Low Viability of Cholera Toxin-Producing Vibrio cholerae O1 in the Artificial Low Ionic Strength Aquatic Solution

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It has been well known that Vibrio cholerae inhabit in environmental water. As many patients infected with cholera toxin-producing V. cholerae O1 (toxigenic V. cholerae O1) emerge in Kolkata, India, it has been thought that toxigenic V. cholerae O1 is easily detected in environmental water in Kolkata. However, we could not isolate toxigenic V. cholerae O1 from environmental water in Kolkata, though NAG Vibrio (generic name of V. cholerae non-O1/non-O139) is constantly detected. To clear the reason for the non-isolation of toxigenic V. cholerae O1, we examined the viability of V. cholerae O1 and NAG Vibrios in the artificial low ionic strength aquatic solution. We found that the viability of toxigenic V. cholerae O1 in the solution is low, but that of NAG Vibrios is high. Subsequently, we examined the viability of NAG Vibrios possessing cholera toxin gene (ctx) in the same condition and found that the viability of these NAG Vibrios is low. These results indicate that the existence of ctx in V. cholerae affects the viability of V. cholerae in the aquatic solution used in this experiment. We thought that there was closely relation between the low viability of toxigenic V. cholerae O1 in the artificial low ionic strength aquatic solution and the low frequency of isolation of the strain from environmental water.

Key words Vibrio cholerae; cholera toxin; aquatic solution; viability

INTRODUCTION

Cholera disease is a life-threatening acute diarrheal disease caused by Vibrio cholerae.1) In V. cholerae, there are 206 serogroups based on the polysaccharide O-antigen.2) Of these 206 serotypes of V. cholerae, the serotype of strains causing cholera disease with severe diarrhea is limited to 2 serotypes, O1 and O139. V. cholerae O139 induced endemic of cholera disease with severe diarrhea is limited to 2 serotypes, O1 and O139.1) In V. cholerae O139 has been recognized as pathogenic strain of V. cholerae since then.3) However, the number of patients infected with V. cholerae O139 has been low in recent years in the world (WHO Cholera, http://www.who.int/mediacentre/factsheets/fs107/en/index.html). In contrast, many patients infected with V. cholerae O1 has emerged in the world. Especially, the patients have constantly emerged in Kolkata, India in all ages.3,4)

The cholera toxin (CT) produced by these virulent strains play an essential role in emergence of symptom by the infection of V. cholerae O1 and O139. Therefore, V. cholerae causing endemic and pandemic is limited to CT-producing (toxigenic) V. cholerae O1 and O139.1)

As V. cholerae is regarded as a bacteria living in environmental water,5) we supposed that possible number of toxigenic Vibrio cholerae O1 inhabited in environment water in Kolkata. Then, we examined V. cholerae inhabiting in environment water in Kolkata. For these two years, we examined more than 50000 colonies presenting yellow color on thiosulfate-citrate-bile salts-sucrose (TCBS) agar plate from pond water. Many V. cholerae non-O1/non-O139 strains, which are commonly designated as NAG Vibrio, have been isolated, but we could not isolate toxigenic V. cholerae O1 (data not published). From this result, we inferred that the viability of toxigenic V. cholerae O1 in pond water might be inferior to that of NAG Vibrio. Then, we examined the viability of toxigenic V. cholerae O1 and NAG Vibrios in the artificial low ionic strength aquatic solution. We used the diluted Page's amoeba saline solution (PAS) as the artificial low ionic strength aquatic solution.

The concentration of Na⁺ in almost river water in Japan lies between 3.0 and 7.9 mg/L.6) The concentration of Na⁺ in PAS (under 100% of ionization degree) is 50.6 mg/L. We used the 11 fold-diluted PAS, in which the concentration of Na⁺ is 4.6 mg/L, as the artificial low ionic strength aquatic solution in this experiment.

The result showed that the viability of toxigenic V. cholerae O1 is lower than that of NAG Vibrio. Subsequently, to clear the role of cholera toxin gene (ctx) in the viability of V. cholerae, we examined the viability of NAG Vibrios possessing ctx in the same condition. The result indicated that there is relation between the possession of ctx of V. cholerae and the viability of the bacteria in the solution examined.

MATERIALS AND METHODS

Bacterial Strains Twelve strains of V. cholerae and one

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strains of *Escherichia coli* were used in this study. In 12 strains of *V. cholerae*, 4 strains (IDH-11477, IDH-11494, IDH-11791, IDH-11827) were isolated from the diarrhea patients admitted to the Infectious Diseases Hospital, Kolkata, India and 7 strains (OKA-003, OKA-005, OKA-007, OKA-140, OKA-144, OKA-150 and OKA-155) were isolated from pond water in Kolkata, India. Isolation of these strains was carried out as reported previously.7)

The serotypes of above strains were determined by slide agglutination test using poly-valent antiserum against O1 antigen and O139 antigen of *V. cholerae* (Denka-Seiken, Japan). The results showed that 4 strains (IDH-11477, IDH-11494, IDH-11791, and IDH-11827) are serotype 1 (O1) and other 7 strains (OKA003, OKA-005, OKA-007, OKA-140, OKA-144, OKA-150 and OKA-155) are belonged to NAG-Vibrio.

*V. cholerae* N16961, which is a representative strain of *V. cholerae* O1 containing *ctx*,9) was from our stock culture. *E. coli* E-010 is an isolate from stool of healthy person in India. The species of *E. coli* E-010 was confirmed by the determination of the nucleotide sequence of 16s RNA (data not shown).

**Detection of Cholera Toxin Gene and Cholera Toxin**

The possession of *ctx* of these clinical strains was examined by PCR. The primers used were designed to detect A subunit gene of cholera toxin (*ctxA*) (forward primer: 5’-ctcagacgaggtttgttagga-3’; reverse primer: 5’ctatcttgtgagcctattacg 3’). The length of the PCR product with these primers is 302 base pairs (bp).9)

The DNA samples of the strains examined were extracted by heating method. The bacteria cultured in 2mL of L-broth were collected by centrifugation and suspended in 0.5mL distilled water. The samples were heated in boiling water for 5min, and were centrifuged. The supernatants obtained were used as DNA sample of each strain for PCR.

CT produced into outside of the cell was detected using immunnoassay. The strains were cultured statically in AKI medium9) at 37°C for 24h. The cultures were centrifuged (12000 × g for 15min) and the supernatants were recovered. CT content of these supernatants was measured by GM1-ganglioside enzyme-linked immunosorbent assay (ELISA) method.9) A standard curve was generated simultaneously with known concentrations of purified CT (Sigma-Aldrich, St. Louis, MO, U.S.A.) wherever needed. Rabbit anti-cholera toxin (Sigma-Aldrich) was used as the primary antibody (1:2000) while goat anti-rabbit conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, U.S.A.) used as the secondary antibody (1:8000).

**Viability of Bacteria** Bacteria were grown in 2mL of LB broth (BD Difco, Franklin Lake, NJ, U.S.A.) for about 15h at 37°C with shaking. A100 µL of above culture was re-incubated in 2mL LB broth and incubated at 37°C for 3–5h with shaking. Then, the bacterial culture was centrifuged and the pellet was suspended in PAS to give the turbidity of McFarland O.D of 2.0. A 0.9mL of the bacterial suspension was poured into 9.1mL of filter-sterilized distilled water. The dilution ratio of PAS in the sample is 11-fold. The bacterial suspension was incubated at 25°C for 5d.

The viability of the bacteria in the solution was determined by the plating method. A 100 µL of the seriously diluted solution was plated on L-agar plate and the plate was incubated for 20h at 37°C. The number of colonies formed was counted, and colony forming unit (CFU) of the original solution was calculated from the number obtained. The CFU was used as indicator of the viability of the bacteria in the solution.

**RESULTS AND DISCUSSION**

The possession of *ctx* in *V. cholerae* used in this experiment was examined by PCR. The band with the length of approx. 300bp was emerged from samples of 4 clinical strains (IDH-11477, IDH-11494, IDH-11791, IDH-11827) and of 2 environmental strains (OKA-003 and OKA-007). The band did not emerge in other strains (*V. cholerae* OKA005, OKA-140, OKA-144, OKA-150 and OKA-155). The existence of *ctx* in 2 environmental strains (OKA-003 and OKA-007) was further confirmed by amplification of B subunit gene of CT and by the determination of nucleotide sequence of the amplified gene fragments (data not presented).

We examined the viability of *V. cholerae* in the artificial low ionic strength aquatic solution, which is 11 fold-diluted PAS, by determining the number of living bacteria in the solution over time. The number of living bacteria was measured by plating method as described above. At first, we examined the viabilities of NAG Vibrio (*ctx*) and *V. cholerae* O1 (*ctx*). At the initiating time of incubation (incubation period, 0d), approximately 5×10^5/mL colonies were contained in all bacterial solutions examined (Fig. 1, sample numbers 1-0 and 2-0). However, the big difference in the number of living bacteria among strains of two groups yielded after incubation for 5d. The CFU of the suspension containing of *V. cholerae* O1 (*ctx*) declined from the level of 10^9/mL to the level of 10^7/mL (Fig. 1, sample number 2-5). However, the CFU of NAG Vibrio (*ctx*) after incubation for 5d was approx. 10^6/permL (Fig. 1, sample number 1-5). This means that the viability of NAG Vibrio (*ctx*) is high. The level of viability of NAG Vibrio (*ctx*) is almost equal to that of *E. coli* (Fig. 1, group 4).

The major difference in character of bacteria between group 1 and group 2, lies in serogroup and *ctx*. We thought that these differences might affect the viability of bacteria. Fortunately, we isolated 2 NAG Vibrios possessing *ctx* (NAG-Vibrio (*ctx*)) from ponds in Kolkata. One is NAG Vibrio OKA-003 and another is NAG Vibrio OKA-007. To clear that these strains are NAG-Vibrio, we determined the serogroup of these strains by slide agglutination method using 206 O group-specific serum prepared by the National Institute of Infectious Diseases of Japan.9) The result showed that serogroups of OKA-003 and OKA-007 are O124 and O152, respectively, probing that these two strains are NAG-Vibrios.

As these two strains, OKA-003 and OKA-007, possess *ctx*, the comparison of the data on the viability of these strains with these of other NAG Vibrios (*ctx*) may clarify the role of *ctx* in the viability. Thus, we examined the viability of these two strains (Fig. 1, group 3). The CFU of OKA-003 and OKA-007 decreased vigorously to the level of 3×10^3 CFU per mL and 2×10^2 CFU per mL, respectively after incubation for 5d (Fig. 1, sample number 3-5). The difference in the viability between two groups of NAG-Vibrios, NAG-Vibrio (*ctx*) and NAG-Vibrio (*ctx*'), is clear. This indicates that the presence of *ctx* in *V. cholerae* strains affect detrimentally to the viability of the strains in the artificial low ionic strength aquatic solution.

From the results obtained, we deduced that CT produced
by *V. cholerae* might be involved in the viability of the strain. Thus we measured the amount of CT produced into outside of the cells. Seven *V. cholerae* (ctx⁺) strains were cultured in AKI medium at 37°C for 24 h and the amount of CT in the medium was measured by GM1-ganglioside ELISA method (Fig. 2). The minimum detection value of this method is 0.625 ng/mL. The production of CT from 5 strains of *V. cholerae* O1 (ctx⁺) was clearly demonstrated. The CT from OKA-003 was slightly detected, while that from OKA-007 was at the level of detection limit.

Subsequently, we examined intracellular CT of OKA-003 and OKA-007, in order to clear the production of CT from these cells. The cells obtained by the culture in 10 mL of AKI medium were collected by centrifugation and suspended with 1 mL of TEAN buffer (0.05 M Tris, 0.2 M NaCl, 1 mM EDTA, 3 mM NaN₃ [pH 7.5]). These cells were crushed using vigorous shaking with beads and these turbid solutions were centrifuged. The supernatants were recovered as a cell lysate samples. The amount of CT in these samples was measured by GM1-ganglioside ELISA method. CT was not detected in these cell extracts examined.

In addition, we examined mRNA of ctxA of OKA-003, OKA-007 and N-16961. RNAs of these strains were extracted from cells grown in logarithmic phase and were detected by quantitative (q) RT-PCR. The relative transcriptional levels of ctxA of these cells which was compared with that of N-16961, were estimated by ΔΔCt method. The results showed that relative transcriptional level of ctxA of OKA-003 and OKA-007 were 0.15 ± 0.03 (mean value ± standard deviation) (n = 4) and 0.08 ± 0.03 (n = 4), respectively. This shows the transcriptional level of ctxA of OKA-007 and OKA-003 was very low. These indicate that the amount of CT produced by OKA-007 and OKA-003 is very low. Especially, the production from OKA-007 is almost unobservable. It means that the activity of OKA-007 and OKA-003 to produce CT is low.

However, the viability of these strains in the artificial low ionic strength aquatic solution remains low (Fig. 1, sample number 3-5), in spite of their low activity to produce CT. This indicates that CT produced by *V. cholerae* is not involved in the viability of the bacteria in the artificial low ionic strength aquatic solution.

The role of CT produced in pathogenesis has been clearly demonstrated. CT released into intestinal lumen induces the exudation of body fluid from host into intestinal lumen, which leads to diarrhea. The exudate contains many nutritious substances for *V. cholerae* and the bacteria can survive for long time in the lumen by utilizing these nutrition.
It is considered that toxigenic V. cholerae O1 cannot survive for long time in environmental water with low ionic strength, though the strain can survive in the fluid secreted from intestine. This means that the low viability of V. cholerae might be deeply concerned in the determination of the habitat area of toxigenic V. cholerae O1. Further examination of the viability of the clinical NAG Vibrios in the artificial low ionic strength aquatic solution might give us an important information about the habitat area of V. cholerae. We will report the result of this experiment in the following manuscript.

Our result showed that CT is not involved in the viability of V. cholerae in the artificial low ionic strength aquatic solution. Indeed, the frequency of isolation of toxigenic V. cholerae O1 from environmental water has been reported to be low. Our other investigations confirm this result as described in introduction section. These results indicate that V. cholerae does not need CT to survive in environmental water.

As another pathogenic factor of toxigenic V. cholerae O1, the toxin co-regulated pilus (TCP) has been reported. TCP functions in colonization of V. cholerae in intestinal lumen. TcpA is the major subunit of TCP and toxigenic V. cholerae O1 universally possess tcpA. However, tcpA has also been detected in some strains of non-pathogenic V. cholerae as well. Therefore, it is probable that TcpA is not involved in the viability of V. cholerae described in this manuscript. Actually, we have recently found that V. cholerae O1-140, and O1-150 possess tcpA as well as toxigenic V. cholerae O1 by PCR, but the viability of two strains are different from these of toxigenic V. cholerae O1 (Fig. 1). However, DNA sequences of these tcpAs are divergent (unpublished data). Therefore, it has remained unclear whether these TcpAs of O1-140, and O1-150 have same activity of these of toxigenic V. cholerae O1. Further examination is necessary to get conclusion about the concern of TcpA in the viability of V. cholerae.

As shown in Fig. 1, there is relation between the possession of ctx and the viability in the artificial low ionic strength aquatic solution in V. cholerae (Fig. 1). The ctx is transferred by CTX phage. Bacteriophages recognize the peculiar structure of target bacteria and attack to the bacteria. Therefore, it is possible that V. cholerae possessing ctx might have common structure on the cell surface, and that the common structure is involved in the viability of V. cholerae in the artificial low ionic strength aquatic solution. Further studies are necessary to demonstrate this hypothesis.

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Conflict of Interest The authors declare no conflict of interest.

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