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Induction of Viable but Non-culturable State in *Vibrio cholerae* O139 by Temperature and Its Pathogenicity

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ABSTRACT

In this study, the effect of temperature on the formation of viable but non-culturable (VBNC) pathogenic *Vibrio cholerae* O139 was examined. Furthermore, both the virulence and cellular protein expression of the bacterial cells under culturable, non-culturable and resuscitated states were determined. Following the incubation of bacterial cells in river water microcosms (RWM) with 0.5% NaCl at 4°C for 35-45 days, plate counts declined to less than 1 CFU/mL; but viable counts dropped to 10^4-10^5 cells/mL during the first 2 weeks and maintained at this level till the end of incubation. However, the entry of cells into a VBNC state was not detected after the cells were cultured at either 15 or 25°C. When the 4°C -induced VBNC samples were either inoculated to a plate supplemented with H_2O_2-degrading compounds or shifted to 28°C, non-culturable O139 cells regained their culturability. The adhesion test showed that the number of O139 cells adhering to HeLa cells was highest in culturable cells and significantly lower in either resuscitated cells and non-culturable cells. As for the cytotoxicity test, all HeLa cells died after incubation with culturable O139 cells, whereas 32.1 ± 4.6% and 100% of target cells died after incubation with resuscitated cells for 3 and 5 hr, respectively. No difference was observed between the percentages of cell death after incubation with non-culturable cells and with sterile culture medium. When mice were challenged with culturable O139 cells, the mortality was 80%. However, all mice survived after challenge with either VBNC or resuscitated cells.

Key words: *Vibrio cholerae*, viable but non-culturable, resuscitation, pathogenicity

INTRODUCTION

Aquatic microbial ecologists have long recognized that some of bacterial populations seem to "disappear" from natural water bodies during certain times of the year, only to reappear at other times. They believe that such "disappearance" not due to death, but to their entry into the "viable but non-culturable" (VBNC) state. VBNC bacteria maintain characteristics of viable cells, such as the potential for metabolic activity and respiration and cellular integrity[1], but can no longer be cultured in or on routine media. In other words, there is reproducible loss of culturability. The VBNC state may represent a dormant form that enhances the survival of nonsporulating bacteria in an adverse environment[2]. Numerous bacteria, such as *Escherichia coli*, *Vibrio vulnificus*, *Salmonella enteritidis*, *Shigella sonnei*, *Shigella flexneri*, *Campylobacter jejuni*, and *Legionella pneumophila*[3-8], can enter the VBNC state after exposure to adverse environmental stress[1]. However, subsequent studies[1-2] also indicated that this non-culturable state can be reversed when the stress is eliminated.

Vibrio cholerae, a normal inhabitant of aquatic environments, is one of the bacterial species of the free-living flora found in estuarine areas, in which it survives under a wide range of conditions of pH and salinity[9]. Discoveries of the past decade have revealed the existence of a dormant state into which *V. cholerae* enters in response to environmental parameters such as temperature, salinity[10], and nutrient deprivation[11]. Colwell *et al*. suggested that *V. cholerae* can enter a state in which the cells are VBNC[10,12-13]. Cholera-like epidemics have been reported from India and Bangladesh that were caused by novel strains of *V. cholerae* assigned to a newly designated serogroup O139[14]. Recently, Sung *et al*[15]. showed that change of salinity can cause a reversible inhibitory effect on either the adhesion or virulence of *V. cholerae* O139.

For human public health, the potential risk resulting from the change of environmental parameters includes the induction of the pathogenic strain into a non-culturable state and the recovery of the inhibited virulence after resuscitation. Therefore, the purpose of this study is to clarify the relationship between the change of these parameters and the virulence of *V. cholerae* at VBNC state. We examined the influence of low temperature on the ability of *V. cholerae* O139 to enter the VBNC state in a very low nutrient medium. Thereafter, the relationship between the culturability and the pathogenicity of O139 strain was examined via *in vitro* adherence and cytotoxicity to target cells, and the mortality of the mice after challenge were also examined. These results will be beneficial to the further study of VBNC-related proteins and genes and clarification of VBNC state induction by stress factors.

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MATERIALS AND METHODS

I. *Starvation Media*

In this study, two starvation media with pH 7 combined with different temperatures and salinity were used in inducing bacterial cells to enter a viable but non-culture state. One medium was prepared from river water (RW) that was collected from Sheng-Jen waterfall in Taipei, Taiwan. The medium was then sterilized by filtration through a 0.2 μM filter membrane (Millipore; Billerica, Mass., USA). The other was the modified Morita mineral salt solution (MMS), which was prepared as described by Novitsky and Morita[16]. Before experiment, sodium chloride was added to the both media to a final concentration of 0.5%.

II. *Preparation of Bacterial Cells and Microcosm Conditions*

The clinical isolate of *Vibrio cholerae* O139 used in the study was a gift from the Center for Disease Control (CDC) of Taiwan, R.O.C. It was isolated from a patient in Kaohsiung City, Taiwan, in August 1997 (label No. 372). The O139 cells were incubated in Tryptic Soy Broth (TSB; Difco, USA) at 28ºC and 100 rpm overnight. In order to determine the incubation period required to reach the late log phase, the growth curve of *V. cholerae* O139 was examined. Namely, bacterial solution (0.2 mL) from an overnight culture was cultured in 200 mL of TSB at 28ºC and 100 rpm for 24 hr. Using a spread-plate method and a spectrophotometer, the values of the colony-forming unit (CFU) and the optical density at 600 nm of the bacterial cultures were measured at 1-hr intervals for 24 hr. The late log phase bacterial cells (OD_{600} = 0.8) were harvested by centrifugation at 6,000 × g for 10 min, and then washed twice with sterile saline and once with sterile RW or MMS to prevent carry-over of nutrients on inoculation of the microcosms. The bacterial concentration was adjusted with RW or MMS to 10^8-10^9 cells/mL and determined using a counting chamber and a microscope. To determine the temperature effect, 1 mL of cells was inoculated into 199 mL of sterile 0.5% NaCl-RW or -MMS in pre-cooled (at 4ºC) 500-mL flasks. The microcosms were separately incubated at 4ºC, 15ºC, and 25ºC in the dark. In the experiment on the salinity effect, 1 mL of cells was inoculated into 199 mL of sterile RW or MMS at various salinities (0%, 0.5%, 3%, and 5% NaCl) and the microcosms were then incubated without shaking at 28ºC. Microcosms were prepared in triplicate for each experiment.

III. *Cell Counts*

Samples taken at the time of inoculation (initial time) and at subsequent time intervals were monitored by the plate count and direct viable count. Plate counts were obtained by spreading appropriate dilutions of O139 cells onto 1/5 Tryptic Soy Agar (TSA) plates (1/5 TSB containing 1.5% agar). Plates were incubated at 28ºC and colonies were counted after 2 days. Bacterial viability was ascertained by using a Live/Dead BacLight bacterium viability kit (No. L-7007, Molecular Probes Europe BV, Leiden, Netherlands). Viable cell counts were determined by using a count chamber and an epi-fluorescence microscope (NIKON Eclipse E800; Nikon Co., Tokyo, Japan), and five microscopic fields were counted for each sample.

IV. *Resuscitation of Non-culturable V. cholerae Cells*

In the experiment, two methods, namely plates supplemented with H_2O_2-degrading compounds[17] and temperature upshift[18], were used to perform the resuscitation of non-culturable O139 cells. In the method of plates supplemented with H_2O_2-degrading compounds, sodium pyruvate[19] or catalase[20] was used. Sodium pyruvate (Sigma) was added directly to 1/5 TSA media prior to autoclaving to give a final concentration of 0.1%. Alternatively, 100 μL of sterile catalase (20,000 U/mL; Sigma) was spread on 1/5 TSA plates at a concentration of 2,000 U/plate. Bacterial cells (0.1 mL), which were separately collected at the time of inoculation (initial time) and at subsequent time intervals, were then spread on the agar plates and colonies were enumerated after 4 days of incubation at 28ºC. Media lacking supplements were designated as non-amended controls. In the temperature upshift experiment, 0.5 mL portion of the non-culturable cell solution from RWM, which was collected from a sample that had entered into VBNC state for 7 days, was added to the same volume of RW or TSB, and then incubated at 28ºC in the dark without shaking. For the non-culturable cells from MMS, the samples were separately added to MMS and TSB. In additional experiment, nalidixic acid (100 μg/mL) was added to sterile RW to assay inhibition of bacterial cell multiplication before the temperature upshift[21]. The culturability of different samples was determined by plate count on 1/5 TSA plates at different time interval indicated as in Figures 1 and 2. If the non-culturable cells can be restored either after a temperature upshift or on plates supplemented with H_2O_2-degrading compounds, but not in TSB or on non-amended TSA plates, then the cells are considered to be resuscitated rather than re-grown from the remaining culturable cells.

V. *Adherence and Cytotoxicity Assays*

The procedures for the adherence and cytotoxicity assays were modified as described by Sung *et al*.[15] and Rahman *et al*.[22], respectively. The bacterial cells from different physiological states in RWM, including culturable, non-culturable and resuscitated cells, were washed with 0.01 M phosphate-buffered saline (PBS) and suspended in sterile Dulbecco's modified Eagle medium (DMEM; Biochrom AG, Berlin, Germany) to give a

bacterial concentration of 2×10^7 cells/mL. This experiment used two types of non-culturable cells, which were separately obtained 7 days and 80 days after entering the VBNC state. Similarly two types of resuscitated cells were collected from sample in RWM that had been resuscitated for 48 h and tested.

In this experiment, HeLa cells were used in the adherence assay as the target cells. Before the assays, 0.5 mL of HeLa cell suspension in DMEM containing 10% fetal calf serum (FCS) was spread gently onto a sterile glass slide (18 × 24 mm) laid in a sterile Petri dish (diameter = 10 cm). The plate was kept motionless at room temperature for 1 hr, after which 10 mL of DMEM containing 10% FCS was applied to the glass slide. The cell sample was incubated in a 5% CO_2-incubator at 37ºC for 24-48 hr to obtain a monolayer with 5×10^5 cells/slide. After the slide was washed once with DMEM, 0.5 mL of bacterial suspension was spread on the HeLa cells. In the adherence assay, the co-culture was incubated at 37ºC for 1 hr in a humid chamber. Subsequently, the slides were washed three times and fixed with 10% formalin-0.01 M PBS (pH 7) for 30 min at room temperature. After washing with PBS, the slides were stained by immunofluorescence staining with mouse anti-O139 serum prepared in our laboratory and then with fluorescein-conjugated horse-anti-mouse IgG (Vector Laboratories, Ltd., Burlingame, CA., USA). The slides were then counter-stained with Evan's blue and the numbers of bacteria associated with the HeLa cells were counted for at least five fields with a total of 50-100 HeLa cells using an epi-fluorescence microscope. The data presented in the study represented the number of adhesive cells per 20 HeLa cells.

In the cytotoxicity assay, the co-culture of bacterial cells and HeLa cells was developed at 37ºC in a humid chamber. The slides were then stained with Trypan blue after at 3 hr and 5 hr incubation. Afterwards, the numbers of dead HeLa cells were counted for at least five fields using a microscope. The data presented in the study represented the mean of percentages of dead cells ± the standard error of the triplicate measurements.

VI. *Challenge Experiment*

The procedure was modified as described by Sung *et al.*[15]. Mice (6-week-old; ICR strain) purchased from The Laboratory Animal Center of Taiwan University College Medicine, were divided into six test groups and one control group. One mouse from each test group was challenged intraperitoneally with 100 μL of bacterial suspension (double dose of LD_{50} in 0.01 M PBS; 4×10^9 cells/mouse). Among the six bacterial samples, the first sample was culturable cells from TSB culture at 28ºC; the second to the fourth samples were cells from separate cultures in RW at 4ºC for 8, 16, and 24 days; the fifth sample was viable but non-culturable cells collected 7 days after VBNC formation in RWM; and the last sample was resuscitated cells. Resuscitated cells were collected from sample which had been resuscitated for 2 days in RWM at 28ºC. Before the challenge, all bacterial samples were washed and resuspended in 0.01 M PBS. During the experiment, 20 mice were used for each replicate and each experiment of bacterial challenge was performed in triplicate. The control group was injected with an equal volume of sterile PBS. Bacterial cells were collected from the peritoneal cavities of dead mice within 1 hr of death, and then identified to confirm that death was caused by *V. cholerae* O139. After injection, the number of dead mice was recorded daily. The mortality was calculated using the following formula: number of dead mice/the total number of mice × 100%.

RESULTS

I. *Induction of Viable but Non-culturable State in Vibrio cholerae O139*

Results from the study of *Vibrio cholerae* O139 incubated separately in river water microcosm (RWM) with 0.5% NaCl at different temperatures showed that plate counts declined from 5×10^6 CFU/mL to less than 1 CFU/mL at 4ºC after 45 days. The viable counts decreased to $(1.9 \pm 1.2) \times 10^4$ cells/mL at the end of the second week and maintained in the range of 10^4-10^5 cells/mL during the remaining incubation period (Figure 1A). However, after culturing at either 15ºC or 25ºC plate counts were maintained to be in the range of 10^5-10^6 CFU/mL during the whole cultivation period (Figure 1A), indicating that bacterial cells did not enter VBNC state. The low-temperature effect was also detectable when O139 cells were cultured in Morita mineral salt solution (MMS) with 0.5% NaCl, with the minimum of 80 days for complete entry into a VBNC state (Figure 1B). In addition, the influences of different concentrations of NaCl (0%, 0.5%, 3%, and 5%) on the culturability of the O139 strain were also observed in this study. The results from culture in both RWM and MMS at 28ºC showed that bacterial counts detected by the plate count were no difference from those detected by the viable count during the whole cultivation period (data not shown). Therefore, only O139 cells in RWM from different physiological states were used in further assay.

II. *Resuscitation of Non-culturable Cells*

In order to clarify whether cells were restored due to resuscitation, two independent experiments were conducted. In the first experiment using plates supplemented with H_2O_2-degrading compounds, plate counts on 1/5 TSA of samples collected from RWM decreased to less than 1 CFU/mL after 35 days of inoculation at 4ºC, while there was no decline in the viable counts. After development of the non-culturable state, a portion of the O139 cells regained culturability on media amended with

either sodium pyruvate or catalase (Figure 2A). Similar results were found in non-culturable cells in MMS (data not shown).

In the second experiment, the temperature upshift experiment, 4ºC -induced samples from both RWM and MMS showed detectable bacterial counts after a direct shift to 28ºC. The plate counts increased to 5 × 10^6 CFU/mL after 40 hr in RWM and 2 hr in MMS, respectively (Figure 2B); however, no bacterial count was detected after incubation of non-culturable cells from both microcosms at 4ºC into TSB and then incubation at 28ºC (data not shown). An additional experiment, in which non-culturable cells were inoculated into the RWM containing nalidixic acid before the temperature upshift treatment, showed 1 × 10^4 and 2.5 × 10^6 CFU/mL of resuscitated cells at 30 h and 40 h after inoculation, respectively.

III. *Pathogenicity of V. cholerae O139 in Different Growth States*

In order to determine the pathogenicity of *V. cholerae* O139 in different growth states, the adhesion and cytotoxicity of bacterial cells in RWM to human cells (HeLa cells) and the mortality of mice following challenge with the test strains were examined. The results showed that the adhesion level was high for culturable cells, intermediate for resuscitated cells and low (less than 5 adhesive cells per 20 target cells) for non-culturable cells collected either 7 days or 80 days after entry into a VBNC state (Figure 3A). In the cytotoxicity test shown in Figure 3B, all target cells were dead after challenge with culturable O139 cells. The resuscitated cells collected from samples that had entered VBNC state for 7 days caused 32.1 ± 4.6% and 100% of target cell mortality after challenge for 3 and 5 hr, respectively (Figure 3B (a)). However, the cells collected from samples in VBNC state for 80 days caused 36.0 ± 1.4% and 35.9 ± 2.2% of cell death after challenge for 3 and 5 hr, respectively (Figure 3B (b)). The percentage of target cells that died when incubated with VBNC O139 was the same as that of the control group, in which target cells were incubated with culture medium alone. Furthermore, the mortality of mice challenged with culturable cells was shown to be 80-85% (Table 1). However, the mortality significantly decreased when mice were challenged with the bacterial cells cultured at 4ºC for 8 and 16 days, whereas all mice survived after being challenged with either non-culturable or resuscitated bacterial cells (Table 1).

Table 1. The mortality of mice challenged with *Vibrio cholerae* O139 at different states by intraperitoneal injection.

Cells at different states	Mortality (%)		
	Test 1	Test 2	Test 3
Culturable cells	85	80	85
Cells cultured at 4°C for 8 days	5	0	0
Cells cultured at 4°C for 16 days	0	0	5
Cells cultured at 4°C for 24 days	0	0	0
Nonculturable cells[a]	0	0	0
Resuscitated cells[b]	0	0	0
Control	0	0	0

[a]cells collected from 7 days after the entry of O139 cells into the non-culturable state in river water microcosm (RWM).
[b]cells collected from 7 days after entering the non-culturable state were resuscitated in RWM and collected at the 2nd day after resuscitation.
Control, mice were challenged with 0.01 M PBS (pH 7.0).
Mice were used for each test group and each experiment of bacterial challenge was performed in triplicate.

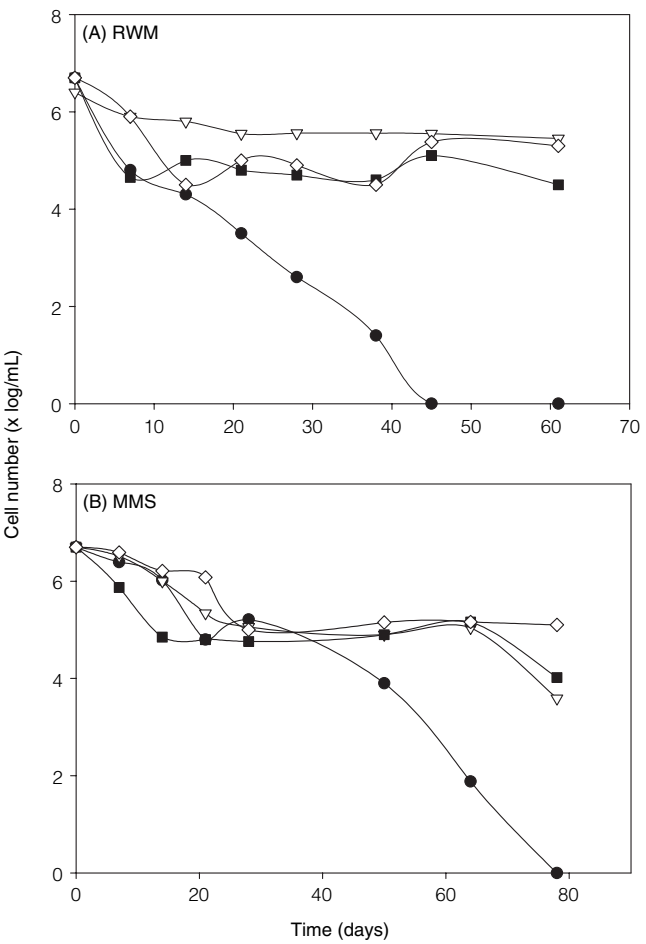

Figure 1. Induction of viable but non-culturable (VBNC) state by *Vibrio cholerae* O139 in the river water microcosm (RWM) containing 0.5% NaCl (A) and in the modified Morita mineral salt solution (MMS) (B) at different temperatures. ●,▽ and ■: plate counts of samples separately from culturing at 4, 15, and 25°C; ◊: viable counts of sample from culturing at 4°C determined by the live/dead staining method.

DISCUSSION

Previous studies had shown that *Vibrio cholerae* exists in a viable but non-culturable (VBNC) state when incubated at low temperature or in different salinities[10], in nutrient deprivation[11], and at low temperature in a

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nutrient-limited artificial seawater microcosm[12]. In this study, only the low temperature had effect on the culturability of the pathogenic *V. cholerae* O139 cultured in nutrient deprivation (Figure 1A and 1B). The study of Morton and Oliver[23] has shown that carbon starvation has a dramatic effect on *V. vulnificus* cells entering into the VBNC state. However, other researches indicated that the starvation inhibits the formation of non-culturable cells in *V. vulnificus*[24-25]. In this study, entry into the VBNC state in the river water microcosm (RWM) required a shorter time than that in the artificial MMS. It was suggested that the RWM may contain a small amount of organic compounds which can be used as a nutrient source, because it was collected from a waterfall frequented by tourists.

For *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*, the VBNC state is induced by a temperature downshift and resuscitation from this state has been reported after the removal of the temperature stress[21,26-28]. However, whether reports of *in vitro* resuscitation depict true resuscitation of non-culturable cells or re-growth of a few culturable cells which escaped detection remains controversial. More recently, there have been publications on resuscitation in conditions where re-growth of culturable cells is less likely[1], for instance, using the detection of intracellular ATP[29], a serial dilution of

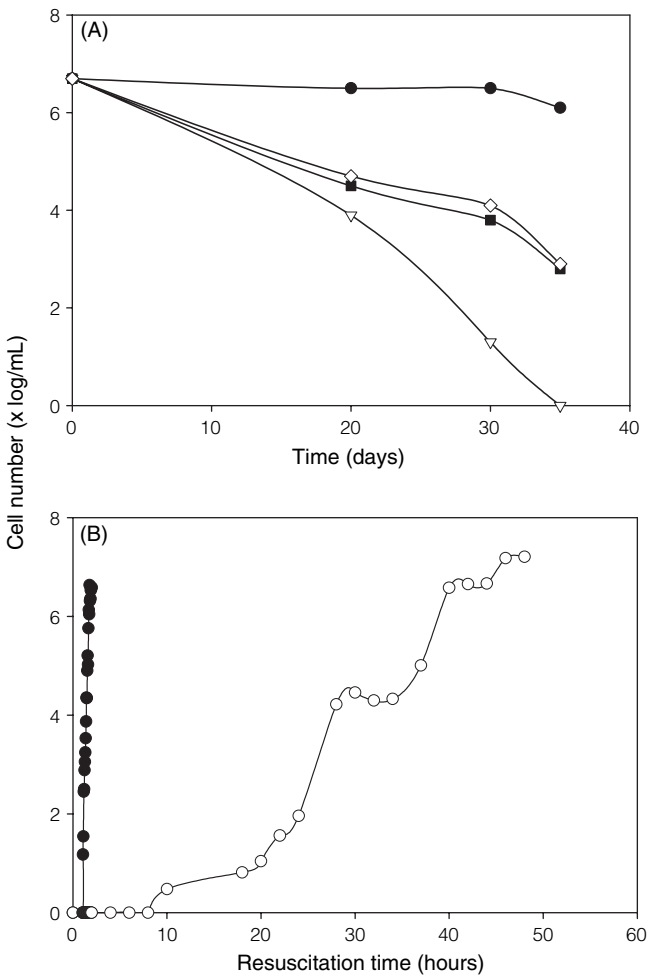

Figure 2. Entry of *Vibrio cholerae* O139 cells into the non-culturable state and resuscitation after incubation on media amended with H_2O_2-degrading compounds at 28°C (A) and after temperature upshift from 4°C to 28°C (B). In figure (A), samples from culturing in river water microcosm (RWM) at 4°C; ●: viable count determined by the live/dead staining method; ▽, ■, and ◊: plate counts of samples separately cultured on 1/5 TSA, 1/5 TSA containing sodium pyruvate and catalase. In figure (B), non-culturable cells collected from samples that had entered VBNC state for 7 days were separately resuscitated in modified Morita mineral salt solution (MMS; solid symbol) and in river water microcosm (RWM; open symbol).

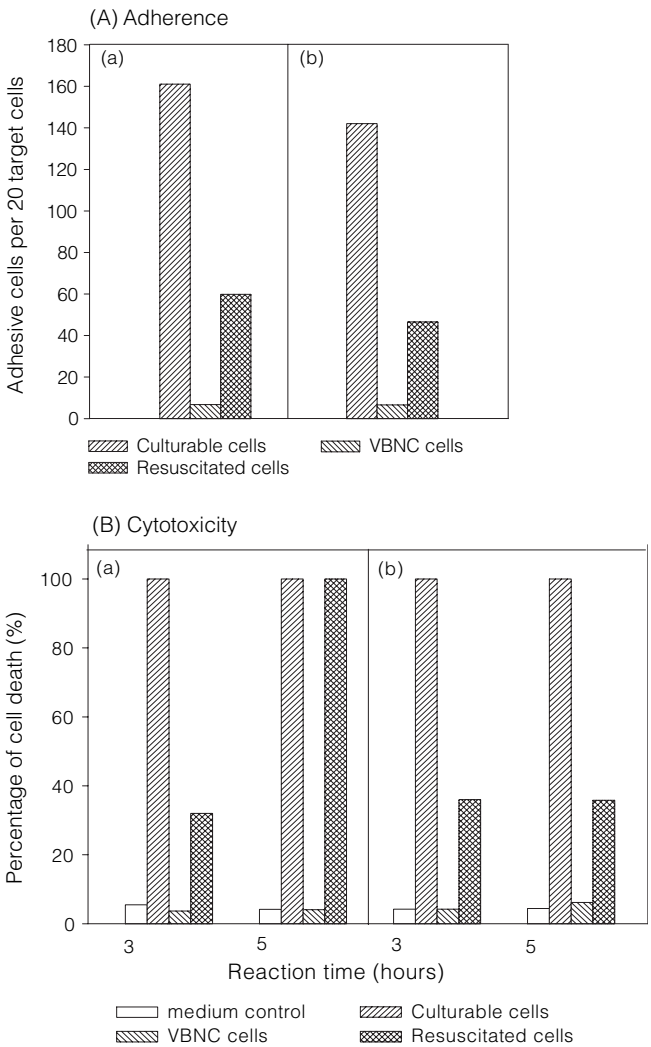

Figure 3. *In vitro* virulence test of *Vibrio cholerae* O139 cells from culturable, viable but non-culturable (VBNC), and resuscitation states in river water. (A) Adherence, determination of adhesion of O139 cells to HeLa cells after co-culturing HeLa cells and O139 cells for 1 hour; (B) Cytotoxicity, examination of the death percentage of HeLa cells after HeLa cells were co-cultured with O139 cells for 3 and 5 hours. (a) O139 cells were obtained 7 days and (b) 80 days after entering the VBNC state. Two types of resuscitated cells were also tested after recovery of the two types of VBNC cells. The sterile Dulbecco's modified Eagle medium (DMEM) without bacterial cells applied to HeLa cells was used as a control.

samples combined with temperature upshift[27], and heat shock treatment[6]. In addition, in order to terminate this controversy, Mizunoe *et al*.[17] have reported that starvation- and low temperature-induced VBNC cells of enterohemorrhagic *E. coli* were resuscitated after spreading from the starvation microcosms directly onto the plates amended with H_2O_2-degrading compounds such as catalase or sodium pyruvate. In this study, both the method of a plate supplemented with H_2O_2-degrading compounds and the temperature upshift demonstrated that non-culturable cells can be restored. Nalidixic acid inhibits proliferation of bacterial cells, so addition of this antibiotic could eliminate effect of re-growth of a few culturable cells on the resuscitation experiment[21]. Inoculation of non-culturable cells into the RWM containing nalidixic acid before the temperature upshift showed the same number of resuscitated cells nalidixic acid-added and unadded groups. These results demonstrated that the culturability of low-temperature stressed O139 cells is regained after removal of the stress. As for the difference between RWM and MMS in the time required for resuscitation, this may be related to the components of the two microcosms. Further experiments are required to clarify this issue. In addition, no plate count was detected after non-culturable cells were directly inoculated into TSB. This indicates that reactive oxygen intermediates produced by cell respiration during resuscitation may not be quickly degraded by anti-oxygen enzymes which are unable to be synthesized in time, such as catalase examined in this study.

Bacterial adherence and colonization are the key steps that help enterotoxigenic and other enteropathogenic organisms to establish infection in the gut[30]. An earlier study showed that differences in temperature, salinity, ion concentrations, and pH values could affect the attachment of enterotoxigenic *V. cholerae* O1 and non-O1[31]. Sung *et al*.[15] have indicated that the effect of salinity on the adhesion of *V. cholerae* O139 is stronger than the effect of the pH value. Furthermore, the level of adhesion may vary according to different growth conditions, and its expression may further affect the pathogenicity of *V. cholerae* O139. As for the VBNC bacteria, Madema *et al*.[32] indicated that a lack of colonization was detected when *Campylobacter jejuni* entered the VBNC state. However, Oliver and Bockian[33] have indicated that VBNC cells of *V. vulnificus* remain virulent, although their virulence is significantly reduced. For pathogenic VBNC cells, the potential risk in human public health can be induced by the recovery of the inhibited virulence[1,22,33-37]. Colwell *et al*.[38] reported that VBNC *V. cholerae* and enteropathogenic *E. coli* regained culturability after animal passage. However, the results from human volunteers who developed clinical symptoms of cholera after ingestion of VBNC *V. cholerae* O1 suggested that maintenance of the infectivity of VBNC cells may be confined to "young cells", while those that have been in the VBNC state longer may lose infectivity[36]. In this study, the culturable *V. cholerae* O139 were shown to be pathogenic; however, the loss of pathogenicity, indicated by a reduction in cellular adheresion and cytotoxicity and a reduction in fatality in infected mice, was detected in non-culturable cells. For resuscitated cells, except of the cells resuscitated from sample that had been entered to VBNC state for 7 days causing all target cells to be dead after challenge for 5 h (Figure 3B), both adhesion and cytotoxicity were partially recovered. These results indicate that adhesive and/or toxic factors could be recovered, but the restored degree of cytotoxicity is in inverse proportion to the duration of the cells in VBNC state. However, the observation that all mice survived after challenge with resuscitated cells (Table 1) indicates that the virulence of resuscitated cells, including cell adhesion and cytotoxicity, is not completely restored. These results suggest that pathogenicity may be reversible after culturability is restored, but the degree of pathogenicity is related to the recovery of adhesion and cytotoxicity of bacterial cells. In order to determine whether the findings observed in either mice or *in vitro* tests coincide with those in humans, further studies are required.

To date, many studies are available on the VBNC state, such as stress factors or conditions which induce microbes to enter VBNC states[39], and resuscitation or re-grown of non-culturable cells after eliminating the stresses[1,6,17,27,29,40-41]. In different growth states, both the physiological characteristics and the pathogenicity of pathogens varied. However, the proteomic and genomic researches on VBNC mechanisms have been done by only a few researchers[42-46], due to the fact that the genomes of most pathogens of interest have not been completely sequenced. In this study, the preliminary analysis of cellular total proteins and the experiment data showed that the expression of many proteins changed after cells entered into the VBNC state (data not shown). We believe these proteins to be related to or necessary for the entry of O139 cells into VBNC state. The whole genome of *V. cholerae* has been completely sequenced[47]. Therefore, in the future, these proteins interest many in genomic research to clarify what genes are related to and involved in either the entry of microbes into the VBNC state or their pathogenicity.

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