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Environmental water in Kolkata is suitable for the survival of *Vibrio cholerae* O1

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ABSTRACT

Many patients with cholera emerge in Kolkata, India throughout the year. Such emergency indicates that cholera toxin-producing *Vibrio cholerae* O1 (toxigenic *V. cholerae* O1) are widespread in Kolkata. This suggests that the suitable conditions for replication of toxigenic *V. cholerae* O1 is provided in Kolkata. In previous studies, we found that the replication rate of toxigenic *V. cholerae* O1 is low in the low ionic aqueous solution. Then we measured the ion concentration in the environmental water of Kolkata. As a control, we measured them in Japanese environmental water. The ion concentration in the environmental water of Kolkata was significantly high. Then, we examined the survival of toxigenic *V. cholerae* O1 in groundwater from Kolkata and found that *V. cholerae* O1 survive for long time in the solution but not in the solution diluted with Milli Q water. In addition, we found that *V. cholerae* O1 proliferated in environmental water of Kolkata to which a small amount of nutrient was added, but did not grow in the environmental water diluted with water to which the same amount of nutrient was added. These results indicate that the environmental water from Kolkata is suitable for survival of *V. cholerae* O1.

1. Introduction

Cholera is a life-threatening acute secretory diarrhea that is caused by cholera toxin-producing *Vibrio cholerae* O1 (toxigenic *V. cholerae* O1) and has been reported around the world for the last 200 years. The number of patients with cholera disease has decreased in developed countries in recent years; however, large epidemics still have appeared in developing and underdeveloped countries of South Asia, Latin America, and Africa (Ali et al. (2015); Kaper et al. (1995). Historically, seven distinct pandemics of cholera have occurred since the onset of the first recorded pandemic in 1817. The 1st through 6th pandemics were shown to originate from the Bengal region of India, near Kolkata (Faruque et al., 1998; Kaper et al., 1995). In contrast, the 7th pandemic was thought to have originated from Indonesia. However, a recent genomic study has shown that the *V. cholerae* O1 isolate that triggered the 7th pandemic also was present in the Kolkata area (Hu et al., 2016; Weill et al., 2017). Therefore, the region around Kolkata is considered to be a hotbed area for cholera.

Because toxigenic *V. cholerae* O1 is regarded as a bacterium that lives in environmental water (Alam et al., 2007; Islam et al., 2020), we postulated that toxigenic *V. cholerae* O1 is able to inhabit environmental water in Kolkata, permitting these bacteria to overwinter in this area. This hypothesis suggests that environmental water in Kolkata is favorable for the maintenance of toxigenic *V. cholerae* O1.

In the previous study, we examined the survival of toxigenic *V. cholerae* O1 in the artificial low ionic strength aquatic solution (Paul et al., 2020). The solution used was Page's solution diluted to a concentration of 4.6 mg/L for Na^+. In this solution used, the viability of toxigenic *V. cholerae* O1 was extremely low. From the result, we thought that the ion concentration in the environmental water had a great influence on the survial of toxigenic *V. cholerae* O1.

People in Kolkata have many opportunities to contact with the environmental water. In Kolkata, many households store well water in their jars and use it as domestic water. People often swim or wash in the

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ponds.

The Kolkata area consists largely of run-off land from the Ganges River. The Kolkata area often is inundated by sea water, and the soils in this area frequently have been polluted with sea water. Thus, the groundwater in the Kolkata area is expected to contain components of sea water (Bricheno et al., 2016). The components of the water in the ponds are also affected by the components of groundwater. In Kolkata, *V. cholerae* excreted from feces has a great chance of contaminating these environmental waters. The survival and growth of *V. cholerae* in the environmental water have a great influence on the transmission of *V. cholerae* in this area.

In the present study, we examined the characteristics of environmental water from the Kolkata area and the activity of the water in supporting the survival and growth of *V. cholerae* O1.

2. Materials and methods

2.1. Sampling of environmental water

Groundwater and pond water samples were collected as environmental water in 2 countries, India and Japan. In India, these water samples were collected in Kolkata, and in Japan from Okayama and Tokyo. In Kolkata, groundwater samples were collected from 2 tube wells in the Beliaghata district (Site-1 and Site-2); pond water was collected from 2 ponds (Pond A in the Sector V district, and Pond B in the Sector A district). In Japan, groundwater was collected from 4 underground springs as Omachi-water in Takashima, Okayama (Site-3); Yamadori-water in Akaiwa, Okayama (Site-4); Otomeyama park-water in Shinjuku, Tokyo (Site-5); and Megurofudoson-water in Meguro, Tokyo (Site-6). Japanese pond water samples were collected from 3 ponds in Okayama (Pond C in the Sawada district, Pond D in the Hara district, and Pond E in Akaiwa). These waters were sampled in October 2018 (Pond A, Pond B), June 2019 (Pond C, Site 1, Site 2, Site 3), September 2019 (Pond D, Pond E, Site 4) and October 2019 (Site 5, Site 6).

2.2. Measurement of the concentrations of ions in water

Water samples were sterilized using a membrane filter (pore size, 0.45 μM). The ion concentrations of these samples were determined by the Indian and Japanese analytical institutions described below. The kinds of ions examined included Na^+, K^+ Ca^{2+}, Mg^{2+}, Ag^+, Zn^{2+}, Al^{3+}, NH_4^+, Cl^-, NO_3^- and SO_4^{2-}.

Samples procured in India were analyzed by SGS India Pvt., Ltd. (Kolkata, India). Analyses were carried out using the methods described in Standard Methods for the Examination of Water, 23rd ed. (American Public Health Association, American Water Works Association, Water Environment Federation) and Indian Standard Methods of Sampling and Testing (physical and chemical) for water and wastewater (Bureau of Indian Standard, 1988; 1986, 1991).

Samples procured in Japan were analyzed by OML Ltd. (Okayama, Japan). Analyses were carried out using the methods described in Testing Methods for Industrial Wastewater (Japanese Standard Association, 2016) and Standard Methods for the Examination of Water (Japan Water Works Association, 2011).

2.3. Bacterial strains and preparation of bacterial suspension to evaluate the survival of bacteria

V. cholerae N16961 was used as a standard strain of *V. cholerae* O1 (Heidelberg et al., 2000). Other strains used in this study (*V. cholerae* IDH11791, *V. cholerae* IDH11494, *Escherichia coli* 1, *E. coli* 2, *E. coli* 3) were isolated in Kolkata, India, by our laboratory. *V. cholerae* were isolated from diarrhea patients and *E. coli* were isolated from a healthy person. Isolation of these strains was carried out as reported previously (Nair et al., 2010). The serotype of *V. cholerae* was determined using a slide agglutination test with polyvalent antiserum against the O1 antigen of *V. cholerae* (Denka-Seiken, Tokyo, Japan). The results showed that these two strains (*V. cholerae* IDH 11494 and IDH 11791) are serotype 1 (O1). The species of *E. coli* used was confirmed by the determination of the nucleotide sequences of the 16S rRNA genes (data not shown).

Water samples used in the survival experiment were derived from the groundwater from Site-1 in Kolkata. Bacteria were cultured in LB-broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 37 °C for 4–5h with shaking. The cultures then were centrifuged for 10 min at $10,000\times g$ to collect the bacteria. The resulting bacterial pellet was suspended in either a filter-sterilized stock solution of groundwater from Site-1 or a 10% solution of the groundwater to give a McFarland turbidity of 2.2. A portion (0.8 mL) of one of these suspensions was added to 9.2 mL of groundwater, or adequately diluted groundwater, or Milli Q water. The final concentrations of groundwater in these samples were 100%, 40%, 10.0%, 4.0%, or 0.8%.

2.4. Number of viable bacteria in solution

The number of viable bacteria in the suspension was determined by a plating method. A 100-μL sample of the serially diluted suspension was plated on LB-agar plate (Becton, Dickinson and Company) and the plate was incubated for 20h at 37 °C. The number of colonies formed was counted, and colony-forming units (CFUs) of the original suspension were calculated from the number obtained. The CFU was used as the number of viable bacteria in the inoculum.

2.5. Growth of bacteria in the diluted groundwater containing limiting nutrients

The bacteria were cultured in LB-broth at 37 °C for 4–5h with shaking. The cultures were centrifuged for 10 min at $10,000\times g$ to collect the bacteria. The resulting bacterial pellet was suspended in LB-broth to give a McFarland turbidity score of 8.0.

The groundwater was serially diluted 5-fold with Milli Q water. These water samples (original groundwater, diluted groundwater, and Milli Q water) and the prepared bacterial suspensions were combined at a ratio of 9:1. These mixtures were cultured at 37 °C with shaking (140 rpm), and the McFarland turbidity of each sample was measured at regular intervals.

3. Results

3.1. Ion concentration in environmental water

Groundwater and pond water were chosen as environmental water sources. Groundwater from six sites (2 in the Kolkata area and 4 in Japan) and pond water from 5 sites (2 in the Kolkata area and 3 in Japan) were collected and filtered through a sterile filter with a pore size of 0.45 μM. The concentrations of 11 ions (Na^+, K^{2+}, Ca^{2+}, Mg^{2+}, Ag^+, Zn^{2+}, Al^{3+}, NH_4^+, Cl^-, NO_3^-, and SO_4^{2-}) in these samples were measured using standard methods. In the groundwater samples, the concentrations of these ions in the samples from Kolkata were higher than these of samples from Japan. Among these ions, the concentrations of Na^+, Ca^{2+}, Mg^{2+}, and Cl^- in Kolkata groundwater were remarkably high (Fig. 1a). Similarly, the concentration of these ions in the pond water were higher than those of Okayama, Japan (Fig. 1b). Therefore, we thought the high ion concentration might relate to the outbreak of cholera disease in this area.

3.2. Survival of V. cholerae O1 in groundwater in Kolkata

To examine the effect of groundwater from Kolkata on the survival of *V. cholerae* O1, the survival of *V. cholerae* N16961 in the water samples containing groundwater from Site-1 in Kolkata at various ratios was

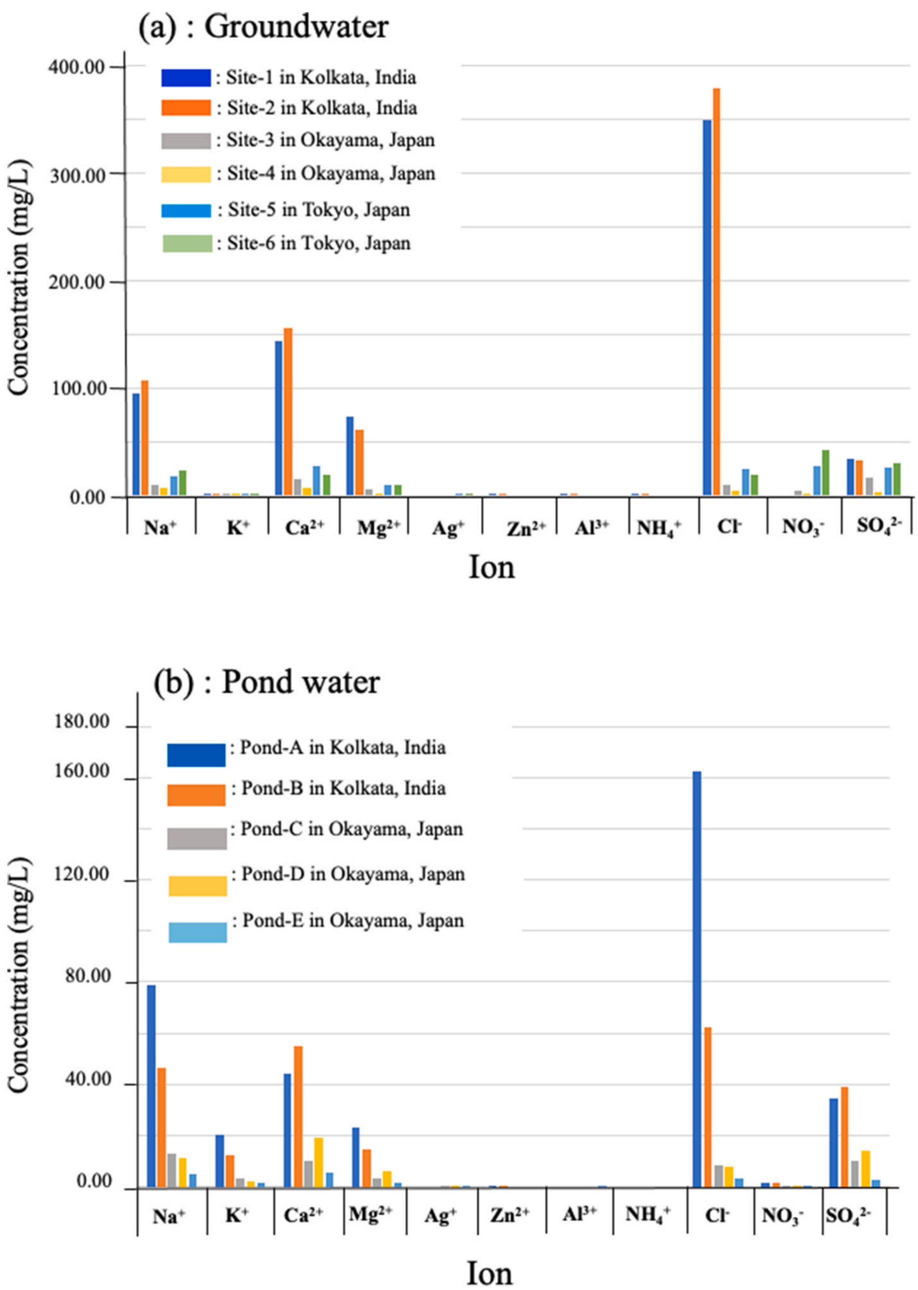

Fig. 1. Ion concentrations of groundwater (a) and pond water (b). Groundwater and pond water samples were collected as environmental water from 2 countries, India and Japan, and these water samples were sterilized using 0.45-μM membrane filters. Ion concentrations in these water samples were determined by specialist companies in the respective countries. Analysis of water collected in India was performed using the methods described in Standard Methods for the Examination of Water, 23rd ed. (American Public Health Association, American Water Works Association, Water Environment Federation) and Indian standard Methods of Sampling and Testing (physical and chemical) for water and wastewater (Bureau of Indian Standard, 1988; 1986, 1991). Water collected in Japan was analyzed using the methods described in Testing Methods for Industrial Wastewater (Japanese Standard Association, 2016) and Standard Methods for the Examination of Water (Japan Water Works Association, 2011).

examined.

V. cholerae N16961 were suspended in a solution containing groundwater at various concentrations. The cell suspension was prepared as described in [Preparation of bacterial suspension to evaluate the survival of bacteria] in the section of Materials and Methods. The final concentrations of groundwater in these samples were 100%, 40%, 10.0%, 4.0%, or 0.8%.

The number of bacteria in these samples just after inoculation with *V. cholerae* N16961 was 2.1 × 10^7/mL (bar labeled "0h" in Fig. 2a). These samples were incubated (without shaking) at 37 °C for 20h. After incubation, the number of cells in these samples was counted using a plating method. The number decreased in every sample. Especially, almost all the bacteria had fallen to the point of death in samples containing low proportions of the groundwater such as 4.0% and 0.8% (Fig. 2a).

To examine whether the decrease observed in *V. cholerae* N16961 incubated in solutions containing low proportions of groundwater from Kolkata was characteristic of *V. cholerae* O1, samples for *E. coli* 1 were prepared in the same way. After incubation for 20h, the number of *E. coli* cells in these samples was measured (Fig. 2b). The number of *E. coli* cells decreased in every sample. However, the phenomenon that the viable cell count disappeared did not occur. That is, a considerable number (1% or more) of bacteria survived even in a 0.8% or 4.0% solution (Fig. 2b). This result suggested that cell death in samples containing low proportions of groundwater from Kolkata was a distinctive feature of *V. cholerae* O1.

The water temperature of Pond A of Kolkata was between 20 °C and 32 °C during our survey (December 2017–June 2018). Then, in order to investigate the survival of toxigenic *V. cholerae* O1 in the field environment water of Kolkata, the survival of the bacteria at 25 °C, which is close to the average water temperature throughout the year in Kolkata, was examined. After incubation for 20h at 25 °C, the number of cells was counted (Fig. 2c). A decrease in viable cell count was observed in all samples, but the decrease was particularly significant in water

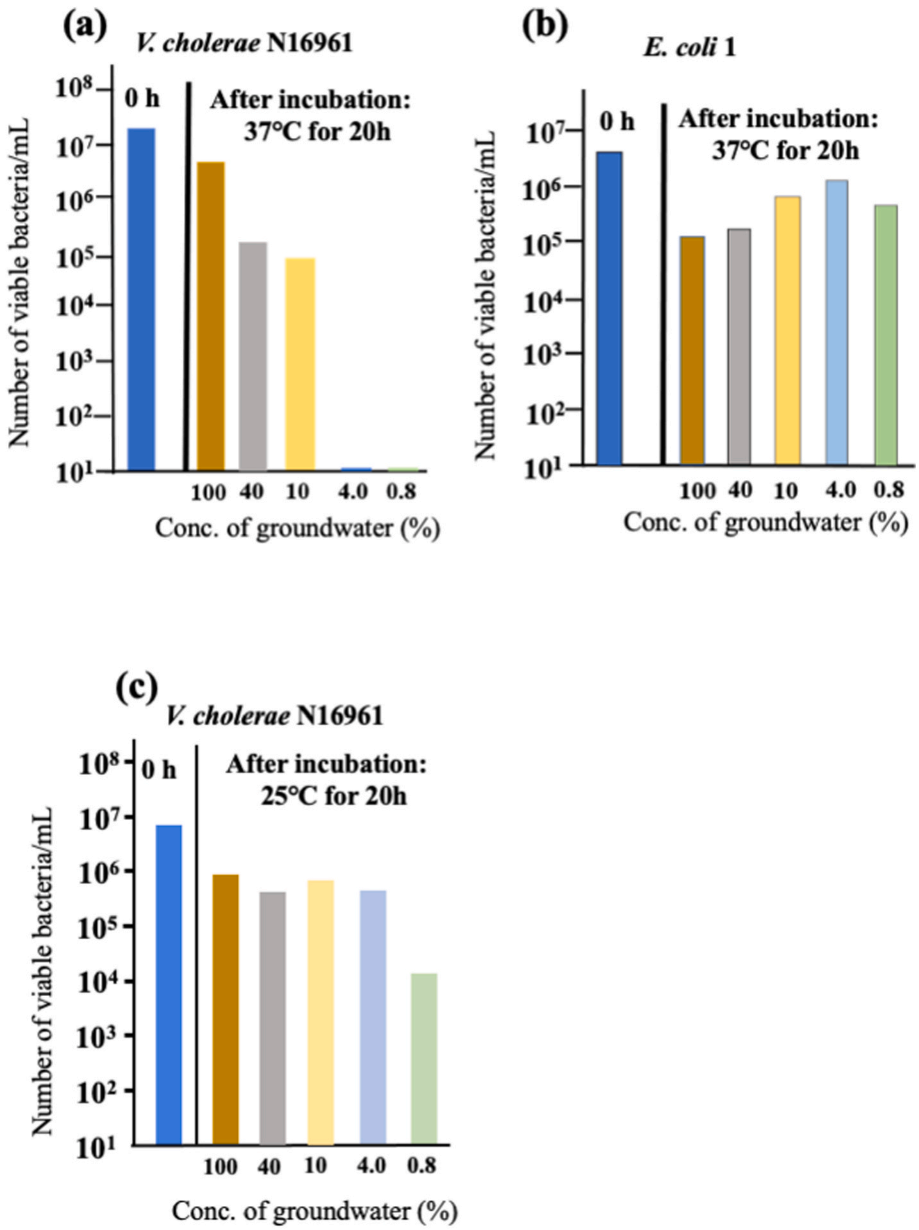

Fig. 2. Survival of V. cholerae O1 and E. coli in solutions containing groundwater diluted to various concentrations with Milli Q water. Bacteria grown to logarithmic phase in LB-broth at 37 °C were collected by centrifugation and suspended in either a sterilized groundwater from Site-1 in Kolkata or a 10% solution of the groundwater. The density of these bacterial suspensions was adjusted to give a McFarland turbidity of 2.2. A portion (0.8 mL) of one of these suspensions was added to 9.2 mL of groundwater, adequately diluted groundwater, and Milli Q water. The final concentrations of groundwater in each sample were 100%, 40%, 10%, 4.0%, and 0.8%. These bacterial suspensions were incubated at 37 °C for 20h (a and b) or 25 °C for 20h (c). The numbers of viable bacteria in the cultures were determined by plating. Samples (100 μL) of the serially diluted cultures were plated on LB-agar plates and the plates were incubated for 20h at 37 °C. The number of colonies formed was counted, and number of viable bacteria in the original suspension was calculated from the number obtained. The bar in the region indicated by 0h in the figure shows the number of viable bacteria at the time of addition of the original bacterial solution.

containing only 0.8% groundwater. The survival rate was 0.4% or less in the 0.8% aqueous solution. On the other hand, more than 15% of the bacteria remained as live bacteria in the undiluted groundwater. From these results, it was considered that Kolkata's environmental water effectively acts to promote the survival of *V. cholerae* in the environmental water throughout the year.

3.3. Survival of V. cholerae O1 in diluted groundwater at low bacterial density

In the above experiments (Fig. 2), suspensions containing more that 4.0×10^6 cells/mL of *V. cholerae* O1 were used. Contamination with such high numbers of *V. cholerae* O1 is unlikely in a normal living environment. Therefore, the survival of *V. cholerae* O1 at a bacterial density of approximately 300 CFU/mL in suspensions containing Site-1 groundwater at various ratios was examined.

The number of viable bacteria fell in every sample (Fig. 3). However, the decrease was small in the undiluted groundwater, and the decrease was larger in diluted groundwater. These data showed that groundwater from Kolkata increases the survival of *V. cholerae* O1 even if the density of bacteria is low.

3.4. Growth of bacteria in groundwater supplemented with limiting nutrients

In Kolkata, pond water and groundwater are often eutrophicated by human waste and feed for farmed fish. To investigate the survival and growth of *V. cholerae* in the water contaminated these materials, the growth of *V. cholerae* in Kolkata groundwater supplemented with limiting nutrients was examined. The nutritional value of these samples was 1/10 of that in standard LB-broth. Bacteria in this medium were incubated at 37 °C with shaking. The growth of bacteria was measured using McFarland turbidity. To determine the contribution of groundwater to cell growth, sample solutions containing various amounts of groundwater from Site-1 in Kolkata were prepared. The concentrations of groundwater in these sample solutions were as follows: 90.00%, 18.00%, 3.60%, 0.72%, 0.14%, 0.03%, and 0.00% (Fig. 4). The growth of *E. coli* was also examined and compared with that of *V. cholerae*.

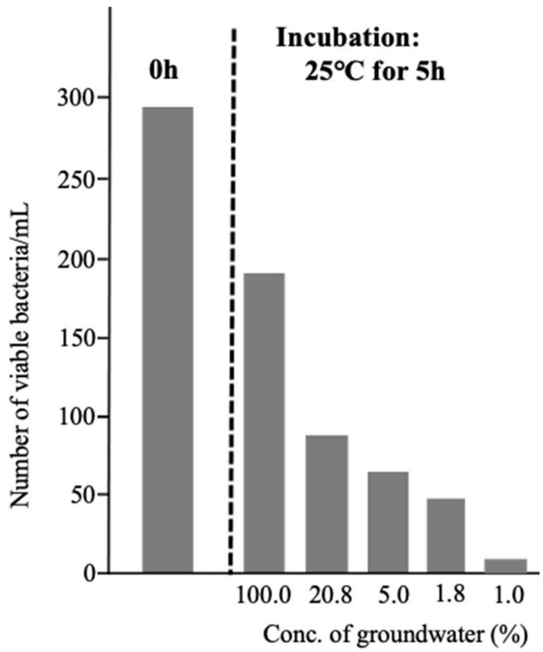

Fig. 3. Survival of *V. cholerae* O1 inoculated at low density in solutions containing groundwater diluted to various concentrations with Milli Q water. *V. cholerae* N16961 was suspended in filter-sterilized Site 1 groundwater to give a McFarland turbidity of 2.2. The bacterial solution was diluted 10^3-fold with the undiluted groundwater from Site-1 in Kolkata. A portion (0.1 mL) of the suspension was added to 9.9 mL of groundwater, adequately diluted groundwater, and Milli Q water. The final concentrations of groundwater in each sample were 100%, 20.8%, 5.0%, 1.8% and 1.0%. After incubation at 25 °C for 5h, the number of viable bacteria in these cultures was measured by plating. The bar in the region indicated by 0h in the figure shows the number of viable bacteria at the time of addition of the original bacterial suspension to each tube.

V. cholerae N16961 grew well in the 90.00% and 18.00% solutions, but did not grow in the 0.72%, 0.14%, 0.03% or 0.00% solutions. Slight growth was observed in the 3.60% solution after incubation for 5h (Fig. 4a). A similar relationship between growth and concentration of groundwater was observed for the growth of other strains of *V. cholerae* O1. These strains also did not grow in the 0.14%, 0.03%, or 0.00% solutions (Fig. 4b and c).

The growth of *E. coli* in the same conditions was examined. *E. coli* grew in all of conditions examined. *E. coli* grew well in solutions containing high ratios of groundwater and grew to a certain level in solutions containing low ratios of groundwater. *E. coli* even grew in the solution that did not contain any groundwater (0.00% solution) (Fig. 4d, e and 4f).

3.5. Bacterial growth in pond water

As shown in Fig. 1b, pond water from the Kolkata area contained higher levels of ions compared to water from ponds in other areas. To examine whether the Kolkata pond water, like Kolkata groundwater, possesses any activity to promote the growth of *V. cholerae* O1, *V. cholerae* N16961 was cultured in diluted pond water from Pond A of Kolkata supplemented with limiting nutrients, with conditions matching those used for the experiment shown in Fig. 4.

V. cholerae N16961 grew in samples containing a high proportion of pond water, but not in samples containing a low proportion of pond water (Fig. 5a). In contrast, and as observed in the cultures using groundwater, *E. coli* 1 grew even in solutions containing a low proportion of pond water (Fig. 5b).

4. Discussion

As shown by the measurements in this manuscript, ion concentrations in groundwater and pond water in Kolkata were higher than those in Japanese water which we examined (Fig. 1). Furthermore, the comparison with the ion concentrations of environmental waters f reported from around the world showed that the ion concentrations of the environmental waters examined in Kolkata were higher than those reported (Chen et al., 2019; Mora et al., 2017; Nwankwo et al., 2020; Saeki and Tase, 1988; Yamanaka, 2017; Yoshikawa et al., 2020). Subsequently, our study in this manuscript presented that groundwater in the Kolkata area is suitable for the survival of *V. cholerae* O1 (Fig. 2a, c, and 3). Furthermore, it was shown that *V. cholerae* O1 grows in groundwater from Kolkata supplemented with limiting nutrients (Fig. 4). The survival and growth of *V. cholerae* O1 was observed in undiluted Kolkata groundwater, but not in diluted groundwater. Given the elevated concentrations of some ions (including Na^+, Ca^{2+}, Mg^{2+}, and Cl^-) in Kolkata groundwater compared to water from other areas (Fig. 1), some of these ions may be responsible for the longer survival and growth of *V. cholerae* O1.

It has been reported that *V. cholerae* O1 does not grow in low-nutrient medium in the absence of added NaCl (Kaper et al., 1979; Singleton et al., 1982) and maximal growth of *V. cholerae* O1 occurs in medium of 2.5% salinity (Grant et al., 2015; Singleton et al., 1982). In addition, we have found and reported that *V. cholerae* O1 cells in low ionic strength aquatic solutions die within a short interval (Paul et al., 2020). Thus, it indicates that the concentration of salt greatly affects the growth of *V. cholerae* O1 *in vitro*. It is also known that temperature and salinity have a great influence on the survival of Vibrionaceae in the natural world, but the properties and condition of water in the natural world are influenced by various factors in a complicated manner depending on the region. Its complexity affects the kinds of bacteria inhabiting the area (Takemura et al., 2014). Under such circumstances, *V. cholerae* O1 sometimes has been isolated from brackish water (Islam et al., 2020; Nair et al., 1988). However, to our knowledge, few studies have reported on the isolation of *V. cholerae* O1 from seawater or freshwater, though non-O1/non-O139 *V. cholerae* frequently are isolated from such water sources (Ceccarelli et al., 2015; Colwell et al., 1977; Daboul et al., 2020; Fernandez and Waters, 2019; Kaper et al., 1979). These observations indicate that *V. cholerae* O1 is an autochthonous resident of estuaries and support our hypothesis that the ions contained in the groundwater around Kolkata permit the survival and growth of *V. cholerae* O1.

The occurrence of cholera patients usually shows explosive growth in a fixed period. However, after a few years, the outbreak of cholera cases from the infected area disappears. Such mode of cholera epidemic has been the mode of cholera outbreaks around the world since John Snow first reported outbreaks of cholera (Grant et al., 2015; Snow, 1856). Cholera patients in Kolkata, however, have been reported as emerging throughout the year. Additionally, the type of occurrence is not explosive. The number of patients decreases in the winter season (from December to February), but a certain number of the patients appear throughout the year, even in winter (Nair et al., 2010). In addition, the emergence of cholera patients is sporadic, and the collective outbreak of cholera disease in a certain area has not been observed in Kolkata. However, cholera patients occur annually (Kanungo et al., 2010; Nair et al., 2010). Such a type of occurrence of cholera patients indicates that the source of infection of cholera disease, namely toxigenic *V. cholerae* O1, is widely scattered in the Kolkata area and the *V. cholerae* O1 live on in secret.

As shown in this report, environmental water around Kolkata, including groundwater and pond water, might facilitate the survival of *V. cholerae* O1 (Figs. 2–5). *V. cholerae* O1 can survive for a long time in the area around Kolkata. However, long-term survival may be difficult in other areas where the ion concentration of environmental water is low. The unique properties of environmental water around Kolkata may be related to the habitat of *V. cholerae* O1 in the Kolkata area throughout

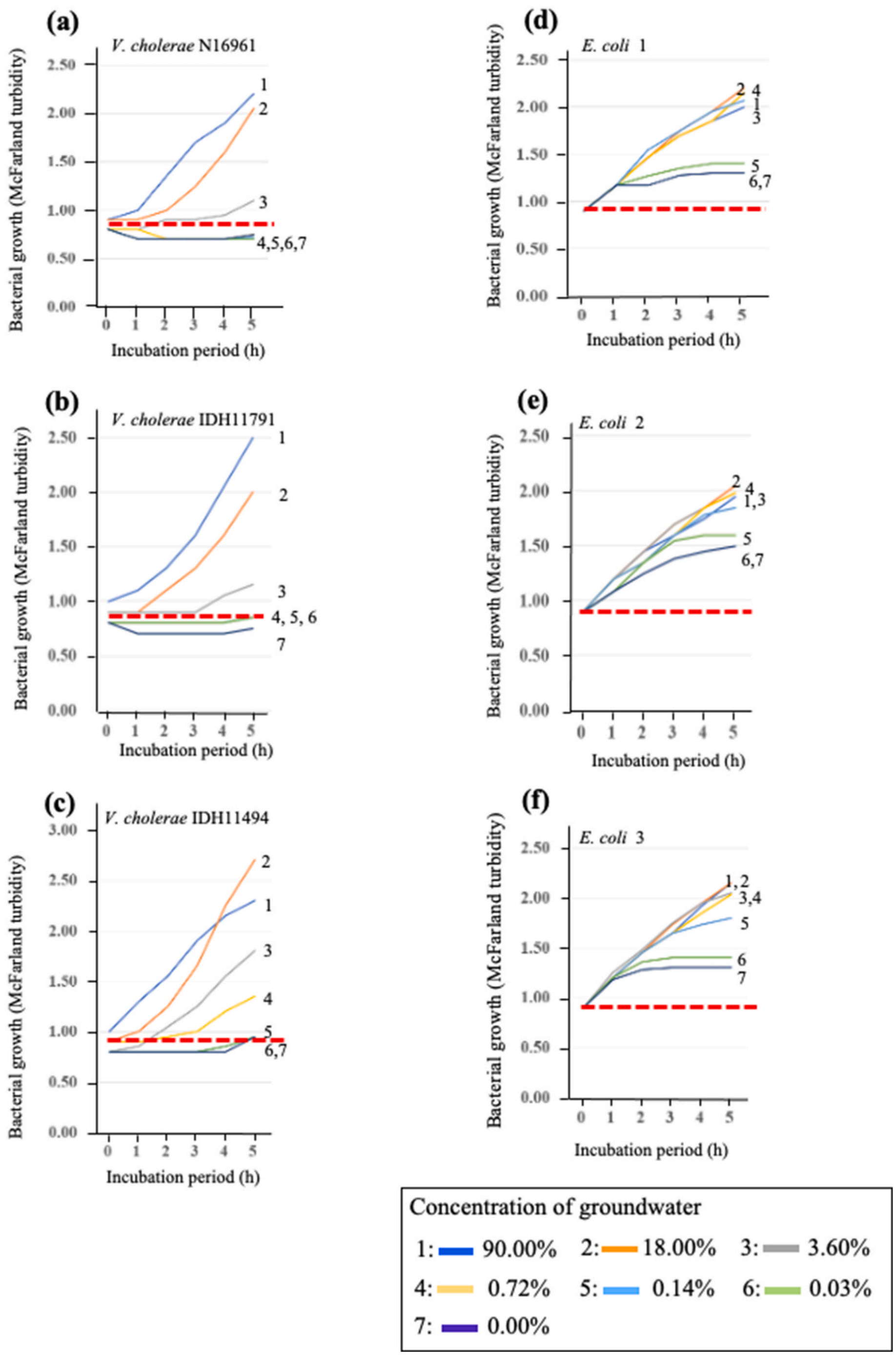

Fig. 4. Growth of bacteria in diluted groundwater containing limiting nutrients. Three strains of *V. cholerae* O1 (N16961, IDH11791, and IDH11494) and 3 strains of *E. coli* (1, 2, and 3) were cultured (separately) in LB-broth to logarithmic phase. These bacteria were collected by centrifugation. The bacterial pellets were suspended (separately) in LB-broth to give a McFarland turbidity of 8.0. The groundwater from Site-1 was subjected to serial 5-fold dilution with Milli Q water. The resulting water samples (original groundwater, diluted groundwater, and Milli Q water) and the bacterial suspensions were mixed at a ratio of 9:1. These mixtures were cultured at 37 °C with shaking (140 rpm) and the McFarland turbidity of each sample was measured at regular intervals for 5 h. The concentration of groundwater in each culture is shown in the figure.

the year and the chronic generation of cholera patients in this area.

In India, cholera outbreak has been reported to occur frequently in areas with coastlines (Muzembo et al., 2022). As described, *V. cholerae* O1 requires salt for survival and growth. Therefore, the high incidence of cholera outbreak in areas with coastlines may be due to the influence of seawater on the ion concentration of well water and pond water, which are related to people's lives. However, the relationship between this outbreak of cholera diseases and the ion concentration of water related to people's lives has not been examined. In addition, the frequency of small, sporadic onset of cholera disease in India, not large outbreaks of cholera infection, is not well documented. The survey across India has not been fully done.

Despite this situation, the work of Pal and Nayak in Odisha is valuable. Odisha, adjacent to Kolkata, is located on the eastern coast of India and has a long coastal line. Kolkata and Odisha are geographically similar. The large area in Odisha is affected by seawater (https://www.researchgate.net/publication/331299104_Ground_water_quality_of_Odisha_State). It has been reported that *V. cholerae* O1 was often isolated from environmental water in Odisha, and cholera cases often occurred in all seasons (Pal et al., 2021; Nayak et al., 2020). These reports support our hypothesis that the quality of environmental water around this area is related to chronic cholera outbreaks in this area.

On the other hand, the incidence of cholera patients in Japan is extremely low. The Japanese water analyzed in this study was sampled in 2019. The numbers of cholera patients in Japan in 2018, 2019 and 2020 were 4, 5 and 1, respectively. All patients are sporadic patients (http://idsc.nih.go.jp/iasr/27/311/tpc311-j.html). Therefore, we can conclude that "no situation has occurred in Japan in which many people

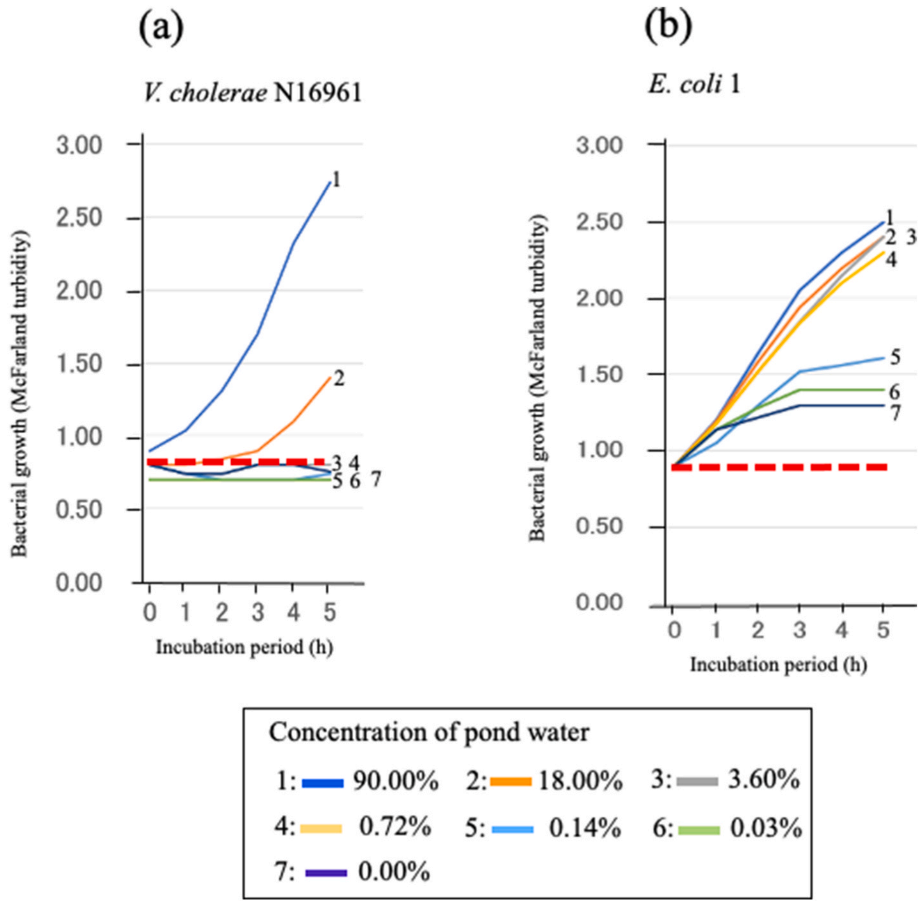

Fig. 5. Bacterial growth in pond water. Bacterial suspensions containing diluted pond water and limiting nutrients were prepared as described in Fig. 4. The water used for the experiment in Fig. 5 was obtained from Pond A, and the strains analyzed were *V. cholerae* O1 (N16961) (a) and *E. coli* 1 (b). The concentrations of the pond water in each culture are shown in the figure. Bacterial growth was measured using McFarland turbidity.

have been infected with *V. cholerae* O1 through the environmental water, and the water analyzed in this manuscript is not associated with the transmission of *V. cholerae* O1".

In addition, as shown by the measurements of water of Lake Biwa (Hayakawa and Fujii. 2010), Lake Inawashiro (https://tenbou.nies.go.jp/science/institute/region/journal/JELA_3501033_2010.pdf) and groundwater (https://www.gytek.co. jp/news/hitokuchi_200812go.htm), the ion concentration of environmental water in many places in Japan is low. The values are at the same level as the ion concentrations in the environmental waters which we analyzed in this manuscript and are lower than those in the environmental waters of Kolkata. Therefore, the environmental water used in daily life in Japan is not suitable for the survival and growth of c *V. cholerae* O1. The research on the ecology of *V. cholerae* presented in this manuscript will contribute to research on the eradication of cholera infections.

The effect of the groundwater from Kolkata on the survival of *E. coli* was not as significant as that on *V. cholerae* O1. (Figs. 2b, 4d and 4e, 4f and 5b). The ion requirement of *E. coli* is lower than that of *V. cholerae* O1. Therefore, it was speculated that *E. coli* can live in environmental water with low ion concentration, and that it may cause *E. coli* infection even in areas with low ion concentration in environmental water including Japan.

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Author contributions

Eizo Takahashi: Designation of research and collection of sample water. Kei Kitahara: Measurement of number of bacterial in sample water. Shin-ichi Miyoshi: Funding acquisition. Goutam Chowdhury: Measurement of number of bacterial in sample water. Asish K. Mukhopadhyay: Measurement of ion concentration in sample water. Shanta Dutta: Drafting the research plan. Sadayuki Ochi: Assistance in ion concentration measurement. Keinosuke Okamoto: Drafting the research plan and writing the manuscript.

Ethical approval

Not applicable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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References

Alam, M., Sultana, M., Nair, G.B., Siddique, A.K., Hasan, N.A., Sack, R.B., et al., 2007. Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. Natl Acad Sci USA 104, 17801–17806. https://doi.org/10.1073/pnas.0705599104.

Ali, M., Nelson, A.R., Lopez, A.L., Sack, D.A., 2015. Updated global burden of cholera in endemic countries. PLoS Neglected Trop. Dis. 9, e0003832 https://doi.org/10.1371/journal.pntd.0003832.

Bricheno, L.M., Wolf, J., Islam, S., 2016. Tidal intrusion within a mega delta: an unstructured grid modelling approach. Estuar. Coast Shelf Sci. 182 https://doi.org/10.1016/j.ecss.2016.09.014. PartA 12-26.

Ceccarelli, D., Chen, A., Hasan, N.A., Rashed, S.M., Huq, A., Colwell, R.R., 2015. Non-O1/non-O139 *Vibrio cholerae* carrying multiple virulence factors and *V. cholerae* O1 in the Chesapeake bay, Maryland. Appl. Environ. Microbiol. 81, 1909–1918. https://doi.org/10.1128/AEM.03540-14.

Chen, Y., Wang, L., Liang, T., Xiao, J., Li, J., Wei, H., et al., 2019. Major ion and dissolved heavy metal geochemistry, distribution, and relationship in the overlying water of Dongting Lake, China. Environ. Geochem. Health 41, 1091–1104. https://doi.org/10.1007/s10653-018-0204-y.

Colwell, R.R., Kaper, J., Joseph, S.W., 1977. *Vibrio cholerae, Vibrio parahaemolyticus* and other vibrios: occurrence and distribution in Chesapeake bay. Science 198, 394–396. https://doi.org/10.1126/science.198.4315.394.

Daboul, J., Weghorst, L., DeAngelis, C., et al., 2020. Characterization of *Vibrio cholerae* isolates from freshwater sources in northwest Ohio. PLoS One 15, e0238438. https://doi.org/10.1371/journal.pone.0238438.

Faruque, S.M., Albert, M.J., Mekalanos, J.J., 1998. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiol. Mol. Biol. Rev. 62, 1301–1314. https://doi.org/10.1128/mmbr.62.4.1301-1314.1998.

Fernandez, N.L., Waters, C.M., 2019. Cyclic di-GMP increases catalase production and hydrogen peroxide tolerance in *Vibrio cholerae*. Appl. Environ. Microbiol. 85 https://doi.org/10.1128/AEM.01043-19 e01043-19.

Grant, S.L., Tamason, C.C., Hoque, B.A., Jensen, P.K.M., 2015. Drinking cholera: salinity levels and palatability of drinking water in coastal Bangladesh. Trop. Med. Int. Health 20, 455–461. https://doi.org/10.1111/tmi.12455.

Hayakawa, K., Fujii, S., 2010. Long-term trends of chloride concentrations in Lake Biwa and why they are increasing (1) Changes in chloride concentration in lake water from 1920 to 2005. Jpn. J. Limnol. 71, 165–183. https://agriknowledge.affrc.go.jp/RN/2010800094.

Heidelberg, J.F., Eisen, J.A., Nelson, W.C., Clayton, R.A., Gwinn, M.L., Dodson, R.J., et al., 2000. DNA sequence of both chromosomes of the cholera pathogen. Vibrio cholerae. Nature 406, 477–483. https://doi.org/10.1038/35020000.

Hu, D., Liu, B., Feng, L., Ding, P., Guo, X., Wang, M., et al., 2016. Origins of the current seventh cholera pandemic. Proc. Natl. Acad. Sci. U.S.A. 113, e7730–e7739. https://doi.org/10.1073/pnas.1608732113.

Islam, M.S., Zaman, M.H., Islam, M.S., Ahmed, N., Clemens, J.D., 2020. Environmental reservoirs of *Vibrio cholerae*. Vaccine 38, A52–A62. https://doi.org/10.1016/j.vaccine.2019.06.033.

Kanungo, S., Sah, B.K., Lopez, A.L., Sung, J.S., Paisley, A.M., Sur, D., et al., 2010. Cholera in India: an analysis of reports, 1997–2006. Bull. World Health Organ. 88, 185–191. https://doi.org/10.2471/BLT.09.073460.

Kaper, J., Lockman, H., Colwell, R.R., Joseph, S.W., 1979. Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake bay. Appl. Environ. Microbiol. 37, 91–103. https://doi.org/10.1128/aem.37.1.91-103.1979.

Kaper, J.B., Morris Jr., J.G., Levine, M.M., 1995. Cholera. Clin Microbiol Rev. 8, 48–86. https://doi.org/10.1128/CMR.8.1.48.

Mora, A., Mahlknecht, J., Rosales-Lagarde, L., Hernández-Antonio, A., 2017. Assessment of major ions and trace elements in groundwater supplied to the Monterrey metropolitan area, Nuevo León, Mexico. Environ. Monit. Assess. 189, 394. https://doi.org/10.1007/s10661-017-6096-y.

Muzembo, B.A., Kitahara, K., Debnath, A., Ohno, A., Okamoto, K., Miyoshi, S., 2022. Cholera outbreaks in India, 2011-2020: a systematic review. Int. J. Environ. Res. Publ. Health 19, 5738. https://doi.org/10.3390/ijerph19095738.

Nair, G.B., Ramamurthy, T., Bhattacharya, M.K., Krishnan, T., Ganguly, S., Saha, D.R., et al., 2010. Emerging trends in the etiology of enteric pathogens as evidenced from an active surveillance of hospitalized diarrhoeal patients in Kolkata, India. Gut Pathog. 2, 4–16. https://doi.org/10.1186/1757-4749-2-4.

Nair, G.B., Sarkar, B.L., De, S.P., Chakrabarti, M.K., Bhadra, R.K., Pal, S.C., 1988. Ecology of *Vibrio cholerae* in the freshwater environs of Calcutta, India. Microb. Ecol. 15, 203–215. https://doi.org/10.1007/BF02011713.

Nayak, S.R., Nayak, A.K., Biswal, B.L., Jena, R.P., Samal, S.K., Pal, B.B., 2020. Incidence of bacterial enteropathogens among diarrhea patients from tribal areas of Odisha. Jpn. J. Infect. Dis. 73, 263–267. https://doi.org/10.7883/yoken.JJID.2019.407.

Nwankwo, C.B., Hoque, M.A., Islam, M.A., Dewan, A., 2020. Groundwater constituents and trace elements in the basement aquifers of Africa and sedimentary aquifers of Asia: Medical hydrogeology of drinking water minerals and toxicants. Earth Syst Environ 4, 369–384. https://doi.org/10.1007/s10661-017-6096-y.

Pal, B.B., Nayak, S.R., Biswal, B., Das, B.K., 2021. Environmental reservoirs of *Vibrio cholerae* serogroups in the flowing freshwater environs from the tribal areas of Odisha, Eastern India. Environ Microbiol Rep 13, 119–125. https://doi.org/10.1111/1758-2229.12914.

Paul, S.S., Takahashi, E., Chowdhury, G., Miyoshi, S., Mizuno, T., Asish, K.M., et al., 2020. Low viability of cholera toxin-producing *Vibrio cholerae* O1 in the artificial low ionic strength aquatic solution. Biol. Pharm. Bull. 43, 1288–1291. https://doi.org/10.1248/bpb.b20-00350.

Saeki, A., Tase, N., 1988. Water quality of groundwater and spring on the northern foot of Mt. Asama. Bull Envir Res Center (Univ of Tsukuba) 12, 57–63.

Singleton, F.L., Attwell, R., Jangi, S., Colwell, R.R., 1982. Effects of temperature and salinity on *Vibrio cholerae* growth. Appl. Environ. Microbiol. 44, 1047–1058. https://doi.org/10.1128/aem.44.5.1047-1058.1982.

Snow, J., 1856. On the mode of communication of cholera. Edinb. Med. J. 1, 668–670.

Takemura, A.F., Chien, D.M., Polz, M.F., 2014. Asociations and dynamics of Vibrionaceae in the environment, from the genus to the population level. Front. Microbiol. 5, 38. https://doi.org/10.3389/fmicb.2014.00038.

Weill, F.X., Domman, D., Njamkepo, E., Tarr, C., Rauzier, J., Fawal, N., et al., 2017. Genomic history of the seventh pandemic of cholera in Africa. Science 358, 785–789. https://doi.org/10.1126/science.aad5901.

Yamanaka, M., 2017. Visit to valuable water springs (117): valuable waters in France, Swizerland and Italy. J. Geophys. Res. 68, 4795–4812. https://doi.org/10.5917/jagh.59.159.

Yoshikawa, T., Takagi, A.P., Ishikawa, S., Hori, M., Nakano, T., Shin, K.C., et al., 2020. Major and trace elements in the surface water of Tonle Sap Lake, Mekong River, and other tributary rivers in Cambodia. Environ. Monit. Assess. 192, 467. https://doi.org/10.1007/s10661-020-08292-4.