MOLECULAR CHARACTERISATION OF BACTERIA

Prevalence of *Vibrio cholerae* with heat-stable enterotoxin (NAG-ST) and cholera toxin genes; restriction fragment length polymorphisms of NAG-ST genes among *V. cholerae* O serogroups from a major shrimp production area in Thailand

A. DALSGAARD, O. SERICHANTALERGS*, T. SHIMADA†, O. SETHABUTR* and P. ECHEVERRIA*

Section of Fish Diseases, Department of Veterinary Microbiology, Royal Veterinary and Agricultural University, DK-1870 Frederiksberg C, Denmark, * Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand and † Department of Bacteriology, National Institute of Health, Shinjuku-ku, Tokyo 162, Japan

Summary. A total of 148 *Vibrio cholerae* isolates from a major shrimp production area in Southern Thailand were examined by colony hybridisation for genes encoding heat-stable enterotoxin (NAG-ST) and cholera toxin (CT). Only non-O1 *V. cholerae* strains were found to harbour NAG-ST (14 of 146) whereas no strains hybridised with the CT probe. NAG-ST-positive *V. cholerae* non-O1 strains were isolated from shrimp farms situated close to urban areas. Five different O serogroups were found among NAG-ST positive non-O1 strains. Southern blot and restriction endonuclease analysis of NAG-ST-positive strains revealed a high degree of genetic divergence. A total of seven classes of enterotoxin gene patterns were found with *Hind*III and *Eco*RI restriction endonucleases. Enterotoxin gene patterns correlated with O-antigen expression in 84% of isolates tested. In combination with other molecular techniques Southern blot analysis with an NAG-ST oligonucleotide probe could be useful for studying the molecular epidemiology of *V. cholerae* non-O1 strains.

Introduction

Vibrio cholerae is an autochthonous bacterium found in aquatic environments^{1,2} and has been associated with cholera and cholera-like diseases as well as extra-intestinal infections.^{3,4} Human infections with *V. cholerae* are most often associated with seafood consumption, exposure to polluted fresh water, brackish water or seawater, and foreign travel.^{4,5}

The production and export of cultured shrimps from developing countries are important commercial activities.⁶ Thus, the presence of *V. cholerae* in marine waters is of great concern for both seafood exporting and importing countries since most importing countries will detain any shrimp products that contain pathogenic *V. cholerae*. A previous study of brackish water shrimps for the presence of human pathogenic vibrios showed *V. cholerae* to be part of the normal microflora in shrimp ponds.^{7,8} Unfortunately, there is

no information about the pathogenicity and serology of these *V. cholerae* isolates.⁷ There is little information about the epidemiology of enterotoxigenic *V. cholerae* in areas where shrimp culture is practised.

Environmental strains of *V. cholerae* O1 and non-O1 are often less pathogenic than clinical isolates, i.e., do not produce cholera toxin (CT) or heat-stable enterotoxin (NAG-ST).^{9,10} Thus, before evaluating whether the presence of *V. cholerae* in shrimp culture is a public health risk, the pathogenic properties of the isolates were determined.

This study was performed to determine the prevalence of *V. cholerae* containing genes that encode two major enteric toxins, NAG-ST and CT,^{11–13} among a collection of strains isolated previously from a major shrimp production area in Southern Thailand. The O serogroups of NAG-ST positive *V. cholerae* non-O1 strains were determined and these strains were examined with chromosomal digests and Southern blot analysis to determine their relationship to each other and to similar clinical and environmental isolates.

Materials and methods

Bacterial strains

A total of 146 *V. cholerae* non-O1 and two O1 isolates from 16 sites located in a major shrimp production area in Southern Thailand was included in colony hybridisation studies. The characterisation and identification of the strains has been reported.¹⁴ All NAG-ST-positive *V. cholerae* non-O1 strains¹⁵ were submitted to Southern blot analyses (table). In addition, the following NAG-ST-positive isolates were included in the Southern blot analyses: strains OAE60-1, L48B, D82, C211, C677, C711 and IC-210, all of which were isolated from patients with diarrhoea in Thailand;¹⁵ strains 1005 and 1145 were isolated in Thailand from food samples (table).

Colony hybridisation

All isolates were examined by the colony hybridisation technique for DNA sequences that encode NAG-ST and CT with oligonucleotide probes consisting of 16 and 23 bp, respectively.¹⁶⁻¹⁸ The probes were labelled with either alkaline phosphatase¹⁸ or [γ -³²P]ATP as described earlier.^{15,16} Pre-hybridisation and hybridisation were performed as reported previously.^{15,20,21} Colony hybridisation filters were exposed to X-Omat X-ray film (Kodak, Rochester, NY, USA) or developed colorimetrically²² for [γ -³²P]ATP and alkaline phosphatase-labelled probes, respectively. NAG-ST-producing *V. cholerae* non-O1 strains, NRT36S²³ and A5¹² and CT-producing *V. cholerae* strains, 889²⁰ and MS371²⁴ were used as positive controls on all filters. An *Escherichia coli* K12 strain was used as a negative control.

Serotyping

V. cholerae non-O1 strains were examined serologically by the slide agglutination test and designated according to the O serogroup system established by Shimada *et al.* (O1-O155).²⁵

Chromosomal DNA preparation and Southern blots

Chromosomal DNA was extracted by the method of Murray and Thompson.²⁶ The DNA preparations were digested with *Eco*RI and *Hind*III restriction endonucleases (Gibco BRL). A 1-kb DNA ladder (Gibco BRL) was used as a molecular size standard. After electrophoresis, the DNA fragments were transferred to a nylon membrane (Hybond N, Amersham International plc, Amersham) by the technique of Southern²⁷ with a vacuum blotter (BioRad Laboratories, Richmond, CA, USA) and an alkali blotting procedure (0.4 M NaOH). Nylon membranes were baked, pre-hybridised and hybridised with the [γ -³²P]ATP-labelled NAG-ST probe as previously described.¹⁵ The membranes were exposed to X-Omat X-ray film overnight at -70°C and the film was

developed according to the manufacturer's instructions.

Results

Of the 148 *V. cholerae* strains tested by the colony hybridisation technique, none hybridised with the CT probe. Fourteen (9.6%) of 146 *V. cholerae* non-O1 isolates hybridised with the NAG-ST or CT probes. The two *V. cholerae* O1 isolates contained no genes that hybridised with the NAG-ST probe.

NAG-ST positive *V. cholerae* non-O1 strains were not isolated from any shrimp samples (16 sampling sites), but were isolated from water and sediment samples at sites 1, 3 and 8. All three sites are close to major urban areas. Site 1 is in an open freshwater lake. Sites 3 and 8 are coastal, with a small river flowing into the sea at site 8. The river received large amounts of domestic waste from a fishing village. Furthermore, site 8 is a popular recreational area.

The O serogroups of NAG-ST positive non-O1 *V. cholerae* are shown in the table. Of the 25 *V. cholerae* non-O1 isolates studied, 23 (92%) of 25 were serotyped, representing five different O serogroups.

The results of Southern blot analyses with the NAG-ST probe are shown in the table. Twenty *V. cholerae* non-O1 strains contained a single copy of the NAG-ST gene with one *Hind*III fragment and one *Eco*RI fragment possessing sequences homologous to the gene probe. When digested with *Eco*RI, strain 63 appeared to contain a duplication of the NAG-ST gene, with two fragments hybridising with the gene probe. Strains 109, 116 and 117 contained no sequences homologous to the NAG-ST probe. In the colony hybridisation studies, neither of these strains hybridised with the alkaline phosphatase-labelled probe and they showed only a weak signal when hybridised with the ³²P-labelled NAG-ST probe (data not shown). All three strains were confirmed to be NAG-ST positive in subsequent studies when nitrocellulose filters with large colony spots were used in colony hybridisation with the ³²P-labelled probe.

Several enterotoxin gene patterns were seen among the *V. cholerae* non-O1 strains. A total of seven classes of enterotoxin gene patterns was suggested (table). The first class consisted of three strains (strains 3, 4 and 17) isolated from coastal water samples obtained at shrimp farm 1, all of which possessed a *Hind*III fragment of c. 1.2 kb. Strains 3 and 4 were from different coastal water samples, whereas strains 17 and 4 were from the same coastal water sample. The second class consisted of two strains isolated from different coastal water samples obtained at shrimp farm 1. Both strains possessed a *Hind*III fragment of c. 4.1 kb and were isolated at a different sampling time compared to the first class of gene patterns. Two strains (63 and 1145) are included in the third class and possessed a *Hind*III fragment of c. 1.8 kb. Strain 63

Table. Serology and results of colony hybridisation and Southern blot analysis of *V. cholerae* non-O1 strains with an NAG-ST oligonucleotide probe

Strain no.	Source	Hybridisation with an NAG-ST probe	Restriction patterns			O serogroup†
			Class*	<i>Hind</i> III (kb)	<i>Eco</i> RI (kb)	
3	Coastal water	+	1	1.2	1.7	O14
4	Coastal water	+	1	1.2	1.7	O14
17	Coastal water	+	1	1.2	1.7	O14
33a	Coastal water	+	2	4.1	5.8	O144
33b	Coastal water	+	2	4.1	5.8	O144
63	Coastal water	+	3	1.8	3.8; 14.0	O6
101	Coastal water	+	4	18.0	3.8	O14
106	Shrimp hatchery water	+‡	4	18.0	§	O47
107	Shrimp hatchery water	+	4	18.0	3.8	O14
109	Shrimp hatchery water	+‡	—	—	—	O47
114	River bed	+	5	1.5	15.0	O69
116	River bed, sediment	+‡	—	—	—	O47
117	River bed, sediment	+‡	—	—	—	O47
126	River bed, sediment	+	5	1.5	15.0	O69
1005	Food	+	7	2.4	17.5	ND
1145	Food	+	3	1.8	3.0	O6
IC-210	Stool	+	4	18.0	15.0	O14
C-711	Stool	+	6	3.2	3.8	O6
C-677	Stool	+	6	3.2	3.8	O6
C-211	Stool	+	6	3.2	3.8	O6
D-82	Stool	+	6	3.2	3.8	O6
L-48B	Stool	+	4	18.0	15.0	O14
OAE-60-1	Stool	+	6	3.2	3.8	ND
A5	Shrimp	+	4	18.0	15.0	O14
NRT-36S	Stool	+	4	18.0	15.0	O14

ND, not determined.

*Designation of enterotoxin gene patterns.

†O serogroup designations according to Shimada *et al.*²⁴‡Strains hybridised with [γ -³²P]ATP-labelled NAG-ST probe only.

§Strains did not hybridise with the NAG-ST probe in the Southern blot analyses.

was from a coastal water sample obtained at shrimp farm 8, whereas strain 1145 was from a food sample with no geographical relationship to strain 63. The fourth class consisted of three coastal water isolates (strains 101, 106 and 107), two stool isolates (strains IC-210 and L-48B) and two reference strains (strains A5 and NRT36S), all of which possessed *Hind*III fragment of *c.* 18.0 kb. Strain 101 was isolated from coastal water, whereas strains 106 and 107 were isolated from shrimp hatchery water samples with all samples obtained at shrimp farm 3. Two strains (114 and 126) with a *Hind*III fragment of *c.* 1.5 kb were included in the fifth class. Both strains were isolated from water and sediment samples from a river bed, respectively. The sixth class contained five epidemiologically unrelated stool isolates,¹⁴ all of which possessed a *Hind*III fragment of *c.* 3.2 kb. The seventh class consisted of strain 1005, a food isolate, which possessed a 2.4-kb *Hind*III fragment. The results of the Southern blot analysis, when chromosomal DNA was digested with the restriction endonuclease *Eco*RI and probed with the ³²P-labelled NAG-ST probe, are shown in the table. Seven classes of enterotoxin gene patterns were found in the *Eco*RI digests.

When comparing results from serotyping and Southern blot analysis, nine isolates belonging to serogroup O14 showed enterotoxin gene class 1

(strains 3, 4 and 17) and six were class 4 (strains 101, 107, IC-210, L-48B, A5 and NRT-36S). Enterotoxin gene class 4 was also shown by one of four isolates of serogroup O47. Six isolates that showed two different classes of enterotoxin gene patterns belonged to serogroup O6. Isolates belonging to serogroups O69 and O144 presented enterotoxin gene classes 5 and 2, respectively.

Discussion

We previously reported a low prevalence of *V. cholerae* O1 (two of 111 sites) in all sample types obtained from a shrimp-producing area in Southern Thailand.¹⁴ A low prevalence of *V. cholerae* in brackish water environments has been described earlier.^{1,2} The two *V. cholerae* O1 isolates were CT-negative and were isolated from a shrimp pond water sample and a coastal water sample, respectively. Hence, toxigenic *V. cholerae* O1 does not seem to constitute part of the normal bacteriological flora of the aquatic environments where shrimp culture is practised in Southern Thailand.

In all, 14 of 146 non-O1 *V. cholerae* isolates hybridised with the NAG-ST probe. NAG-ST-positive strains were isolated from samples obtained at sites

situated close to urban areas only, indicating that shrimp production in these areas might constitute a possible threat to public health. However, it should be noted that NAG-ST non-O1 strains were not isolated from any shrimp samples. Furthermore, the infectious dose for *V. cholerae* is usually high ($\geq 10^6$ organisms).²⁷ The finding of identical O serogroups (O6 and O14) among previously isolated clinical strains¹⁴ and strains from coastal water samples is interesting and may be of public health relevance.

We reported earlier that seven of 103 clinical *V. cholerae* non-O1 isolates from Thailand and none of 78 clinical and environmental isolates from Mexico and the USA hybridised with an NAG-ST probe.¹⁵ We isolated non-O1 *V. cholerae* also from 15 of 215 patients with diarrhoea from an epidemic of a cholera-like disease in a refugee camp in Thailand. Five of 15 of these non-O1 isolates produced NAG-ST.²⁰ Most previous studies have reported lower rates of NAG-ST positivity amongst *V. cholerae* non-O1 isolates.^{8,15,29} Pal *et al.*²⁹ found that 2.3% and none of 521 environmental non-O1 isolates hybridised with an NAG-ST and a CT probe, respectively,²⁹ whereas Nair *et al.*⁸ reported that none of 107 *V. cholerae* non-O1 isolates from brackish water shrimps hybridised with a CT probe or produced NAG-ST. The epidemiological importance of the relatively high prevalence of NAG-ST-positive *V. cholerae* non-O1 reported in the present study and in our previous studies in Thailand, compared with other geographical areas, needs further investigation.

V. cholerae O139, the cause of the present epidemic of diarrhoea in India and Bangladesh, exhibits most characteristics of *V. cholerae* O1 including the ability to produce cholera toxin.^{30,31} Since none of the 146 *V. cholerae* non-O1 isolates included in this study contained genes that encode cholera toxin, agglutination of these isolates with O139 antisera was not performed.

The establishment of consistent classes of enterotoxin gene patterns with *Hind*III and *Eco*RI restriction endonucleases suggests that both enzymes could be used to study the epidemiology of *V. cholerae* non-O1 strains.

The finding of seven classes of enterotoxin gene patterns indicates a high evolutionary diversity among *V. cholerae* non-O1 strains. This is supported by a

study of *V. cholerae* non-O1 restriction digest patterns of chromosomal DNA and Southern blot analysis with a cholera toxin gene probe and reveals that *V. cholerae* non-O1 strains exhibited greater genetic diversity than the highly conserved *V. cholerae* O1.³² In a recent study based on Southern hybridisation with an NAG-ST probe of 12 *V. cholerae* non-O1 strains, two groups of hybridisable *Hind*III fragments were reported.²⁹ One group of strains exhibited a hybridisable fragment similar to that of the NRT36S reference strain and a smaller *Hind*III fragment hybridised with the probe in the other group of strains. The possibility that different chromosomal positions of NAG-ST genes influence the expression of the genes needs further investigation.

We reported earlier that strains D-82, C-211, C-677 and C-711 were epidemiologically unrelated.¹⁵ However, since all strains showed identical enterotoxin gene patterns (class 6) and belonged to the same serogroup (O6), a very close genetic relationship among these strains is likely.

The results of the present study show for the first time a correlation between O serogroups and classes of NAG-ST enterotoxin gene patterns. On the basis of Southern blot analyses and serotyping, the isolates were divided into seven and five groups, respectively. In one case only, isolates that showed an identical enterotoxin gene class 4 belonged to two different O serogroups (O14 and O47). Serogroups O6 and O14 both contained isolates that showed two enterotoxin gene classes.

All *V. cholerae* non-O1 strains belonging to class 1 or class 2 were isolated only from site 1 whereas strains belonging to class 5 were isolated only from site 3. However, since environmental and clinical *V. cholerae* non-O1 isolates, without any known epidemiological relatedness, were grouped in the same gene pattern classes (classes 3 and 4), Southern blot analysis with an NAG-ST oligonucleotide probe should be used in combination with other molecular techniques when studying the epidemiology of *V. cholerae* non-O1.

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