interfering suckling mouse-active heat-labile El Tor haemolysin elaborated by the majority of the *V. cholerae* non-O1 strains (11, 19). Although there is a great deal of similarity between amino acid sequences of STs produced by different enteric pathogens, it has now become evident that there are distinct differences in the nucleotide sequences of the genes encoding the STs of *E. coli* and *V. cholerae* non-O1 (22). These differences explain the failure of probes derived from the sequence of *E. coli* STs to hybridize with NAG-ST-producing strains of *V. cholerae* non-O1 (26). Recently, the NAG-ST gene has been cloned and sequenced (22), enabling the construction of a DNA probe encoding a 271-bp cloned fragment of the NAG-ST gene (31). The availability of the *nag-st* DNA probe formed the impetus to reassess a large collection of environmental strains of *V. cholerae* non-O1 which were previously examined by the suckling mouse assay and found to be negative for NAG-ST (17, 19).

### MATERIALS AND METHODS

**Bacterial strains.** A total of 521 environmental strains of *V. cholerae* non-O1, which consisted of 363 strains isolated from water, sediment, and plankton from a lacustrine environment during an ecological study in Calcutta conducted between July 1984 and June 1985 (20), 141 strains isolated from five different species of brackish water and freshwater prawns cultured in paddy fields during June to November

\* Corresponding author.

1985 (17), 4 strains from estuarine waters, and 13 strains of uncertain sources of isolation but of environmental origin were included in this study.

**DNA probes.** A recombinant plasmid, pAO111 containing the coding sequence for NAG-ST (31) (the accession number of the NAG-ST sequence is M36061 in the GenBank data base), was used as the source of the DNA probe for the toxin. The digestion of pAO111 with restriction endonucleases *EcoRI* and *BamHI* released a 271-bp DNA fragment specific for the NAG-ST gene. This fragment was isolated through 1% agarose gel electrophoresis and used for labeling the probe. Similarly, *EcoRI* fragment (554 bp) of the plasmid pKTN901 (14) containing the A1 subunit of cholera toxin (CT) was used to screen all of the *V. cholerae* non-O1 strains for the presence of the gene encoding CT. The DNA fragments were labelled enzymatically according to a protocol supplied by the manufacturer (enhanced chemiluminescence gene detection system; Amersham International). Briefly, the double-stranded DNA fragments were completely denatured by boiling for 5 min and immediately cooled on ice. These were incubated (at 37°C for 10 min) with horseradish peroxidase, previously complexed with a positively charged polymer to form a loose attachment by charge attractions. Addition of glutaraldehyde resulted in the formation of chemical cross-links so that the probe remained covalently labelled with horseradish peroxidase. These labelled probes were used in subsequent hybridizations.

**Colony hybridization.** The test organisms were inoculated on positively charged sterile nylon filters layered over nutrient agar plates and incubated at 37°C overnight. The membranes containing the freshly grown strains were placed successively on the top of a denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 7 min, twice on a neutralization solution (0.5 M Tris-HCl [pH 7.2], 1.5 M NaCl) for 4 min each, and finally on 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The membranes were treated with proteinase K (1 mg/ml) for 30 min at room temperature, air dried, and baked at 80°C for 2 h. The membranes were hybridized with labelled probes at 42°C overnight. After hybridization, the membranes were washed to remove unbound probe. Bound probe was detected by an enzymatic reaction followed by oxidation of luminol in the presence of an enhancer. The emitted blue light was visualized on X-ray film.

**Suckling mouse assay and NAG-ST ELISA.** The biological activity and immunological reactivity of a representative of the NAG-ST DNA probe-positive *V. cholerae* non-O1 strains were examined by the conventional suckling mouse assay (32) and by a recently developed NAG-ST monoclonal antibody (30, 31) based-competitive enzyme-linked immunosorbent assay (ELISA) (18). Because of the documented low in vitro production of NAG-ST (2), the test strains were grown in 1 liter of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% NaCl for 24 h at 37°C and concentrated approximately 30-fold as described previously (31). In each plate, synthesized NAG-ST (1 µg/ml) and phosphate-buffered saline (pH 7.2) containing 0.05% Tween were used as the positive and negative controls, respectively.

**CT bead ELISA.** Production of CT by the probe-positive strains in Casamino Acids medium supplemented with lincomycin was examined by an ultrasensitive bead ELISA (23, 33). Various dilutions of purified CT (List Biological Laboratories, Campbell, Calif. [lot no. CVX-034]; positive control) and uninoculated medium or buffer (negative control) were run concurrently whenever the CT bead ELISA was

TABLE 1. Hybridization of environmental *V. cholerae* non-O1 isolates with the NAG-ST and CT DNA probes

| Origin                               | No. of strains | No. CT DNA gene positive | No. NAG-ST gene positive |
|--------------------------------------|----------------|--------------------------|--------------------------|
| Lacustrine environment               | 363            | 5                        | 8                        |
| Freshwater and brackish water prawns | 141            | 0                        | 4 <sup>a</sup>           |
| Estuarine waters                     | 4              | 0                        | 0                        |
| Miscellaneous environmental sources  | 13             | 1                        | 0                        |
| Total                                | 521            | 6                        | 12                       |

<sup>a</sup> One strain which hybridized with the NAG-ST probe was excluded from this collection since it was identified as *V. mimicus*.

performed. None of the negative controls (buffers and media) yielded a net optical density (OD) of more than 0.039 in 10 different runs performed on different days (mean, 0.026; standard deviation [SD], ± 0.009). The mean OD for 10 pg of pure CT per ml by this assay was 0.06 (SD, ± 0.017) while the mean OD for 39 pg/ml was 0.17 (SD, ± 0.055). On the basis of these data, an OD of 0.10 or greater was arbitrarily selected as the cutoff for evidence of a positive CT test.

**Determination of hemolytic activity.** The level of hemolytic activity of the *V. cholerae* non-O1 strains was also determined with washed rabbit erythrocytes diluted to a final concentration of 1% in 10 mM phosphate buffer (pH 7.0) containing 1.3% NaCl as described previously (19).

**Serotyping.** Serovars of the strains which hybridized with the NAG-ST and CT DNA probes were examined by the somatic O-serogrouping scheme (25) to determine whether the presence of the toxin genes clustered with any given serovar.

**Southern hybridization.** Representative strains of NAG-ST probe-positive environmental *V. cholerae* non-O1 were selected for this analysis. A NAG-ST-producing strain, NRT 36, isolated from a patient with traveller's diarrhea at Narita Airport quarantine station (Tokyo, Japan), was taken as the reference organism. Chromosomal DNA was isolated by lysing the organisms with sodium dodecyl sulfate and then by proteinase K treatment and finally extracted with phenol-chloroform (4). A total of approximately 1 µg of DNA was digested with restriction endonuclease for 18 to 20 h at 37°C. Electrophoresis was performed through a horizontal gel with 0.7% agarose in 1× TAE (Tris acetate-EDTA) buffer. The DNA fragments in the gel were stained with ethidium bromide and visualized with a UV transilluminator. The DNA fragments were blotted from the gel onto a nitrocellulose filter by the method described by Southern (27). Transfer was allowed to proceed for 16 to 18 h with 20× SSC buffer. The filters were baked and hybridized with the NAG-ST probe as described earlier.

## RESULTS

Results revealed that 12 (2.3%) of the 521 environmental isolates of *V. cholerae* non-O1 examined hybridized with the NAG-ST probe. One additional strain which was positive by the NAG-ST probe was not included in this series, since on subsequent characterization, the strain was identified as *Vibrio mimicus* (Table 1). The CT gene was detected in 6 (1.2%) of the 521 strains examined and was detected mainly from the collection of strains recovered from the lacustrine environment but not from freshwater or brackish water prawns. None of the *V. cholerae* non-O1 strains examined

TABLE 2. Results of the suckling mouse assay, NAG-ST ELISA, CT bead ELISA, and hemolysin assay of the *V. cholerae* non-O1 strains which hybridized with the NAG-ST and CT DNA probes

| Strain no. | Hybridized with |             | Suckling mouse assay<br>(FA ratio) <sup>a</sup> | NAG-ST ELISA<br>(% inhibition) <sup>b</sup> | CT bead ELISA<br>(OD <sub>450</sub> ) <sup>c</sup> | Production of<br>hemolysin |
|------------|-----------------|-------------|---|---|--|----------------------------|
|            | NAG-ST<br>probe | CT<br>probe |   |   |  |                            |
| VCE-89     | +               | -           | 0.09  | 86.5  | ND <sup>d</sup>                                    | +                          |
| VCE-100    | +               | -           | 0.09  | 84.0  | ND   | +                          |
| VCE-101    | +               | -           | 0.11  | 86.7  | ND   | +                          |
| S-18       | -               | +           | ND  | ND  | 3.2320   | +                          |
| VCE-232    | -               | +           | ND  | ND  | 0.8440   | +                          |
| VCE-233    | -               | +           | ND  | ND  | 1.5930   | +                          |
| 353        | -               | +           | ND  | ND  | 2.7660   | +                          |
| VCE-227    | -               | +           | ND  | ND  | 0.2420   | +                          |
| VCE-228    | -               | +           | ND  | ND  | 0.2810   | +                          |

<sup>a</sup> Fluid accumulation (FA) ratios (and gut-body weight ratios) of  $\geq 0.090$  were considered positive.

<sup>b</sup> Strains giving  $>40\%$  inhibition of the binding of anti-NAG-ST monoclonal antibody to the microtiter plate-bound NAG-ST were regarded as positive.

<sup>c</sup> An OD at 450 nm (OD<sub>450</sub>) of 0.10 or greater was taken as evidence for a positive CT test, and this OD corresponded to about 25  $\mu$ g of CT per ml when the bead ELISA was performed with various dilutions of pure CT (List Biological Laboratories; lot no. CVX-034).

<sup>d</sup> ND, not done.

hybridized with both of the toxin probes. In this study, 65.5% of the strains of *V. cholerae* non-O1 examined produced hemolysin when tested with rabbit blood. All of the NAG-ST and CT probe-positive strains were hemolysin positive (Table 2). Of the 13 NAG-ST gene-possessing strains, 3 belonged to serovar O14, 1 each belonged to serovars O40, O99, O2, and O67, and, for the remaining strains, serogrouping is under continued investigation. Of the six CT probe-positive *V. cholerae* non-O1 strains, four belonged to serovar O109 (provisional serovar) and one belonged to O53, while one strain was categorized as rough.

Thirty-fold-concentrated supernatants of the three representative NAG-ST DNA probe-positive *V. cholerae* non-O1 strains (VCE89, VCE100, and VCE111) gave positive fluid accumulation ratios in the suckling mouse assay even after heating (100°C for 5 min) and also inhibited the binding of NAG-ST monoclonal antibody to the bound NAG-ST in the competitive ELISA procedure (Table 2). Likewise, all of the six CT probe-positive *V. cholerae* non-O1 strains produced *in vitro* CT when examined by the CT bead ELISA.

The electrophoretic patterns of the *Hind*III digest of chromosomal DNA from the representative NAG-ST gene-positive strains were visualized by staining the gel with ethidium bromide (Fig. 1A). Although it is hard to draw firm conclusions about the differences among strains in the digestion patterns from the stained gel, careful examination revealed differences in the pattern exhibited by Fig. 1A, lane 1 (reference culture, NRT 36), compared with the others. However, a clear picture emerged from the Southern hybridization experiment with the NAG-ST probe, which revealed that this gene is located in one *Hind*III fragment. Between the groups of strains examined, there was a variation in the hybridizable fragments (Fig. 1B). One group of strains exhibited a hybridizable fragment similar to that of the NRT 36 reference strain; a smaller *Hind*III fragment hybridized with the probe in the other group of strains.

## DISCUSSION

In previous studies of the toxin profiles of environmental *V. cholerae* non-O1 conducted in this laboratory (17, 19), we were unable to detect NAG-ST in the currently examined collection of strains using the suckling mouse assay. Culture supernatants of strains which elicited fluid accumulation in the suckling mice in the earlier studies were retested by

heating the supernatant at 100°C for 5 min to destroy the activity of the heat-labile El Tor hemolysin. Heating the culture supernatant resulted in loss of the suckling mouse factor in all of the strains examined, leading us to believe that none of the strains produced NAG-ST. From the present study, it is evident that the hemolysin obliterated the results of NAG-ST probe-positive strains in the conventional suckling mouse assay. By heating the culture supernatant, the activity of the heat-labile hemolysin is destroyed, but in the process, the NAG-ST molecule is coprecipitated and masked by heat-denaturing products in crude preparations, as has been documented while purifying NAG-ST (2). Therefore, the DNA probe is the only reliable method to detect NAG-ST strains of *V. cholerae* non-O1.

From this study, it appears that NAG-ST strains of *V. cholerae* non-O1 are sparsely distributed in the environment, although in numbers slightly higher than those of CT-producing *V. cholerae* non-O1 strains. Using a synthetic oligonucleotide probe for NAG-ST, Hoge et al. (8) reported

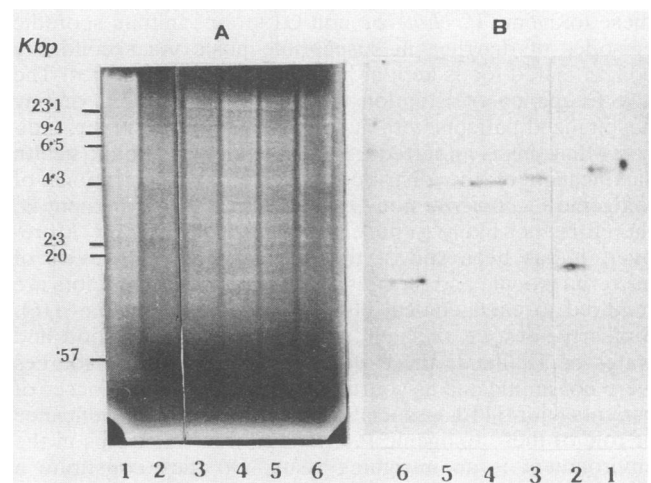


FIG. 1. *Hind*III digests of chromosomal DNA (A) and Southern blot hybridization (B) with the 271-bp DNA fragment containing the NAG-ST gene. Lanes 1 to 6 are all *V. cholerae* non-O1 strains as follows: 1, NRT 36 (reference strain); 2, SF 68; 3, VCE 103; 4, VCE 101; 5, VCE 100; 6, VCE 89.

that 6.8% of the clinical *V. cholerae* non-O1 isolates from Thailand and none of the 78 isolates from Mexico and the United States hybridized with the probe. Independent studies in Japan have demonstrated that among the vibrios, *V. mimicus* strains are apparently the reservoirs of the NAG-ST gene, since 13.7% of the environmental strains and 22.6% of the clinical strains harbored the gene, compared with 1.2% and 3.6% of the environmental and clinical strains, respectively, of *V. cholerae* non-O1 (29). Because of the small number of probe-positive NAG-ST *V. cholerae* non-O1 strains, it would be too early to extrapolate any correlations between serovar and toxin production, but these data would be useful for comparing the serovar patterns as and when information on serovars of NAG-ST strains from other geographical areas becomes available.

Southern blot analysis of the NAG-ST gene-positive strains indicated the absence of any internal *Hind*III site within the coding sequence for NAG-ST in *V. cholerae* non-O1 strains recovered from the local environment. This is in agreement with the reported sequence analysis of the NAG-ST gene (22). The observation of two groups of strains on the basis of two distinct hybridizable *Hind*III fragments is interesting. This restriction length polymorphism with respect to the NAG-ST gene could be of epidemiological importance. The strains tested in these experiments were collected from a geographical location quite different from those reported in the literature. The distribution of strains showing such restriction length polymorphism is yet to be seen. However, since the coding sequence of NAG-ST does not contain any *Hind*III site, the use of another restriction endonuclease which cleaves the NAG-ST gene internally will provide more information regarding restriction length polymorphism. These analyses are in progress. Whether the variation in the position of the NAG-ST gene in the chromosome has any impact on the expression of the toxin remains unexplored.

With the available data, it is difficult to speculate on the importance of these toxigenic, environmental *V. cholerae* non-O1 strains in the epidemiology of non-O1 gastroenteritis. In areas like Calcutta, where there is extensive interaction between humans and aquatic bodies for a variety of purposes, there is the possibility, although remote, that these toxigenic *V. cholerae* non-O1 strains initiate sporadic episodes of diarrhea in susceptible hosts via secondarily contaminated foods kept at high ambient temperatures. The low frequency of isolation of *V. cholerae* non-O1 among hospitalized patients with acute diarrhea (6), however, indicates that this is an infrequent happening. The public health significance of the distribution of low concentrations of toxigenic *V. cholerae* non-O1 strains in the environment is, therefore, not likely to pose a serious health hazard. Moreover, it has been shown that ingestion of high levels of bacterial inocula and the presence of colonization factors are required to elicit clinical illness in human volunteers (16). Similarly, despite the identification of ETEC in food and water in Thailand, these potential environmental sources were not implicated as sources of ETEC in the 52 houses of patients with ETEC-associated diarrhea (6). The significance or role of these toxigenic *V. cholerae* non-O1 strains in the environment is an intriguing issue. Do they constitute a reservoir of the toxin genes in the environment or do they form a minuscule, independent population of the entire *V. cholerae* non-O1 flora in the environment, which, during hitherto unknown favorable environmental conditions, selectively outgrow their toxin-negative counterparts? More

epidemiological and ecological studies are apparently required before we will be able to answer these questions.

#### ACKNOWLEDGMENT

The award of a Junior Research Fellowship to A.P. from the Council of Scientific and Industrial Research (CSIR), New Delhi, India, is gratefully acknowledged.

#### REFERENCES

1. Aldova, E., K. Laznickova, E. Stepankova, and J. Lietava. 1968. Isolation of nonagglutinable vibrio from an enteritis outbreak in Czechoslovakia. *J. Infect. Dis.* **118**:25-31.
2. Arita, M., T. Takeda, T. Honda, and T. Miwatani. 1986. Purification and characterization of *Vibrio cholerae* non-O1 heat-stable enterotoxin. *Infect. Immun.* **52**:45-49.
3. Bonner, J. R., A. S. Coker, C. R. Berryman, and H. M. Pollock. 1983. Spectrum of *Vibrio* infections in a Gulf Coast community. *Ann. Intern. Med.* **99**:464-469.
4. Brenner, D. J., G. R. Fanning, K. E. Johnson, R. V. Citrella, and S. Falkow. 1969. Polynucleotide sequence relationships among members of Enterobacteriaceae. *J. Bacteriol.* **98**:637-650.
5. Craig, J. P., K. Yamamoto, Y. Takeda, and T. Miwatani. 1981. Production of cholera-like enterotoxin by a *Vibrio cholerae* non-O1 strain isolated from the environment. *Infect. Immun.* **34**:90-97.
6. Echeverria, P., J. Serivatana, D. N. Taylor, S. Yanggratoke, and C. Tirapat. 1985. A comparative study of enterotoxigenic *Escherichia coli*, *Shigella*, *Aeromonas*, and *Vibrio* as etiologies of diarrhoea in North Eastern Thailand. *Am. J. Trop. Med. Hyg.* **34**:547-554.
7. El-Shawi, N., and A. J. Thewaini. 1969. Non-agglutinable vibrios isolated in the 1966 epidemic of cholera in Iraq. *Bull. W.H.O.* **40**:163-166.
8. Hoge, C. W., O. Sethabutr, L. Bodhidatta, P. Echeverria, D. C. Robertson, and J. G. Morris, Jr. 1990. Use of a synthetic oligonucleotide probe to detect strains of non-O1 *Vibrio cholerae* carrying the gene for heat-stable enterotoxin (NAG-ST). *J. Clin. Microbiol.* **28**:1473-1476.
9. Hoge, C. W., D. Watsky, R. N. Peller, J. P. Libonati, E. Israel, and J. G. Morris, Jr. 1989. Epidemiology and spectrum of *Vibrio* infections in a Chesapeake Bay community. *J. Infect. Dis.* **160**:985-993.
10. Honda, T., M. Arita, T. Takeda, M. Yoh, and T. Miwatani. 1985. Non-O1 *Vibrio cholerae* produces two newly identified toxins related to *Vibrio parahaemolyticus* haemolysin and *Escherichia coli* heat-stable enterotoxin. *Lancet* **ii**:163-164.
11. Ichinose, Y., K. Yamamoto, N. Nakasone, M. J. Tanabe, T. Takeda, T. Miwatani, and M. Iwanaga. 1987. Enterotoxicity of El Tor-like hemolysin of non-O1 *Vibrio cholerae*. *Infect. Immun.* **55**:1090-1093.
12. Kamal, A. M. 1971. Outbreak of gastroenteritis by non-agglutinable (NAG) vibrios in the Republic of Sudan. *J. Egypt. Public Health Assoc.* **46**:125-173.
13. Kaper, J., H. Lockman, R. R. Colwell, and S. W. Joseph. 1979. Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. *Appl. Environ. Microbiol.* **37**:91-103.
14. Kaper, J. B., J. G. Morris, Jr., and M. Nishibuchi. 1988. DNA probes for pathogenic *Vibrio* species, p. 65-77. *In* F. C. Tenover (ed.), *DNA probes for infectious disease*. CRC Press, Inc., Boca Raton, Fla.
15. McIntyre, O. R., J. C. Felley, W. B. Greenough III, A. S. Bennenson, S. I. Hassan, and A. Saad. 1965. Diarrhoea caused by non-cholera vibrios. *Am. J. Trop. Med. Hyg.* **14**:412-418.
16. Morris, J. G., Jr., T. Takeda, B. D. Tall, G. A. Losonsky, S. K. Bhattacharya, B. D. Forrest, B. A. Kay, and M. Nishibuchi. 1990. Experimental non-O group 1 *Vibrio cholerae* gastroenteritis in humans. *J. Clin. Invest.* **85**:697-705.
17. Nair, G. B., R. K. Bhadra, T. Ramamurthy, A. Ramesh, and S. C. Pal. 1991. *Vibrio cholerae* and other vibrios associated with paddy field cultured prawns. *Food Microbiol.* **9**:203-208.
18. Nair, G. B., S. K. Bhattacharya, and T. Takeda. Submitted for publication.

19. Nair, G. B., Y. Oku, Y. Takeda, A. Ghosh, R. K. Ghosh, S. Chattopadhyay, S. C. Pal, J. B. Kaper, and T. Takeda. 1988. Toxin profiles of *Vibrio cholerae* non-O1 from environmental sources in Calcutta, India. *Appl. Environ. Microbiol.* **54**:3180-3182.
20. Nair, G. B., B. L. Sarkar, S. P. De, M. K. Chakrabarti, R. K. Bhadra, and S. C. Pal. 1988. Ecology of *Vibrio cholerae* in the fresh water environs of Calcutta, India. *Microb. Ecol.* **15**:203-215.
21. O'Brien, A. D., M. E. Chen, R. K. Holmes, J. B. Kaper, and M. M. Levine. 1984. Environmental and human isolates of *Vibrio cholerae* and *Vibrio parahaemolyticus* produce a *Shigella dysenteriae* 1 (Shiga-like) cytotoxin. *Lancet* **i**:77-78.
22. Ogawa, A., J. Kato, H. Watanabe, G. B. Nair, and T. Takeda. 1990. Cloning and nucleotide sequence of a heat-stable enterotoxin gene from a patient with traveller's diarrhea. *Infect. Immun.* **58**:3325-3329.
23. Oku, Y., Y. Uesaka, T. Hirayama, and Y. Takeda. 1988. Development of a highly sensitive bead ELISA to detect bacterial protein toxins. *Microbiol. Immunol.* **32**:807-816.
24. Safrin, S., J. G. Morris, Jr., M. Adams, V. Pons, R. Jacobs, and J. K. Conte, Jr. 1988. Non-O1 *Vibrio cholerae* bacteremia: a case report and review. *Rev. Infect. Dis.* **10**:1012-1017.
25. Sakazaki, R., and T. Shimada. 1977. Serovars of *Vibrio cholerae*. *Jpn. J. Med. Sci. Biol.* **30**:279-282.
26. Seriwatana, J., P. Echeverria, D. N. Taylor, T. Sakuldaipeara, S. Changchawalit, and O. Chivoratanond. 1987. Identification of enterotoxigenic *Escherichia coli* with synthetic alkaline phosphatase-conjugated oligonucleotide DNA probes. *J. Clin. Microbiol.* **25**:1438-1441.
27. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.
28. Takao, T., T. Hitouji, S. Amito, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani. 1983. Amino acid sequence of a heat-stable enterotoxin isolated from enterotoxigenic *Escherichia coli* strain 18D. *FEBS. Lett.* **152**:1-5.
29. Takeda, T. Unpublished data.
30. Takeda, T., G. B. Nair, K. Suzuki, and Y. Shimonishi. 1990. Production of a monoclonal antibody to *Vibrio cholerae* non-O1 heat-stable enterotoxin (ST) which is cross-reactive with *Yersinia enterocolitica* ST. *Infect. Immun.* **58**:2755-2759.
31. Takeda, T., Y. Peina, A. Ogawa, S. Dohi, H. Abe, G. B. Nair, and S. C. Pal. 1991. Detection of heat-stable enterotoxin in a cholera toxin gene positive strain of *Vibrio cholerae* O1. *FEMS Microbiol. Lett.* **80**:23-28.
32. Takeda, Y., T. Takeda, T. Yano, K. Yamamoto, and T. Miwatani. 1979. Purification and partial characterization of heat-stable enterotoxin of enterotoxigenic *Escherichia coli*. *Infect. Immun.* **25**:978-985.
33. Uesaka, Y., Y. Otsuka, M. Kashida, Y. Oku, K. Horigome, G. B. Nair, S. C. Pal, S. Yamasaki, and Y. Takeda. 1992. Detection of cholera toxin by a highly sensitive bead-enzyme linked immunosorbent assay. *Microbiol. Immunol.* **36**:43-53.
34. Yamamoto, K., Y. Takeda, T. Miwatani, and J. P. Craig. 1983. Evidence that a non-O1 *Vibrio cholerae* produces enterotoxin that is similar but not identical to cholera enterotoxin. *Infect. Immun.* **41**:896-901.
35. Yanik, B. S., and B. G. Prasad. 1954. A note on vibrios isolated in Kumbh Fair, Allahabad. *Indian Med. Gazette* **89**:341-349.
36. Yoh, M., T. Honda, and T. Miwatani. 1985. Production by non-O1 *Vibrio cholerae* of hemolysin related to thermostable direct hemolysin of *Vibrio parahaemolyticus*. *FEMS. Microbiol. Lett.* **29**:197-200.
37. Yoshimura, S., T. Takao, Y. Shimonishi, M. Arita, T. Takeda, H. Imashi, T. Honda, and T. Miwatani. 1986. A heat-stable enterotoxin of *Vibrio cholerae* non-O1: chemical synthesis, and biological and physicochemical properties. *Biopolymers* **25**:S69-S83.