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# Cholera Enterotoxin Production in *Vibrio cholerae* O1 Strains Isolated from the Environment and from Humans in Japan

AKIHIRO MINAMI,[1]† SATORU HASHIMOTO,[1] HISAO ABE,[1]‡ MICHIKO ARITA,[2] TOORU TANIGUCHI,[2] TAKESHI HONDA,[2] TOSHIO MIWATANI,[2] AND MITSUAKI NISHIBUCHI[2]§*

*Osaka Quarantine Station, 4-10-3 Chikko, Minato-ku, Osaka 552,[1] and Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565,[2] Japan*

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***Vibrio cholerae* O1 strains isolated from various sources in Japan over the years 1977 through 1987 were examined to confirm the presence or absence of the cholera enterotoxin (CT) gene and production of CT and to determine the kappa-phage type. The CT gene was detected in none of 225 isolates from natural waters but was present in all of the 10 isolates from environmental waters implicated in domestic cholera cases, in 64 strains (26.6%) of the 241 isolates from imported seafoods, in 43 strains (95.6%) of the 45 isolates from domestic cholera cases, and in 119 strains (93.7%) of the 127 isolates from imported cholera cases. The results suggest that the CT gene-positive strains of *V. cholerae* O1 have been imported into Japan through seafoods and/or by travelers. Sporadic cholera cases have resulted in contamination of the surrounding environment, but the CT gene-positive strains may not have persisted in natural waters to serve as a reservoir for epidemic cholera. The commercially available VET-RPLA kit (a latex agglutination kit for immunological detection of CT) detected production of CT in all of the CT gene-positive strains, indicating that there was no silent CT gene in the test strains. There was a strong correlation between the kappa-phage type and the presence or absence of the CT gene, suggesting a significant clonal difference between CT gene-positive and -negative strains. Five CT gene-negative strains isolated from imported cholera cases (travelers with mild diarrhea) induced a considerable amount of fluid accumulation in rabbit and/or suckling mouse intestines, indicating production of an enterotoxic factor(s) other than CT. It is considered necessary to characterize the fluid accumulation factor(s) and to study dissemination of the CT gene-negative O1 strains producing the enterotoxic factor(s) to assess the public health significance of the CT gene-negative O1 strains distributed in the environment.**

Cholera enterotoxin (CT) has been considered a major virulence factor of *Vibrio cholerae* (9, 11, 28). However, strains of *V. cholerae* O1 in which production of CT could not be detected have been isolated, principally from environmental sources, in various parts of the world (28). The failure to detect CT production could be attributed to various factors such as in vitro culture conditions and sensitivity of the detection method. Therefore, a more definitive gene probe method was employed recently. Initially, this method was successfully used to rule out the genetic potential of the nontoxigenic strains of *V. cholerae* O1 to produce CT (14). The method was subsequently used for screening many isolates of *V. cholerae* from the Louisiana Gulf Coast environment to confirm the presence of the CT gene and also used in the epidemiological studies of *V. cholerae* O1 in the United States (12), Hong Kong (29), and Australia (8). Of these studies, the one carried out in Australia (8) indicated that most strains of *V. cholerae* O1 isolated from water, sewage, and food contained the CT gene.

The presence of *V. cholerae* O1 strains (particularly those with the potential to produce CT) in the environment could become a major public health problem. In this study, *V. cholerae* O1 strains isolated from environmental waters, imported seafoods, and imported and domestic cholera cases in Japan over a period of 11 years were examined to confirm the presence or absence of the CT gene and CT production and to detect the associated characteristics. All of the tests were helpful in assessing the public health significance of these isolates.

## MATERIALS AND METHODS

**Bacterial strains.** The strains of *V. cholerae* O1 isolated by us and other workers in Japan over a period of 11 years (1977 through 1987) were studied. These included 225 strains isolated from natural waters at 26 sampling stations (Fig. 1) during the period of 1978 through 1987, 10 strains isolated from environmental waters (human waste from airplane toilets, septic-tank water, river water, and estuarine water) presumed to be contaminated with feces in four domestic cholera cases over the years 1977 through 1986, 241 strains isolated from imported seafoods (frozen shrimp, frozen cuttlefish, frozen octopus, fresh fish, and fresh turtles) collected in 55 independent samplings during the period of 1980 through 1987, 45 strains isolated from stool samples implicated in 45 domestic cholera cases over the years 1977 through 1986, and 127 strains isolated from stool samples implicated in 127 imported cholera cases over the years 1978 through 1987. In principle, a cholera case was defined as a diarrhea case in which *V. cholerae* O1 could be isolated from stools. Two exceptional strains of *V. cholerae* O1 were isolated from healthy carriers when a domestic cholera outbreak was investigated. These two strains were also counted as the isolates from domestic cholera cases. The stool samples implicated in the imported cholera cases were

* Corresponding author.
† Present address: Narita Airport Quarantine Station, 1-1 Goryobokujo Sanrizuka, Narita, Chiba 282, Japan.
‡ Present address: Chiba Branch Office, Tokyo Quarantine Station, 1-12-2 Chuo-minato, Chiba 260, Japan.
§ Present address: Department of Microbiology, Faculty of Medicine, Kyoto University, Konoe-cho, Yoshida, Sakyo-ku, Kyoto 606, Japan.

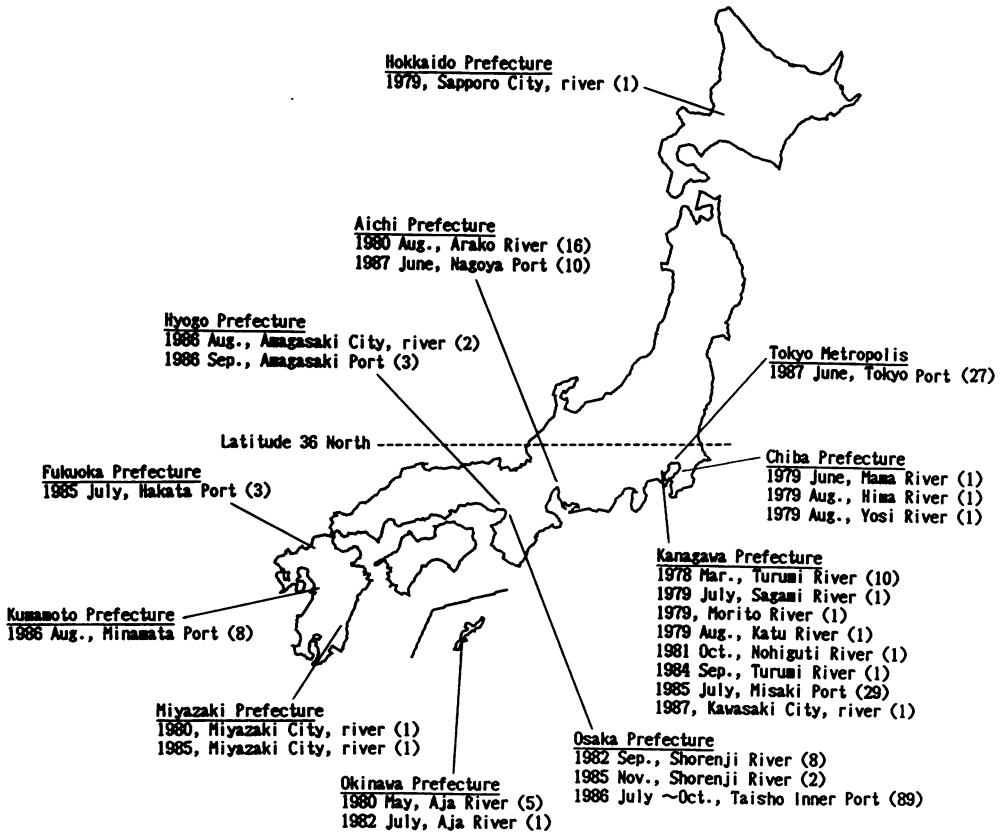


FIG. 1. Place and time of isolation of *V. cholerae* O1 strains from natural waters in Japan. The number in parenthesis denotes the number of strains isolated and used in this study.

collected from travelers at various quarantine stations upon their arrival into Japan. The strains were preserved by subculturing in semisolid medium (0.5 or 1% tryptone, 0.5% NaCl, 0.5% agar [pH 7.0 to 7.2]) prepared in tightly stoppered tubes at room temperature. The intervals between subcultures were 1 to 3 years. The biochemical characteristics of the test strains were examined by the standard methods (2, 15, 16). The serovar of the test strain was first determined by the slide agglutination test with serovar-specific antisera. The serovar-specific antisera were prepared by immunizing rabbits with the heat-killed cells of *V. cholerae* O1 NIH41 (serovar Ogawa) or NIH35A3 (serovar Inaba) followed by absorption of the sera thus obtained with the heat-killed cells of a different serovar. The polyvalent O-1 antiserum used for identification of the isolates was prepared by mixing the two serovar-specific antisera and then diluting appropriately. The serovar determination was further confirmed by using a latex agglutination kit commercially available from Denka Seiken Co. Ltd. (Tokyo, Japan). This test (*Vibrio cholerae* Antigen Typing & Detection Kit by Slide Latex Agglutination) utilizes a latex agglutination method with a monoclonal antibody raised to each of the a, b, and c factors of the O1 antigen. The test was carried out as specified by the manufacturer.

**Detection of the CT gene.** The presence or absence of a nucleotide sequence homologous to that of the CT gene was examined by the DNA colony hybridization method with a CT gene-specific DNA probe. The DNA probe used was the 0.56-kb *Eco*RI fragment isolated from the recombinant plasmid pCVD27 (13). This probe was derived from the sequence encoding 94% of the A-subunit gene. The plasmid pCVD27 was supplied by James B. Kaper. The procedure employed to purify the plasmid and to isolate and purify the DNA fragment has been described before (21). The probe DNA was labeled with [$\alpha$-$^{32}$P]dATP; the colony blot was prepared and hybridization was performed under high-stringency conditions as described previously (20).

**Detection of CT production.** Test strains were grown by using a modification of the method of Craig et al. (5). The test organism was initially propagated in 3 to 5 ml of 1% Trypticase peptone (BBL)–1% NaCl for 8 h at 30°C. A loopful of the culture was inoculated into 10 ml of modified CAYE broth (3% Casamino Acids [Difco], 0.3% yeast extract, 0.05% $K_2HPO_4$ [pH 7.0]) prepared in a petri dish with an inner diameter of 9 cm. After stationary incubation overnight at 30°C, a loopful of the culture was transferred to 10 ml of the CAYE medium with added 0.2% glucose and 90 μg of lincomycin (Upjohn) per ml prepared in the petri dish. The culture was incubated under stationary conditions for 24 h at 30°C. Half of the culture thus obtained was saved, polymyxin B (Pfizer) was added to the remaining culture to a final concentration of 20,000 U/ml, and the culture was incubated for 2 h at 37°C. The cultures with and without polymyxin B treatment were centrifuged (1,600 × *g*, 30 min), and the supernatants were filtered through sterile 0.45-μm-pore-size membrane filters (Millex-HA; Millipore). The CT in the filtered culture supernatant was detected by using the VET-RPLA kit commercially available from Denka Seiken (distributed outside of Japan by Unipath Ltd., Hampshire, England). This kit uses the latex agglutination method with

TABLE 1. Biochemical characteristics of 648 strains of *V. cholerae* O1 examined in this study

| Test | Result[a] | Test | Result[a] |
|---|---|---|---|
| Cytochrome oxidase | + | Acid from: | |
| Oxidation-fermentation | Fermentative | Arabinose | − |
| Gas from glucose | − | Cellobiose | − |
| Motility (Bacto SIM) | + | Dulcitol | − |
| Indole (Bacto SIM) | + | Glucose | + |
| Methyl red | − | Inositol | − |
| Sensitivity to O/129 | + | Lactose | + |
| Arginine dihydrolase (Moeller) | − | Maltose | + |
| Lysine decarboxylase (Moeller) | +[b] | Mannitol | + |
| Ornithine decarboxylase (Moeller) | + | Mannose | + |
| $H_2S$ (Bacto SIM) | − | Raffinose | − |
| Cholera red | + | Rhamnose | − |
| Citrate (Simmons) | + | Salicin | − |
| Citrate (Christensen) | + | Sorbitol | − |
| Tartrate (Jordan) | + | Sucrose | + |
| Malonate | − | Trehalose | + |
| Voges-Proskauer | +[c] | Xylose | − |
| Hemolysis of sheep erythrocytes | + | Growth in NaCl (%) | |
| Agglutination of chicken erythrocytes | + | 0 | + |
| Sensitivity to: | | 3 | + |
| Polymyxin B (50 U) | − | 7 | − |
| Mukerjee phage IV | − | 10 | − |
| Agglutination with polyvalent O1 antiserum | + | | |

[a] Unless otherwise specified, all the test strains gave the indicated results: +, positive; −, negative.
[b] Eleven isolates from imported frozen shrimp were negative.
[c] Five isolates from imported cholera cases were negative.

rabbit immunoglobulin G against the CT. The test was performed according to the manufacturer's specifications.

**Kappa-phage type.** Test organisms were classified into two subtypes on the basis of their lysogenicity with or sensitivity to the kappa-type phage as described by Takeya et al. (27). The Celebes-type strain either liberates the kappa-type phage or is sensitive to the kappa-type phage, but the classic Ubon-type strain neither liberates the phage nor is sensitive to the phage. For examining the lysogenicity of the test strains with kappa-type phage, strain H218 was used as a positive control. Strain C5, which liberates kappa-type phage particles into the culture medium, was employed for the spot test to examine the sensitivity of the test strain. The two standard strains were provided by Shoichi Shimodori.

**Enterotoxicity tests.** The rabbit ileal loop test was performed essentially as described by De and Chatterje (6). The test organism grown on brain heart infusion agar (Difco) for 6 h at 37°C was suspended in brain heart infusion broth to a final concentration of ca. $10^{10}$ CFU/ml. Then 1 ml of the cell suspension was inoculated into a 10-cm ligated rabbit ileal loop, and the fluid accumulation (FA) ratio (volume of accumulated fluid [milliliters] per centimeter of loop) was measured 18 to 22 h later. One sample was tested on three to four rabbits. The loops for a negative control (brain heart infusion broth) and a positive control (culture supernatant of CT gene-positive strain BT36) were always included when preparing the loops in a rabbit. The suckling mouse assay was carried out by the method of Dean et al. (7). The test organism was propagated in two test media (tryptic soy broth [Difco] and brain heart infusion broth with 0.5% NaCl added [22]) overnight at 37°C, and the supernatant obtained by centrifugation (13,000 × *g*, 1 min) was filtered through a 0.45-μm-pore-size sterile membrane filter (Chromatodisk; Biofield). Samples of 0.1 ml of the filtered supernatant were orally administered to two suckling mice (each aged 2 to 3 days and weighing between 2.0 and 2.3 g). The mice were sacrificed 3 h later, and the FA ratio (the ratio of the weight of the intestines to the remaining body weight) was determined for the individual animals. When the FA ratios of the two animals differed by more than 0.01, the experiment was repeated until satisfactory FA ratios were obtained. The average FA ratio was then obtained. The test samples that induced significant FA (average FA ratio of >0.08) were heated for 10 min at 100°C and subjected to the same test to assess the effect of heat on the mouse enterotoxic factor(s).

## RESULTS AND DISCUSSION

We confirmed identification of the test strains by a battery of biochemical tests (Table 1) and by determination of the serovars. Of the 648 strains identified as *V. cholerae* O1, 11 strains isolated from frozen shrimp were atypical in that they yielded negative results on the lysine decarboxylase test. Some strains of *V. cholerae* O1 with atypical biochemical characteristics had been isolated from the environment previously (28). All of the test strains belonged to the El Tor biovar. Distribution of the serovars in the strains isolated from natural waters, environmental waters implicated in domestic cholera cases, imported seafoods, stools from domestic cholera cases, and stools from imported cholera cases were 58 Inaba and 167 Ogawa, 1 Inaba and 9 Ogawa, 62 Inaba and 179 Ogawa, 5 Inaba and 40 Ogawa, and 12 Inaba and 115 Ogawa, respectively.

The presence or absence of the CT gene in the test strains was examined by using the DNA colony hybridization test (Table 2). We examined 225 strains isolated from natural waters. These strains had been isolated from freshwater and estuarine water samples collected at stations near densely populated areas (Fig. 1). In the summer of 1986, in particular, we conducted extensive samplings at Taisho Inner Port in Osaka Prefecture, resulting in isolation of 89 strains of *V. cholerae* O1. None of the 225 strains contained the CT gene.

TABLE 2. Results of the tests for CT gene, CT production, and kappa-phage type of *V. cholerae* O1 strains isolated in Japan

| Source of isolate | No. of strains | CT gene[a] | Ct production[b] | Kappa-phage type[c] |
|---|---|---|---|---|
| Natural water | 162 | − | − | U |
| | 1 | − | − | C |
| | 16 | − | $+^{w}$ | U |
| | 46 | − | ND | U |
| Environmental water implicated in domestic cholera cases | 10 | + | + | C |
| Imported seafoods | 62 | + | + | C |
| | 2 | + | + | U |
| | 164 | − | − | U |
| | 5 | − | − | C |
| | 4 | − | $+^{w}$ | U |
| | 4 | − | ND | U |
| Stool from domestic cholera cases | 43 | + | + | C |
| | 2 | − | − | C |
| Stool from imported cholera cases | 119 | + | + | C |
| | 8 | − | − | U |

[a] +, present; −, absent.
[b] +, strongly positive; $+^{w}$, weakly positive; −, negative; ND, not determinable because of agglutination of control latex particles.
[c] C, Celebes type; U, classic Ubon type.

However, all of the 10 strains isolated from environmental waters associated with domestic cholera cases carried the CT gene. Of the 241 strains isolated from imported seafoods, 177 strains (73.4%) did not contain the CT gene. There was no remarkable year-to-year variation in the ratio of the CT gene-positive to -negative strains isolated from seafoods (data not shown). Two strains (4.4%) of the 45 stool isolates from domestic cholera cases and eight strains (6.3%) of the 127 stool isolates from imported cholera cases did not contain the CT gene.

CT production by the test strains was also examined. It is known that CT production varies among strains of *V. cholerae* O1 (4). Craig (4) reported that classical strains produced more CT when grown with shaking, whereas El Tor strains produced more CT when grown without shaking. Since all of the test strains were of the El Tor biotype, we employed a stationary culture method to maximize the CT production. A recent report (1) demonstrated that the VET-RPLA, a latex agglutination kit developed for rapid and easy detection of CT, gave results that were nearly identical to those obtained with a sensitive sandwich enzyme-linked immunosorbent assay. We employed the relatively convenient VET-RPLA method. Two test samples (culture supernatants with and without polymyxin B treatment) were prepared for each test strain. When CT was produced, the sample prepared with polymyxin B treatment induced stronger agglutination of the latex particles coated with anti-CT immunoglobulins than did the sample prepared without polymyxin B treatment. This indicates that polymyxin B treatment stimulated the release of CT into the spent culture medium. Occasionally, one or both of the sample preparations induced agglutination of the control latex particles coated with nonimmune rabbit globulins. Therefore, the results listed in Table 2 were recorded according to the following criteria. If both preparations induced nonspecific agglutination, the result was recorded as "not determinable." If one of the two preparations induced nonspecific agglutination, the result obtained with the other preparation was recorded. If neither of the two preparations induced nonspecific agglutination, the result obtained with the sample prepared with polymyxin B treatment was recorded. The results of the VET-RPLA test paralleled those of the CT gene detection (Table 2). All of the CT gene-positive strains produced CT at detectable levels. The results indicate that there was no silent CT gene in the test strains and that the VET-RPLA method is sensitive enough to detect the CT gene-positive strains. However, 20 strains (4.9%) induced a weak positive reaction and 50 strains (12.1%) induced a weak agglutination of control latex particles. Therefore, special attention to avoid possible false-positive (weakly positive) readings and to nonspecific agglutinations is advised when the VET-RPLA method is employed.

El Tor strains can be classified into the Celebes or classic Ubon type by the kappa-phage typing proposed by Takeya and co-workers (26, 27). Among clinical strains, the classic Ubon-type strains are associated with very mild infections (26). Nontoxigenic strains isolated from the natural environments in the United States, Brazil, and England belong to the classic Ubon type (25). We determined the kappa-phage type of the test strains and found a strong correlation between the kappa-phage type and the presence or absence of the CT gene (Table 2). Among 412 CT gene-negative strains, 404 (98.1%) belonged to the classic Ubon type and 234 (99.2%) belonged to the Celebes type. These results suggest that a significant clonal difference may exist between CT gene-positive and -negative strains and that the kappa-phage typing may be utilized to presume the presence or absence of the CT gene.

Molecular epidemiologic studies with DNA probes have suggested that a single strain of enterotoxigenic *V. cholerae* O1 is resident in the U.S. Gulf Coast-coastal Mexico area (3, 12, 18). However, CT gene-positive strains of *V. cholerae* O1 were isolated only from sewage samples when over 2,500 strains of *V. cholerae* isolated from Louisiana environments were examined (24). In contrast, most strains of *V. cholerae* O1 isolated from sewage and water samples in Australia contained the CT gene (8), indicating a wide distribution of CT gene-positive strains of *V. cholerae* O1 in the Australian environment. Our results summarized in Table 2 indicate that both CT gene-positive and -negative strains of *V. cholerae* O1 have been imported into Japan through seafoods and by travelers; and sporadic cholera cases have resulted in contamination of the surrounding environment with CT gene-positive strains. The failure in detecting CT gene-positive strains of *V. cholerae* O1 in natural water samples suggests that the imported CT gene-positive strains of *V. cholerae* O1 may not have persisted in natural waters in Japan to serve as a reservoir for epidemic cholera. However, it is important to continue surveying the environmental waters for CT gene-positive strains of *V. cholerae* O1. In the facilities where the DNA probe assay cannot be performed, the VET-RPLA method and/or the kappa-phage typing method may be employed for presumptive detection of CT gene-positive strains.

This study confirmed there is wide distribution of CT gene-negative strains of *V. cholerae* O1 in imported seafoods and in natural waters in Japan (Table 2). Detailed comparative characterization of the CT gene-negative O1 strains isolated from imported seafoods and from natural waters over the 11 years (1977 through 1987) may reveal whether the imported CT gene-negative O1 strains have adapted to the Japanese natural waters recently or whether they have been resident in the natural waters in Japan for many years. Two of the stool isolates from domestic cholera cases and eight of the stool isolates from imported cholera cases did

TABLE 3. Clinical data associated with the isolation of the CT gene-negative strains of *V. cholerae* O1 from stools

| Strain no.[a] | Source[b] | Serotype[c] | Kappa-phage type[d] | Frequency of diarrhea per day during symptomatic period | No. of days from onset to stool examination | Symptoms when stool was sampled | Condition of stool | Other possible pathogen isolated simultaneously |
|---|---|---|---|---|---|---|---|---|
| AQ1002 | I | O | U | 2–3 | 7 | Diarrhea | Unknown | None |
| AQ1003 | I | O | U | 2 | 2 | Diarrhea, abdominal pain | Mucous | None |
| AQ1028 | I | O | U | 2 | 10 | None | Solid | None |
| NAQ103 | I | O | U | 1–4 | 1 | Diarrhea | Soft | None |
| NAQ104 | I | O | U | 1–4 | 1 | Diarrhea | Muddy | None |
| NAQ117 | I | I | U | 1–4 | 1 | None | Unknown | None |
| NAQ118 | I | O | U | 1–4 | 2 | None | Unknown | Non-O1 *V. cholerae* |
| NAQ136 | I | O | U | 1–4 | 1 | Diarrhea | Unknown | *V. parahaemolyticus* |
| M11 | D | I | C | None | Not applicable | None | Solid | None |
| M15 | D | O | C | None | Not applicable | None | Solid | None |

[a] AQ and NAQ indicate isolates at Osaka Airport and Narita Airport quarantine stations, respectively.
[b] I, imported cholera cases; D, domestic cholera cases (isolated from healthy carriers during a cholera outbreak).
[c] I, Inaba; O, Ogawa.
[d] C, Celebes; U, classic Ubon.

not contain the CT gene (Table 2). Three CT gene-negative strains of *V. cholerae* O1 were also isolated from travelers with diarrhea at an international airport in Japan recently (10). If the CT gene-negative strains of human origin are enteropathogenic and are able to persist in the natural environment, they can pose a potential threat to the public. One CT gene-negative strain of *V. cholerae* O1 was isolated from a patient with severe gastroenteritis in the United States, but its enteropathogenicity was not demonstrated by FA in rabbit ileal loops (19). The 10 CT gene-negative strains from domestic and imported cholera cases (Table 2) were isolated from patients with mild diarrhea or from healthy carriers exposed to cholera outbreaks (Table 3). Other possible enteropathogens were isolated simultaneously from the stool samples in two cases (Table 3). We examined the enterotoxicity of these 10 CT gene-negative strains in the rabbit ileal loop and suckling mouse models (Table 4). Five strains isolated from mild diarrheal cases (AQ1002, NAQ103, NAQ104, NAQ117, and NAQ136) produced an extracellular factor(s) that induced significant FA in the rabbit ileal loops (FA ratio, >0.7), but the amounts of the fluid were much less than that caused by the cultue filtrate containing CT (strain BT36, Table 4). Three (NAQ103, NAQ104, and NAQ117) of these five strains also produced an extracellular factor(s) in one or both of the two test media and thus stimulated considerable FA in the intestines of the suckling mice (FA ratio, >0.08). The factor(s) causing intestinal FA in the suckling mice was heat labile (Table 4). The FA factors of strains NAQ103 and NAQ104 that were produced in brain heart infusion broth containing 0.5% NaCl (22) gave the highest FA ratios. Similar but more potent heat-labile suckling mouse factors were produced by clinical strains of non-O1 *V. cholerae*, *Vibrio mimicus*, and *Vibrio fluvialis* (22). A factor(s) other than CT that induces FA in suckling mice (23) or causes mild diarrhea in human volunteers (17) was shown to have been produced by the CT gene-positive strains of *V. cholerae* O1. More detailed characterization of the FA factor(s) of the CT gene-negative

TABLE 4. Results of the rabbit ileal loop and suckling mouse assays of the CT gene-negative strains of *V. cholerae* O1 isolated from stools

| Strain no.[a] | FA ratio in rabbit ileal loop | FA ratio in the suckling-mouse intestines obtained with: | | | |
|---|---|---|---|---|---|
| | | Tryptic soy broth[b] | | Brain heart infusion broth plus 0.5% NaCl[b] | |
| | | Unheated | Heated | Unheated | Heated |
| AQ1002 | 0.91 ± 0.34 | 0.064 | NT[c] | 0.074 | NT |
| AQ1003 | 0.64 ± 0.22 | 0.061 | NT | 0.062 | NT |
| AQ1028 | 0.25 ± 0.32 | 0.060 | NT | 0.056 | NT |
| NAQ103 | 0.77 ± 0.28 | 0.079 | NT | 0.101 | 0.061 |
| NAQ104 | 0.85 ± 0.25 | 0.081 | 0.056 | 0.101 | 0.061 |
| NAQ117 | 1.13 ± 0.04 | 0.098 | 0.057 | 0.090 | 0.064 |
| NAQ118 | 0.31 ± 0.34 | 0.070 | NT | 0.069 | NT |
| NAQ136 | 0.87 ± 0.51 | 0.057 | NT | 0.067 | NT |
| M11 | 0.02 ± 0.03 | 0.060 | NT | 0.060 | NT |
| M15 | 0.00 ± 0.00 | 0.053 | NT | 0.060 | NT |
| BT36[d] | 1.68 ± 0.30 | NT | NT | NT | NT |
| None[e] | 0.00 ± 0.00 | NT | NT | NT | NT |

[a] See Table 3.
[b] The test organism was grown at 37°C overnight with shaking, and the filtered culture supernatant with or without heat treatment (100°C, 10 min) was administered.
[c] NT, not tested.
[d] A CT gene-positive clinical strain isolated in Bangladesh (a positive control for the rabbit ileal loop assay).
[e] Brain heart infusion broth not containing bacterial cells (a negative control for the rabbit ileal loop assay).

O1 strains (e.g., the relationship between the enterotoxic factor[s] of the CT gene-positive strains of *V. cholerae* O1 and those of related vibrios) and study of the dissemination of the CT gene-negative O1 strains producing the enterotoxic factor(s) are needed to assess the public health significance of the CT gene-negative strains of *V. cholerae* O1 distributed in the environment.

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