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Use of a Synthetic Oligonucleotide Probe To Detect Strains of Non-Serovar O1 *Vibrio cholerae* Carrying the Gene for Heat-Stable Enterotoxin (NAG-ST)

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A synthetic oligonucleotide probe was developed to identify the gene for the heat-stable enterotoxin (NAG-ST) of non-serovar O1 *Vibrio cholerae*. Of 103 non-O1 *V. cholerae* isolates from Thailand, 31 isolates from Mexico, and 47 isolates from patients in the United States, only 7 (all from Thailand) hybridized with the probe. Probe-positive strains produced significantly higher fluid accumulations in infant mice than probe-negative strains.

Strains of *Vibrio cholerae* other than serovar O1 (non-serovar O1 *V. cholerae*) have increasingly been recognized as the causative agents of diarrheal disease. The clinical spectrum includes mild, self-limited infections as well as severe dehydrating diarrhea and dysentery (1, 3, 5, 11, 11a, 12). Less common but dramatic in appearance are extraintestinal infections, including bacteremic wound infections and primary septicemia (16). Non-O1 *V. cholerae* strains are ubiquitous in estuarine environments and are frequently found in shellfish. In one Food and Drug Administration study, non-O1 *V. cholerae* strains were cultured from 111 (14%) of 790 raw oyster samples (21).

The mechanisms responsible for the pathogenesis of non-serovar O1 *V. cholerae*, particularly in diarrheal disease, are only beginning to be elucidated. While some strains produce a cholera toxin-like toxin, these strains appear to constitute only a fraction of non-O1 *V. cholerae* isolates outside the Indian subcontinent. Other proposed virulence factors include the El Tor and Kanagawa hemolysins (7, 8), a Shiga-like toxin (15), and cell-associated hemagglutinins (4). Non-O1 strains may also produce a 17-amino-acid heat-stable enterotoxin (NAG-ST) that is closely related to the heat-stable toxins produced by enterotoxigenic *Escherichia coli* and other enteric pathogens (2, 20; S. Yamasaki, H. Ito, T. Hirayama, Y. Takeda, and Y. Shimonishi, Proc. 24th U.S.-Japan Coop. Med. Sci. Progr. Cholera Rel. Diarrheal Dis. Panel, Tokoyo, 1988) (Fig. 1).

In one Japanese study, 55% of clinical isolates gave a positive fluid accumulation response in infant mice, suggesting that the strains produced NAG-ST (2). In recent volunteer studies, illness was seen only after administration of a non-serovar O1 *V. cholerae* strain that produced NAG-ST, leading to the hypothesis that NAG-ST was a critical virulence factor for the organism (11a). The current study was undertaken to determine what percentage of clinical non-O1 *V. cholerae* isolates carried the NAG-ST gene and to see whether the frequency of carriage differed among geographic areas.

While the amino acid sequence for NAG-ST is known (20), the gene for this toxin has not yet been sequenced. In our preliminary studies, a probe derived from the sequence of *E. coli* heat-stable enterotoxin STh (17) failed to hybridize with non-serovar O1 *V. cholerae* A5 and NRT36S, both of which were known to produce NAG-ST on the basis of results from studies with infant mice and from a competitive NAG-ST enzyme-linked immunosorbent assay (11a). We then constructed a family of oligonucleotides on the basis of the amino acid sequence of NAG-ST and the known nucleotide sequences of *E. coli* heat-stable toxins STh (13) and STp (18). Information on codon preferences in vibrio species and *E. coli* was used to decide which nucleotides would be more likely for a given amino acid. Degeneracies were introduced at points in the sequence where more than one nucleotide seemed equally likely. One probe with three internal degeneracies (i.e., a pool of six oligonucleotides), designated NAG-ST1, gave excellent results in initial testing with control strains A5 and NRT36S. The nucleotide sequence of NAG-ST1 is shown in Fig. 2, together with the sequences of STh and STp. In Southern blot analysis, the probe hybridized with a single chromosomal restriction fragment of A5 and NRT36S after digestion with either *Hin*dIII or *Pst*I.

In subsequent experiments using colony blots, we screened collections of clinical non-serovar O1 *V. cholerae* isolates from Thailand, Mexico (6), and the United States with the NAG-ST1 probe. Colony blots were prepared on nitrocellulose. The NAG-ST probe was end labeled with [α-^{32}P]ATP by using a forward kinase protocol (10). Hybridization buffer consisted of either 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% bovine serum albumin, 0.5% polyvinylpyrrolidone, and 1% sodium dodecyl sulfate or 6× SSC, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin, 1 mM EDTA (pH 8), and boiled salmon sperm (100 μg/ml). Optimal results (determined by using a checkerboard procedure) were obtained with 4 to 6 h of hybridization at 50°C followed by a wash in 3× SSC at room temperature (30 min), two 15-min washes in 3× SSC with 0.1% sodium dodecyl sulfate at 50°C, and a final wash in 2× SSC for 1 h at room temperature. NRT36S

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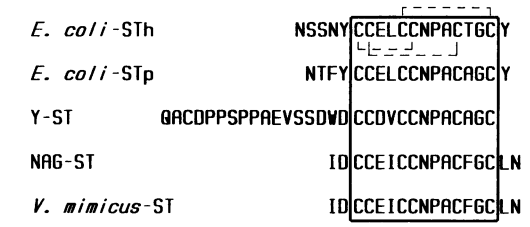

FIG. 1. Amino acid sequences of related heat-stable enterotoxins (data from Yamasaki et al. [Proc. 24th U.S.-Japan Coop. Med. Sci. Progr. Cholera Rel. Diarrheal Dis. Panel]). Box encloses regions showing a high degree of homology; intramolecular disulfide bonds are indicated by dashed lines.

and A5 were used as positive controls on all filters. An *E. coli* K-12 strain was used as the negative control.

Results are shown in Table 1. Of 181 non-serovar O1 *V. cholerae* strains tested, only 7 (4%) were positive. These seven strains were all isolated from patients in Thailand but were unrelated epidemiologically; they were isolated between 1982 and 1985 from seven children ranging in age from 1 to 9 years at three different hospitals in metropolitan Bangkok. The higher probe-positive rate among Thai strains (7 of 103) was significantly different from the rate among all other clinical strains (0 of 66) ($P = 0.043$, two-tailed Fisher's exact test).

The probe also hybridized with three of six enterotoxigenic *E. coli* strains, all three of which produced STp. This was not unexpected, given the close similarity of the probe to the gene sequence encoding STp (Fig. 2). While the probe was also closely related to STh, the single additional nucleotide difference between STp and STh, in the region corresponding to the probe, may have accounted for the lack of hybridization with STh-producing *E. coli*. Cross-reactivity with other vibrio species or other gram-negative rods was negligible: of 49 such isolates, only 1 (a *Citrobacter freundii* isolate) hybridized with the probe. Production of a heat-stable toxin has not been described for *C. freundii*, and the incidental hybridization may reflect some lack of specificity due to the internal degeneracies included in the probe design. To pursue this observation, further work using a number of *Citrobacter* isolates is necessary, since the infant mouse assay has not been standardized for this organism.

Eight strains that hybridized with the probe (including control strains NRT36S and A5) were assayed for fluid accumulation (FA) in infant mice, together with six randomly selected probe-negative Thai strains. The technique for the suckling mouse assay was modified from the method used by Nishibuchi and Seidler (14), except that inoculations were done directly into the stomach via a hypodermic needle through the body wall. Optimal results were obtained when strains were grown in Trypticase soy broth with yeast

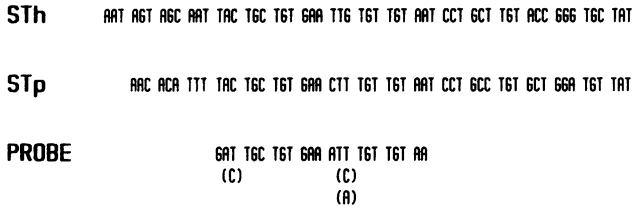

FIG. 2. Nucleotide sequences of STh and STp compared with the sequence of the NAG-ST oligonucleotide probe. Degeneracies introduced into the probe are shown in parentheses.

TABLE 1. NAG-ST synthetic oligonucleotide probe: results of colony hybridizations

Organism	No. of isolates hybridizing with probe/no. tested
Non-serovar O1 *V. cholerae* clinical isolates (no.)	
U.S.: stool (25), blood (5), wound (2), sputum (2), ear (1)	0/37[a]
U.S. (foreign travel associated): stool (8), blood (2)	0/10
Mexico (stool)	0/19
Thailand (stool)	7/103
Non-O1 *V. cholerae* environmental isolates from Mexico	0/12
Vibrio cholerae O1	0/9
Other *Vibrio* species (*V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. damsela*, and *V. fluvialis*)	0/8
Enterotoxigenic *E. coli* producing	
STh, STp, and LT	2/2
STh and LT	0/3
STp only	1/1
Other *E. coli* strains	0/8
Salmonella spp.	0/5
Shigella spp.	0/3
Other gram-negative rods (*Citrobacter*, *Edwardsiella*, *Klebsiella*, *Kluvera*, *Serratia*, *Yersinia*, *Aeromonas*, and *Enterobacter* spp. and Enteric Group 11)	1/16

[a] The source of 2 of the 37 clinical isolates was unknown.

extract at 30°C. Each strain was tested in five suckling mice. The FA ratio was defined as gut weight divided by remaining body weight. Table 2 shows the mean FA ratios obtained for each strain. Results showed some overlap in mean FA index between groups and in some cases wide standard deviations, preventing us from establishing a definitive cutoff value for NAG-ST production. However, overall the FA ratios for probe-positive strains were significantly higher than those

TABLE 2. Non-serovar O1 *V. cholerae*: fluid accumulation in suckling mice

Strain	Probe result	Mean FA ratio (SD)[a]
NRT36S	Positive	0.078 (0.014)
A5	Positive	0.080 (0.014)
OAE60-1	Positive	0.082 (0.010)
L48B	Positive	0.069 (0.012)
D82	Positive	0.082 (0.013)
C211	Positive	0.081 (0.011)
C677	Positive	0.088 (0.016)
C711	Positive	0.083 (0.012)
OAE07-1	Negative	0.067 (0.011)
L48A	Negative	0.062 (0.006)
D240	Negative	0.062 (0.003)
C351	Negative	0.068 (0.004)
C590	Negative	0.074 (0.013)
C689	Negative	0.063 (0.007)

[a] Mean for five mice per determination.

for probe-negative strains ($P = 0.0001$, two-level nested analysis of variance on rank data).

In summary, we have developed a synthetic oligonucleotide probe on the basis of the amino acid sequence of NAG-ST that appears to identify non-serovar O1 *V. cholerae* strains carrying the NAG-ST gene. In Southern blot analysis, the probe detected what appeared to be a single gene copy in control strains. As a group, probe-positive strains produced significantly greater fluid accumulation in infant mice than did probe-negative strains. The overlap in mean FA index between probe-positive and probe-negative strains may reflect problems with gene expression in vitro or difficulties with the suckling mouse assay for detection of the heat-stable toxin from non-O1 *V. cholerae*. While Japanese investigators have been successful in using suckling mouse assays for the detection of NAG-ST (2), questions about the optimal conditions for these assays remain.

Overall, only 4% of the clinical non-serovar O1 *V. cholerae* strains in this series hybridized with the probe. Previous studies, using suckling mouse assays designed for enterotoxigenic *E. coli*, have also reported low rates of NAG-ST production among non-O1 *V. cholerae* isolates (12, 19). These data differ from the previously noted reports from Japan, in which up to 55% of clinical non-O1 isolates gave positive fluid accumulation responses in suckling mice (2, 7). It is possible that the fluid accumulation responses seen in these studies were in some cases due to toxins other than NAG-ST. Alternatively, there may be regional differences in the production of NAG-ST among non-O1 strains. Of note, the Japanese strains in many instances were obtained from patients with traveler's diarrhea returning from Southeast Asia; if NAG-ST is an important virulence factor for non-O1 *V. cholerae*, one might expect to find an increased frequency of NAG-ST–producing strains among symptomatic persons returning from an area (such as Thailand) where such strains are known to be present.

While case series and volunteer studies have confirmed that non-serovar O1 *V. cholerae* strains are important pathogens (11a, 12) and suggested that NAG-ST production may be associated with virulence (11a), the critical virulence factors remain ill defined. If NAG-ST is the essential virulence factor for non-O1 *V. cholerae*, it would appear that only a small percentage of clinical strains in many geographic areas are potentially pathogenic. Alternatively, and perhaps more likely, virulent non-O1 *V. cholerae* strains produce one or more of a variety of different toxins (with a variety of regional distributions), analogous to the heterogeneity seen among diarrhea-associated *E. coli* strains (9). Further studies, including studies of isogeneic mutants from which specific toxin genes have been deleted, are necessary to clearly define the role of NAG-ST as well as other toxins in the pathogenesis of non-O1 strains.

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