Identification of Heat-Stable Enterotoxin-Producing Strains of Yersinia enterocolitica and Vibrio cholerae Non-O1 by a Monoclonal Antibody-Based Enzyme-Linked Immunosorbent Assay

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Abstract: Using a mouse monoclonal antibody (MAb) 2F raised against Vibrio cholerae non-O1 heat-stable enterotoxin (NAG-ST) which also recognizes a shared epitope of Yersinia enterocolitica heat-stable enterotoxin (Y-ST), a competitive enzyme-linked immunosorbent assay (ELISA) was developed for independent detection of NAG-ST and Y-ST. There was good concordance between the Y-ST ELISA and the sucking mouse assay (SMA) for detection of Y-ST from test strains of Y. enterocolitica, and the Y-ST ELISA can effectively replace the SMA for routine detection of Y-ST. On the contrary, evaluation of the NAG-ST ELISA and the SMA using 139 strains of V. cholerae non-O1 showed discordant results and this was attributed to the presence of the sucking mice active factor(s) such as El Tor hemolysin and to the production of low amounts of NAG-ST. Concentration of culture supernatants of V. cholerae non-O1 followed by heating at 100°C was essential to obtain reproducible results by both the NAG-ST ELISA and the SMA. The ELISA developed in this study can be used for the identification of biologically active strains. While recently genetic methods such as polymerase chain reaction became available and were very reliable and simple techniques, the ELISA in this study has an advantage in detecting biologically toxic gene products of the strains. The genetic methods cannot differentiate silent STa genes which we often encounter in the case of Y. enterocolitica.

Key words: Heat-stable enterotoxin, Enzyme-linked immunosorbent assay (ELISA), Vibrio cholerae, Yersinia enterocolitica

Yersinia enterocolitica and Vibrio cholerae non-O1 are frequently associated with human gastroenteritis (4, 7, 9, 13). Among other virulence factors, both these enteropathogens are known to produce an infant mouse active, methanol-soluble heat-stable enterotoxin (STa). The Y. enterocolitica STa (Y-ST) is a 30 amino acid peptide (16) while the V. cholerae non-O1 STa (NAG-ST) is a 17 amino acid peptide (17) and both the toxins exhibit remarkable similarity, especially in the carboxyl terminal toxic domain, to the STas (STh and STp) produced by enterotoxigenic Escherichia coli (16, 17). It is now clear that the STas comprise an expanding family of structurally, functionally and immunologically related peptide toxins which bind specifically and reversibly to a receptor found in the microvillus membranes of the intestinal cell brush border (5, 14) stimulating fluid secretion via receptor-mediated activation of guanylate cyclase (3).

The production of Y-ST by strains of Y. enterocolitica has been associated with yersiniosis (7, 13). Likewise, a recent human volunteer study has clearly shown that NAG-ST plays a role in the pathogenesis of non-O1 V. cholerae gastroenteritis (8). However, the epidemiological importance of

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Abbreviations: BHIB, brain heart infusion broth; ELISA, enzyme-linked immunosorbent assay; MAb, monoclonal antibody; MED, minimum effective dose; NAG-ST, Vibrio cholerae non-O1 heat-stable enterotoxin; NRS, normal rabbit serum; PB, phosphate buffer; PBS, phosphate buffered saline; SMA, sucking mouse assay; STa, heat-stable enterotoxin; Y-ST, Yersinia enterocolitica heat-stable enterotoxin.
Y-ST- and NAG-ST-producing strains of *Y. enterocolitica* and *V. cholerae* non-O1, respectively, and the magnitude of occurrence of these strains in the environment, foods and among clinical cases are still uncertain. This hiatus in information stems, in part, from the lack of a simple, accurate and highly sensitive assay system for detection of Y-ST and NAG-ST. Currently, the only available test for identification of Y-ST- and NAG-ST-producing strains is the suckling mouse assay (SMA), which is not suitable for screening several strains at a time. In addition, the presence of a suckling mouse active heat-labile El Tor hemolysin in most *V. cholerae* non-O1 isolates (6, 10) complicates the interpretation of the results.

Immunodetection of heat-stable enterotoxin has always been a problematic issue owing to the non-immunogenic nature of the toxin in its natural state and to the consequent difficulties in producing homogeneous, high-titered antibody preparations. Monoclonal antibodies (MAbs) against *E. coli* STh and STp have proven to be valuable and sensitive reagents in the development of immunoassays for *E. coli* STas (2, 15, 21). However, these assays cannot be used for the detection of Y-ST or NAG-ST due to the high specificity of the MAbs. The present study is a continuation of our previous efforts in which we successfully raised a MAb against NAG-ST that fortuitously cross-reacted with Y-ST since the MAb recognized a shared epitope (19). In this study, the MAb was used to develop a competitive enzyme-linked immunosorbent assay (ELISA) to identify Y-ST- and NAG-ST-producing strains.

**Materials and Methods**

*Enterotoxin preparation.* NAG-ST and a short analog of Y-ST (Y-ST[14-30]) were synthesized as described in detail elsewhere (18, 22, 23) and the synthetic preparations were used in this study.

*Bacterial strains.* NRT36, a NAG-ST-producing strain (8) of *V. cholerae* non-O1 isolated from the diarrheal stool of a patient at the Narita airport quarantine station and from which the NAG-ST gene was cloned and sequenced previously (11), was used in the preliminary experiments to standardize the competitive ELISA. For the evaluation of the performance of the ELISA developed in this study versus the suckling mouse assay, a total of 137 strains of *Y. enterocolitica* (110 from humans with diarrhea, 20 from porcine and 7 from canine origin) belonging to serotypes O3 (112 isolates), O5 (12 isolates), O8 (9 isolates) and O9 (4 isolates) obtained from the culture collection of T. Maruyama (The National Institute of Public Health, Tokyo) and 139 strains of *V. cholerae* non-O1 isolated from patients with traveler's diarrhea at the Narita airport, Tokyo (obtained from Kensuke Nakajima, Narita Airport Quarantine Station, Chiba, Japan) were used in this study.

**Cultural conditions.** Culture filtrates of the *Y. enterocolitica* and *V. cholerae* non-O1 strains were prepared as described previously (10, 12). For concentration of NAG-ST from culture supernatants, the test strains were grown in 450 ml of brain heart infusion broth (BHIB) containing 0.5 % NaCl using 1 liter Erlenmeyer flasks for 24 hr at 37°C with shaking. The culture supernatant was adjusted to 60% saturation of ammonium sulfate with stirring and the resulting suspension was kept overnight in a refrigerator. After centrifugation (16,300 × g for 30 min at 4°C), the precipitate was dissolved in a small amount of distilled water, extensively dialyzed against distilled water, added with the water up to 15 ml (approximately 30-fold concentration), heated at 100°C for 5 min and this was used as the sample.

**Monoclonal antibody.** A recently raised mouse monoclonal antibody (MAb) 2F against synthetic NAG-ST which recognizes aspartic acid located at position 2 from the N-terminus of NAG-ST (19). The reactivity of the MAb with Y-ST was related to the presence of aspartic acid in Y-ST in the same corresponding position as that of NAG-ST (19).

**Competitive ELISA.** Flexible 96-well polyvinyl chloride microtiter plates (Falcon: Becton Dickinson Labware, Oxnard, Calif., U.S.A.) were directly coated with 0.1 ml per well of NAG-ST (1 μg/ml) at 37°C for 2 hr, and processed as described (19). Solid-phase NAG-ST was used for both the NAG-ST and Y-ST detection. In each plate, synthesized NAG-ST (1 μg/ml) and phosphate buffered saline (PBS) were used as the positive and negative controls, respectively.

**Suckling mice assay.** The SMA was performed as described previously (19) to evaluate the biological activity.

**Determination of hemolytic activity of V. cholerae non-O1.** Washed rabbit erythrocytes diluted to a final concentration of 1 % (v/v) in 10 mM phosphate buffer (PB) (pH 7.0) containing 1.3% NaCl was mixed 1 : 1 with culture supernatants of *V. cholerae* non-O1 and incubated at 37
C for 1 hr. The mixture was subsequently centrifuged at 1,000×g for 5 min and the amount of released hemoglobin in the supernatant was measured spectrophotometrically at 540 nm.

Results

The titration plot of a competitive ELISA for synthetic NAG-ST and Y-ST[14-30] is presented in Fig. 1. The ELISA detects 20 ng/ml and 70 ng/ml (1 ng and 3.5 ng/well) of NAG-ST and Y-ST[14-30] respectively when the cut-off point is fixed at 40% inhibition. The minimum effective dose (MED) in the suckling mouse for NAG-ST and for Y-ST[14-30] were reported to be 5 ng (1) and 25 ng (23), respectively. The ELISA is more sensitive than the SMA, and at a concentration of MED the inhibition was distinct (more than 50%) for both the toxins. The 139 strains of V. cholerae non-O1 and the 137 strains of Y. enterocolitica were then subjected to the ELISA. While standardizing the ELISA, it was found that the addition of 10% volume of 1M Tris-HCl (pH 7.4) to the Y. enterocolitica culture supernatants (data not shown) prior to performing the ELISA vastly improved the efficiency of the assay. Of the 137 strains of Y. enterocolitica examined, 83 (60.6%) strains were identified positive in the mouse assay and 79 strains of these showed more than 40% inhibition in the ELISA (Fig. 2), while 35 (25.5%) strains were negative in the SMA and inhibited less than 40% in the ELISA. There were 19 (13.9%) strains which showed intermediate values by both the assays. There were four strains which were toxic in the mouse but clearly negative in the ELISA. Recently we found these four strains comprise a new subtype of Y-ST (paper submitted). There was fair correlation between results of both the assays for identification of Y-ST-producing strains of Y. enterocolitica.

In striking contrast, culture filtrates of V. cholerae non-O1 were difficult to manage. As shown in Table 1, the ingredients of BHIB (used for culturing V. cholerae non-O1) did not interfere with the binding of MAb2F since a mixture of synthesized NAG-ST and BHIB showed 77% inhibition. However, culture supernatants of the NAG-ST-producing reference strain NRT36 did not adequately inhibit the binding (33% inhibition) while in the presence of heat-inactivated normal rabbit serum (NRS) the inhibition value (57.2% inhibition) of NRT36 culture supernatant was much higher. Addition of NRT36 culture filtrate to

![Graph 1](image1.png)

**Fig. 1.** Standard curve for synthetic Y-ST[14-30] and NAG-ST using the monoclonal antibody-based competitive ELISA. Each point represents mean of three values with standard error obtained by three experiments. Closed box represents Y-ST[14-30] while open box represents NAG-ST.

![Graph 2](image2.png)

**Fig. 2.** Comparison of results obtained with Y-ST ELISA and suckling mouse assay in parallel tests with 137 different Y. enterocolitica strains.
NAG-ST did not increase the inhibition but decreased the value, indicating that V. cholerae non-O1 released something non-specific reacting factor. The addition of 10% volume of NRS to NRT36 culture filtrate improved the efficiency of the ELISA (Table 1). However, other strains of V. cholerae non-O1 gave quite discordant results even after the treatment with NRS. Examination of the 139 strains of V. cholerae non-O1 for the hemolysin revealed that 117 (84.2%) were hemolytic, and we could not optimize the ELISA for NAG-ST-producing strains using culture supernatant.

To avoid the disparity in results between the ELISA and SMA for identification of NAG-ST-producing strains of V. cholerae non-O1, further studies were performed using four NAG-ST DNA probe (during the later part of this study, it became available) (20) positive strains and five randomly selected gene negative strains (see Table 2) of V. cholerae non-O1. Aliquots of 30-fold concentrated culture filtrates of the strains were heated at 100 C for 5 min and were examined by the ELISA and SMA. From the results shown in Table 2, the heated samples of the four gene positive strains gave positive fluid accumulation ratios in the SMA and also inhibited the binding of MAb2F to the solid-phase NAG-ST. On the other hand, the five gene negative strains gave negative reactions in both the assays. The treatment of the filtrate by 60% ammonium sulfate followed by heating was efficient to eliminate the non-specific reacting factor in the filtrate of V. cholerae non-O1. Two out of four NAG-ST gene positive strains were positive by both the assays only after the concentration of the culture filtrate. The results revealed complete concordance between the ELISA and SMA but the need for concentrating culture supernatants

**Table 1. Effect of addition of heat-inactivated normal rabbit serum (NRS) to various combinations of samples prior to performing the competitive ELISA for detection of NAG-ST. Values are the means of three experiments with standard error**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance at 492 nm</th>
<th>Percent inhibition</th>
</tr>
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<tbody>
<tr>
<td>Phosphate buffered saline (pH 7.2)</td>
<td>1.63±0.188</td>
<td>0±11.5</td>
</tr>
<tr>
<td>Brain heart infusion broth (BHB)</td>
<td>1.244±0.254</td>
<td>23.8±15.6</td>
</tr>
<tr>
<td>BHB+Normal rabbit serum (NRS)</td>
<td>1.394±0.169</td>
<td>14.6±10.3</td>
</tr>
<tr>
<td>NRT36 culture supernatant</td>
<td>1.094±0.121</td>
<td>33.0±7.4</td>
</tr>
<tr>
<td>NRT36 culture supernatant+NRS</td>
<td>0.699±0.284</td>
<td>57.2±17.4</td>
</tr>
<tr>
<td>Synthesized NAG-ST*+BHB</td>
<td>0.376±0.097</td>
<td>77.0±5.9</td>
</tr>
<tr>
<td>Synthesized NAG-ST+NRT36 supernatant</td>
<td>0.514±0.108</td>
<td>68.5±6.6</td>
</tr>
<tr>
<td>Synthesized NAG-ST+NRT36 culture supernatant+NRS</td>
<td>0.446±0.095</td>
<td>72.7±5.8</td>
</tr>
</tbody>
</table>

*Amount of NAG-ST was adjusted to 1 µg/ml. The ELISA was performed as given in the “Materials and Methods.”

**Table 2. Results of the NAG-ST ELISA for detection of NAG-ST from 30-fold concentrated and heated culture supernatants of V. cholerae non-O1 as compared with the suckling mouse assay (SMA)**

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>NAG-ST* gene</th>
<th>ELISA% Inhibition</th>
<th>SMA FA ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>+</td>
<td>88.0±6.2</td>
<td>0.157±0.013</td>
</tr>
<tr>
<td>36</td>
<td>+</td>
<td>82.5±10.1</td>
<td>0.101±0.011</td>
</tr>
<tr>
<td>99</td>
<td>+</td>
<td>78.7±11.5</td>
<td>0.104±0.008</td>
</tr>
<tr>
<td>118</td>
<td>+</td>
<td>80.5±5.8</td>
<td>0.143±0.025</td>
</tr>
<tr>
<td>17</td>
<td>−</td>
<td>17.2±9.4</td>
<td>0.067±0.006</td>
</tr>
<tr>
<td>20</td>
<td>−</td>
<td>25.8±13.0</td>
<td>0.062±0.007</td>
</tr>
<tr>
<td>79</td>
<td>−</td>
<td>&lt;0±5.4</td>
<td>0.066±0.002</td>
</tr>
<tr>
<td>80</td>
<td>−</td>
<td>12.2±7.7</td>
<td>0.068±0.010</td>
</tr>
<tr>
<td>117</td>
<td>−</td>
<td>&lt;0±1.3</td>
<td>0.050±0.003</td>
</tr>
</tbody>
</table>

* a) Colony hybridization with a polynucleotide probe was used as described (20).
  b) Mean of four experiments±S.E.
  c) Mean of five mice±S.E.
of *V. cholerae* non-O1 followed by heating was mandatory to obtain reliable results with the NAG-ST competitive ELISA developed in this study.

**Discussion**

This study endeavored to develop a competitive ELISA for screening of Y-ST- and NAG-ST-producing strains of *Y. enterocolitica* and *V. cholerae* non-O1, respectively, using a high affinity MAb. The development of the Y-ST competitive ELISA did not pose any problems. Addition of 10% volume of 1 M Tris-HCl was required to optimize the pH of the culture supernatant of *Y. enterocolitica* before performing the ELISA. There were four inconsistent strains which were positive by the SMA but negative by the ELISA. But they were found to produce a new subtype of Y-ST because their biological activity was heat stable and their molecular weight was less than 10,000 (paper submitted).

On the other hand, the development of the NAG-ST ELISA was a difficult proposition. During standardization of the NAG-ST ELISA, it became evident that some hitherto unidentified factor released by cells of *V. cholerae* non-O1 reacted non-specifically with MAb2F and obliterated the free NAG-ST in the culture supernatant to bind with MAb2F. This problem was surmounted by the addition of heat-inactivated NRS which apparently neutralized the interfering factor. The inherent low production of NAG-ST by wild strains of *V. cholerae* non-O1 (1) and the production of the hemolysin appear to be the two main reasons for the discordant results. The SMA is, obviously, not a suitable reference assay to evaluate the ELISA for detection of NAG-ST. Heating of the culture supernatant of *V. cholerae* non-O1 at 100°C for 5 min to eliminate the heat-labile hemolysis before introducing into the sucking mice may not solve the problem owing to the reported heat fragility of the NAG-ST in its crude state (1). The NAG-ST competitive ELISA developed in this study cannot be used for routine screening of broth cultures of *V. cholerae* non-O1. However, the NAG-ST ELISA can be used to authenticate NAG-ST-producing strains of *V. cholerae* non-O1 for which genetic techniques cannot be substituted.

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**References**


