The study of viability of *Vibrio cholerae* strains in low ionic strength aquatic environment.

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Abstract

It has been regarded that *Vibrio cholerae* O1 inhabit in environmental water. As many cholera patients emerge in Kolkata, it has been thought that *V. cholerae* O1 is easily detected in environmental water in Kolkata. However, the detection of *V. cholerae* O1 is rare, though other *V. cholerae* (NAG Vibrio) is constantly detected. To clear the reason for the difference of the detection rate of two Vibrios, we examined the viability of *V. cholera* O1 and NAG Vibrios in low ionic strength aquatic medium. We observed greater declining viability of *V. cholerae* O1 possessing cholera toxin gene (*ctx*) in low ionic strength solution, but the decline of NAG Vibrios non-possessing *ctx* is small. To evaluate the concerning of *ctx* in the viability, we examined the viabilities of *V. cholerae* O1which do not possess *ctx* and NAG Vibrios possessing *ctx* under the same condition. The result indicated that the existence of the *ctx* induces the decrease the viability of the host in low ionic strength solution. The decrease observed in this experiment might relate with the low detection of *V. cholerae* O1 possessing *ctx* in environmental water, though NAG Vibrio is constantly detected.

KEYWORDS: *Vibrio cholerae*, cholera toxin gene, low ionic strength aquatic solution, viability

Introduction

Cholera is the diarrhoeal disease predominantly infecting humans and is caused by *Vibrio cholerae* O1 and O139 [1]. Ingestion of water or food contaminated with these bacteria is the

main route of infection [2] [3]. Thebacteria is regarded to be inhabited in the aquatic environment.

More than two hundreds serotypes of *V. cholerae* have been reported. Among them, strains casing pandemic is limited to two serotypes, O1 and O139 [4]. As many patients with *V. cholerae* emerge in Kolkata, it was thought that *Vibrio cholerae* O1 and O139 inhabit in environment water in Kolkata at fairly high ratio [5]. We examined *V. cholerae* inhabiting in environment water in Kolkata. For these two years, we examined more than 100, 000 colonies presenting yellow color on TCBS agar plate from environment water. However, although many *V. cholerae* non-O1/ non-O139 strains (NAG Vibrio) were isolated, we could not isolate virulent *V. cholerae* O1 and O139. From this result, we thought that the viability of *V. cholerae* O1 and O139 possessing *ctx* is inferior to that of NAG Vibrios. Then, we examined the viability of *V. cholera* O1 and NAG Vibrios in low ionic strength aquatic medium. Subsequently, to clear the influence of *ctx* to the viability of strains, the viability of Vibrios possessing *ctx* was compared with those of Vibrios without *ctx*. The result indicated that the existence of *ctx* reduce the viability of Vibrios.

METHODS

Bacterial strains

Six clinical strains of *V. cholerae* O1, ten environmental isolates of V. *cholerae* and one strain of *Escherichi coli* were used in this study. In clinical strain of *V. cholerae* O1, five strains (OKA 036, IDH11477, IDH11494, IDH11791, and IDH11827) were isolated from the diarrhea patients admitted to the Infectious Diseases Hospital, Kolkata, India and one strain (N16961), which is a representative strain of *V. cholerae* O1 containing cholera toxin gene (*ctx*), was from our stock culture. The possession of *ctx* of these clinical strains was examined by PCR. The primers used were designed to detect A subnit gene of cholera toxin (CT) (*ctxA*) (Forward primer: 5’-ctcagacgggatttgttaggcacg, reverse primer: 5’tctatctctgtagcccctattacg 3’) [6]. Then we confirmed the existence of *ctx* gene by genome sequencing (data not shown). Strains IDH11477,

IDH11494, IDH11791, and IDH11827 were found to possess *ctx*, but *ctx* was not detected in OKA036.

Other 10 strains of *V. cholerae* used in this experiment were isolated from environmental water around Kolkata area. The serotypes of these strains were examined by slide agglutination test using poly-valent antiserum against *V. cholerae* O1 antigen (Denka-Seiken, Japan). Three strains of them (*V. cholerae* OKA141, OKA501, OKA502) are serotype 1 (O1). Other 7 strains (OKA003, OKA-005, OKA007, OKA140, OKA-144, OKA150 and OKA-155) are strains belonged to nonagglutinating *V. cholerae* (NAG-Vibrio).

ctx in these environmental strains was examined by PCR described as above. By PCR of DNA samples of 10 environmental strains of *V. cholerae*, the fragment corresponding *ctx*A subunit was produced from 2 strains (OKA003 and OKA007), and the fragment was not produced from samples of other 8 strains. (Fig.1).The existence of *ctx* in positive two strains (OKA003 and OKA007) was confirmed by determination of genome sequence (data not shown).

E. coli used is an isolate from healthy person in India. The determination of the sequence of 16s-RNA shows that the strain used is *E. coli*.

Viability study of Vibrio isolates with time

Bacteria were grown in 2ml amounts of LB broth for about 15 hr at 37^{o}C with shaking (150 rpm). A100 µl of above culture of each Vibrio was re-inoculated in 2 ml LB broth and incubated at 37^{o}C for 3 – 5 hr with shaking. The bacterial culture was centrifuged and the pellet was suspended in amoeba saline solution (1X) to give the turbidity of McFarland O.D of 2.0 (~ 1 x10^{8} CFU/ ml). The composition of amoeba saline solution was as follows: NaCl: 0.02M, $MgSO_4.7H_2O$: .016 mM, $CaCl_2$: .054 mM, Na_2HPO_4: 1mM, KH_2PO_4: 0.997 mM. 0.9 ml of the bacterial resuspension was added to 9.1 ml of filter-sterilized distilled water. The viability of the bacterial strains in the solution was measured on 0 days (initiating day of the incubation) and on 10th day of the incubation by plating method using L-agar plate.Viability was determined by counting the number of colonies formed after overnight incubation at 37 C.

Results

The viability of *V.cholerae* in low ionic strength aquatic solution over time was examined by counting the number of colony-forming units after overnight culture at 37^{o}C. At the initiating

time of incubation of these bacterial solution (the data of day 0), approximately $1x10^7$/ml colonies were detected in these solutions (Fig 1A). Slight differences in the numbers of colonies formed among these samples appeared. These differences may come from the difference of the size of each strain. The bacteria with smaller size give lower density in the turbidity. To give the same density with that of larger size bacteria, larger number of the smaller bacteria must be added to the solution. The difference in number of bacteria appeared in Fig 1A may reflect these features. However, the influence of the difference on the measurement of viability of *V. cholerae* was not serious as described later. After incubation for 10 days at 25° C, the viability of these bacteria was measured (Fig. 1B).

V. *cholerae* O1 strains, possessing *ctx* showed greater declining viability (Fig. 1B-4) than that of NAG Vibrio which does not possess *ctxA* (Fig.1B-1). After the incubation for 10 days in this condition, the viability of V. *cholerae* O1 (*ctx*$^+$) decrease from the level of 10^7 CFU per ml to the level of 10^1 CFU per ml. However, the viability of NAG Vibrio (*ctx*$^-$) under the same condition, the decrease of viability was from the level of 10^7 CFU per ml to the level of 10^6 CFU per ml. There is a significant difference in viability of two strains.

One of obvious differences in property of two strains, *V. cholerae* O1 (*ctx*$^+$) and NAG Vibrio (*ctx*$^-$), is possession of *ctx*. To examine the participation of *ctx* in the viability of *V. cholerae,* two types of *V. cholerae, V. cholerae* O1 which does not contain *ctx* (*V. cholerae* O1 (ctx$^-$)) and NAG Vibrio which contain *ctx* (NAG-Vibrio (*ctx*$^+$)), were selected from environmental water and a patient and their viabilities under the same condition were examined. The viability of *V. cholerae* O1(ctx$^-$) differed depending on strains (Fig.1B-3). In strains OKA141 and OKA501, the viability remained as high as that of NAG Vibrio (*ctx*$^-$). While, in strains OKA036 and OKA502, the viability decreased to the level of $4 x10^2$ CFU per ml. Though the viability of the latter two strains, OKA036 and OKA502, was low, but the level was clearly higher than those of *V. cholerae* O1 (*ctx*$^+$) (Fig. 1B-4)

In addition, the viability of NAG-Vibrio (*ctx*$^+$) decreased vigorously to the level of $3 x 10^1$ CFU per ml (in OKA003) and to the level of $2 x 10^2$ CFU per ml (in OKA007) (Fig. 1B-2). The difference between two kinds of NAG-Vibrio, NAG-Vibrio (*ctx*$^-$) and NAG-Vibrio (*ctx*$^+$), is clear. These results indicted that the presence of *ctx* in *V.cholerae* strains affect detrimentally to the viability of the strains in low ionic strength aquatic environment.

Discussion

In general, the main source of virulent *V. cholerae* has been thought to be environment water [7]. To isolate virulent strain of *V. cholerae* O1, we had examined many Vibrios inhabiting environment water in Kolkata multiple number of times. Most of the strains isolated were NAG-Vibrio, wheras *V. cholerae* O1 was almost rarely isolated. Although the Vibrio growth rates in nature, particularly in the marine environment, is an intensively investigated and much discussed [8] [9] [10], studies concerning the kinetics of growth of Vibrios in low ionic aquatic environment are rare. Then, we examined the survival of *V. cholerae* in low ionic strength aquatic environments in this study.

In this experiment, we found that viability of *V. cholerae* O1 (ctx^+) is very low in low ionic strength aquatic environments and that of NAG-Vibrio (ctx^-) is high. The viability of latter strain was almost equal to that of *E. coli* (Fig. 1B-5). Such viabilities of both strains observed might relate to the survival of these strains in environmental water in Kolkata.

Based on the results obtained, we hypothesized that there was some relation between the existence of *ctx* in *V. cholerae* and viability of these strains in low ionic strength aquatic environment. The *ctx* is transferred by CTX phage. Bacteriophages recognize the peculiar structure of target bacteria and attach to the bacteria [11]. The O antigen is said to be the major target of phages [12]. Therefore, it is likely 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*.

The role of CT in pathogenesis has been clearly demonstrated [13], but studies had not been carried out to analyze its effect on the viability of the Vibrios in environmental water. Our result consistently shows that Vibrios containing *ctx* have lower viability in water with low ionic strength, than the Vibrios without *ctx*. This may indicate that the production of CT in poor nutrition condition such as in environment water may be an energy burden for *V. cholerae* and cause extinction of the bacteria. On the other hand, the production of CT might promote the survival of *V. cholerae* in human intestine [14]. CT released into intestinal lumen induces the exudation of body fluid from host into intestinal lumen, which leads to diarrhea [15]. The exudate contains many nutritious substances for *V. cholerae* and the bacteria can survive for long time by utilizing these nutritions [16]. Further studies are necessary to clarify the relationship between the function of CT and the survival of *V. cholerae* in natural ecological system.

References

1. Chatterjee A, Dutta P, Chowdhury R. 2007. Effect of Fatty Acids and Cholesterol Present in Bile on Expression of Virulence Factors and Motility of Vibrio *cholerae*. Infection and Immunity 75:1946-1953.

2. Kaper J, Morris J, Levine M. 1995. Cholera. Clinical Microbiology Reviews 8:48-86.

3. Charles R, Ryan E. 2011. Cholera in the 21st century. Current Opinion in Infectious Diseases 24:472-477.

4. Weil A, Harris J. 2015. Vibrio cholerae. Molecular Medical Microbiology 1079-1098.

5. Mandal S. 2011. Cholera Epidemic in and Around Kolkata, India: Endemicity and Management. Oman Medical journal 288-289.

6. KEASLER S. 1993. Detecting and biotyping Vibrio cholerae 01 with multiplex polymerase chain reaction. The Lancet 341:1661.

7. Salvador almagro-moreno, Ronald k. taylor. 2013. Cholera: Environmental Reservoirs and Impact on Disease Transmission. One Health 149-165.

8. Ducklow H, Carlson C. 1992. Oceanic Bacterial Production.Advances in Microbial Ecology 113-181.

9. Hagström Å, Larsson U. 1984. Diel and Seasonal Variation in Growth Rates of Pelagic Bacteria. Heterotrophic Activity in the Sea 249-262.

10. Robarts R, Zohary T. 1993. Fact or Fiction-Bacterial Growth Rates and Production as Determined by [methyl-3H]-Thymidine.Advances in Microbial Ecology 371-425.

11. Brussow H, Canchaya C, Hardt W. 2004. Phages and the Evolution of Bacterial Pathogens: from Genomic Rearrangements to Lysogenic Conversion. Microbiology and Molecular Biology Reviews 68:560-602.

12. Seed K, Faruque S, Mekalanos J, Calderwood S, Qadri F, Camilli A. 2012. Phase Variable O Antigen Biosynthetic Genes Control Expression of the Major Protective Antigen and Bacteriophage Receptor in Vibrio *cholerae* O1. PLoS Pathogens 8:e1002917.

13. Goodsell D. 2005. Cholera Toxin. RCSB Protein Data Bank.

14. Conner J, Teschler J, Jones C, Yildiz F. Staying Alive: Vibrio cholerae's Cycle of Environmental Survival, Transmission, and Dissemination. Virulence Mechanisms of Bacterial Pathogens, Fifth Edition 593-633.

15. Field M. 2003. Intestinal ion transport and the pathophysiology of diarrhea. Journal of Clinical Investigation 111:931-943.

16. Krebs S, Taylor R. 2011. 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.

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Fig. 1

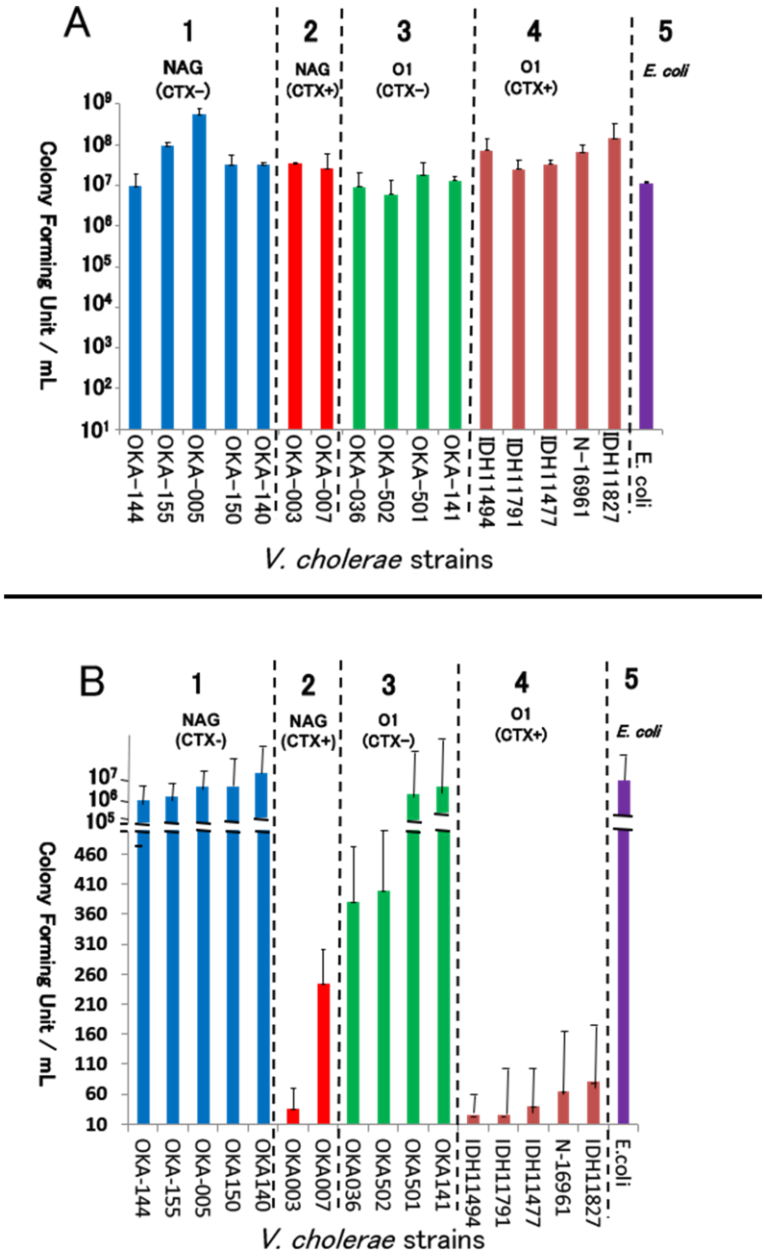

Fig 1: Number of colonies of *V.* cholerae strains formed on LB agar plates after incubation. *V. cholerae* strains were inoculated in low ionic strength aquatic solution and viability was determined by plate count method at (A) 0 day (initiating day of incubation) and at (B) 10th day of incubation. An irrelevant bacteria *E.coli* was used as a positive control. Value at each time-point shows the mean of four separate experiments (Fig. 1B-4) and two separate experiments (others). Data is shown as mean + SD.