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

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Received April 16, 2020; accepted June 2, 2020; advance publication released online June 9, 2020

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. cholera* 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 since 1990’s. *V. cholerae* O139 has been recognized as pathogenic strain of *V. cholerae* since then.1) 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,3) 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.5) 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...
strain of *Escherichia coli* were used in this study. In 12 strains of *V. cholerae*, 4 strains (IDI-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 (IDI-11477, IDH-11494, IDH-11791, and IDH-11827) are serotype O1 (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,8) 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).

**Detections 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’-ctctatctctgtagcccctattacg-3’; reverse primer: 5’-ctcagagctccggttggagaca-3’). The length of the PCR product with these primers is 302 base pairs (bp).5)

The DNA samples of the strains examined were extracted by heating method. The bacteria cultured in 2 mL of L-broth were collected by centrifugation and suspended in 0.5 mL distilled water. The samples were heated in boiling water for 5 min, 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 immunoassay. The strains were cultured statically in AKI medium9) at 37°C for 24h. The cultures were centrifuged (12000 × g for 15 min) 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 toxinn (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 2 mL 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 reincubated in 2 mL 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 0.9 mL of the bacterial suspension was poured into 9.1 mL 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 5 d.

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 20 h 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 (IDI-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, 0 d), approximately 5 × 10⁷/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 5 d. The CFU of the suspension containing of *V. cholerae O1* (*ctx−*) declined from the level of 10⁷/mL to the level of 10³/mL (Fig. 1, sample number 2-5). However, the CFU of NAG Vibrio (*ctx−*) after incubation for 5 d was approx. 10⁷/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.5) The result showed that serogroups of OKA-003 and OKA-007 are O124 and O152, respectively, proving 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 × 10² CFU per mL and 2 × 10² CFU per mL, respectively after incubation for 5 d (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. 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.

Subsequently, we examined intracellular CT 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. 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.2) 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.3) 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.4) Therefore, it is presumable that TcpA is not involved in the viability of *V. cholerae* described in this manuscript. Actually, we have recently found that *V. cholerae* OKA-140, and OKA-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 *tcpA* genes are divergent (unpublished data). Therefore, it has remained unclear whether these TcpAs of OKA-140, and OKA-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 attach to the bacteria.5) 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.

Acknowledgments This work was supported in part by the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), the Ministry of Education, Culture, Sports, Science and Technology in Japan, the Japan Agency for Medical Research and Development (AMED; Grant No. JP18fm0108002).

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Harris JB, LaRocque RC, Qadri F, Ryan ET, Calderwood SB. Cholera. *Lancet*, 379, 2466–2476 (2012).
2) Yama S, Okitsu T, Shimada T, Katsube Y. Distribution of serogroups of *Vibrio cholerae* non-O1 non-O139 with specific reference to their ability to produce cholera toxin, and addition of novel serogroups. *Kansenshogaku Zasshi*, 71, 1037–1045 (1997).
3) Nair GB, Ramamurthy T, Bhattacharya MK, Krishnan T, Ganguly S, Saha DR, Rajendran K, Manna B, Ghosh M, Okamoto K, Takeda Y. Emerging trends in the etiology of enteric pathogens as evidenced from an active surveillance of hospitalized diarrhoeal patients in Kolkata, India. *Gut Pathog.*, 2, 4 (2010).
4) Kanungo S, Saha BK, Lopez AL, Sung JS, Paisley AM, Sur D, Clemens JD, Balakrish Nair G. Cholera in India: an analysis of reports, 1997–2006. *Bull. World Health Organ.*, 88, 185–191 (2010).
5) Islam MS, Zaman MH, Islam MS, Ahmed N, Clemens JD. Environmental reservoirs of *Vibrio cholerae*. *Vaccine*, 38 (Suppl. 1), A52–A62 (2020).
6) Kobayashi J. A chemical study on the average quality and characteristics of river waters of Japan. *Nogaku Kenkyu*, 48, 63–106 (1961).
7) Morita D, Takahashi E, Morita M, Ohnishi M, Mizuno T, Miyoshi S, Datta D, Ramamurthy T, Chowdhury G, Mukhopadhyay AK, Okamoto K. Genomic characterization of antibiotic resistance-encoding genes in clinical isolates of *Vibrio cholerae* non-O1/non-O139 strains from Kolkata, India: Generation of novel types of genomic islands containing plural antibiotic resistance genes. *Microbial. Immunol.*, 64, 435–444 (2020).
8) Heidelberg JF, Eisen JA, Nelson WC, et al. DNA sequence of both chromosones of the cholera pathogen *Vibrio cholerae*. *Nature*, 406, 477–483 (2000).
9) Iwanaga M, Yamamoto K. New medium for the production of cholera toxin by *Vibrio cholerae* O1 bio-type El Tor. *J. Clin. Microbiol.*, 22, 405–408 (1985).
10) Kanjilal S, Citorik R, LaRocque RC, Ramoni MF, Calderwood S, Saha BK, Lopez AL, et al. Genomic characterization of antibiotic resistance-encoding genes in clinical isolates of *Vibrio cholerae* non-O1/non-O139 strains from Kolkata, India: Generation of novel types of genomic islands containing plural antibiotic resistance genes. *Microbial. Immunol.*, 64, 435–444 (2020).
11) Field M. Intestinal ion transport and the pathophysiology of diarrhoea. *J. Clin. Invest.*, 111, 931–943 (2003).
12) Krebs SJ, Taylor RK. Nutrient-dependent, rapid transition of *Vibrio cholerae* to coccoid morphology and expression of the toxin co-regulated pilus in this form. *Microbiology*, 157, 2942–2953 (2011).
13) Herrington JA, Hall RH, Losonsky G, Mekalanos JJ, Taylor RK, Levine MM. Toxin, toxin-coregulated pilus, and the toxR regulon are essential for *Vibrio cholerae* pathogenesis in humans. *J. Exp. Med.*, 168, 1487–1492 (1988).
14) Rivera IN, Chun J, Hug A, Sack RB, Colwell RR. Genotype associated with virulence in environmental isolates of *Vibrio cholerae*. *Appl. Environ. Microbiol.*, 67, 2421–2429 (2001).
15) Brüssow H, Canchaya C, Hardt WD. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiol. Mol. Biol. Rev.*, 68, 560–602 (2004).