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Effects of Salinity and pH on the Adherence and Virulence of *Vibrio cholerae* O139

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ABSTRACT

Vibrio cholerae must first adhere to and colonize the human small intestine in order to cause cholera and this adherence can be affected by several physicochemical factors. The purpose of this study was to examine the adherence and virulence of *V. cholerae* O139 that was grown in experimental broth media with different salinities and pH values. After the bacterial cells were incubated separately in these media, the adherence of *V. cholerae* O139 to HeLa cells was examined. By comparison with bacteria grown in a control medium with 0.5% NaCl and pH 7.0, the percentages of adhesion of bacteria grown in media with 0% and 5% NaCl were found to decline significantly by 56% ($P = 0.0007$) and 69% ($P = 0.0008$) respectively, and those in media with pH 6 and 5.5 decreased to 91% ($P = 0.016$) and 82% ($P = 0.006$) respectively. After the bacterial cells were sub-cultured in the experimental media serially, no difference in adherence was observed between the first, the fifth and the tenth generations ($P = 0.182$). However, after the tenth generation, when the treated bacterial cells were re-incubated in a control medium, the level of adherence was found to become similar to that of the control bacteria. In order to determine the effect of adherence on the pathogenicity of *V. cholerae* O139, mice were challenged with a double dose of the LD_{50} of the test strains incubated in 0% NaCl, 5% NaCl, and pH 5.5, and the survival of rates were at 50%, 60% and 20%, respectively. These results suggest that changes of salinity can cause a reversible inhibition effect on either adhesion or virulence of *V. cholerae* O139, and those effects resulting from changes in salinity are stronger than those of different pH values.

Key words: *Vibrio cholerae* O139, salinity, pH, adherence, virulence

INTRODUCTION

Vibrio cholerae is a normal inhabitant of aquatic environments, 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[1]. Many researchers reported that various environmental and physicochemical factors, such as temperature, sunlight, pH and salinity were associated with the toxigenicity of *V. cholerae* O1[2-6]. In order to contract cholera[7] through ingestion of contaminated water[8] or food[9], *V. cholerae* must pass the gastric acid barrier, colonize the small intestine, and produce cholera toxin (CT), a potent enterotoxin responsible for causing acute severe and dehydrating diarrhea, which has been well characterized[10]. The adhesion and colonization processes are essential to the pathogenesis of cholera, as strains that are unable to colonize the gut are also unable to cause disease in volunteers[11]. It is also known that the antigens involved in either adhesion or colonization induced protection in experimental cholera[12-14]. However, the adhesion responsible for small-bowel colonization is not yet fully characterized. Numerous surface factors have been implicated in adhesion to the mucosa of the proximal small intestinal epithelial cells[15], including lipopolysaccharide (LPS)[16], various hemagglutinins[17,18] toxin-coregulated pilus in *V. cholerae* of classical biotype[13,19] and several outer-membrane proteins (OMPs)[20-22]. Hood and Winter[23] have shown that temperature, pH, various ions and nutrient starvation could all influence the attachment of *V. cholerae*.

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[24]. The organism has been characterized with respect to its hemagglutination (HA) activity, plasmid content, CT production, cell surface protein and LPS profiles, and antigenic properties[25]. All the results indicated that the O139 isolates possess certain characteristics that make them distinct from their O1 counterparts[25]. Adhesion and subsequent colonization are also important activities in the infection by *V. cholerae* O139. Yamamoto *et al*.[26] found that capsulated *V. cholerae* O139 tends to autoagglutinate and, can effectively adhere to the intestinal mucosa that seems to be an important step in its infection. Since the studies related to the influence of physiochemical conditions on the relationship between adhesion and pathogenicity of *V. cholerae* O139 are limited, the purpose of this study was to attempt to examine the effect of experimental media with different salinities and pH values on the adhesion and virulence of *V. cholerae* O139. The results can be then used as a reference for future pathogenicity studies and may be beneficial when developing vaccine or food safety regulations.

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MATERIALS AND METHODS

I. *Preparation of Bacterial Cells*

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 Taiwan in August 1997, and the labeling number is 372. To examine the effect of experimental media with different salinities or pH values on bacterial growth, bacterial solution (1 mL) from an overnight culture was individually cultured in 50 mL of trypticase soy broth (TSB; Difco, USA) with different NaCl (Wako, Japan) concentrations (0%, 0.5% and 5%, w/v) and pH values (pH 5.5, 6, 8 and 8.5) at 37˚C and 170 rpm for 24 hr. The values of the colony-forming unit (CFU) and the optical density at 600 nm of the bacterial cultures were measured, using the spread-plate method and a spectrophotometer, respectively, at 1-hr interval for 24 hr. The growth curves resulting from this process were used to determine the culture time required to reach the late log phase. Late log-phase bacterial cells cultured in different salinity or pH media were prepared for bacterial subculture, adherence assay and virulence test conducted in the study. For all the tests, the control bacterial cells were grown in the control medium (0.5% NaCl and pH 7). For the bacterial subculture, after the bacterial cells from each generation had reached the late log phase (OD_{600} of 0.6), 100 μL of each of the bacterial solutions was sub-cultured into 100 mL of media with different salinities or pH values.

II. *Adherence Assays*

The late log-phase bacterial cells were washed with 0.01 M phosphate-buffered saline (PBS) and suspended in sterile Dulbecco's modified Eagle medium (DMEM; BIOCHROM KG, Berlin) to give a concentration of bacterial cells of 2×10^7 cells/mL. Many *in vitro* models have used epithelial cell lines, such as HeLa, in evaluating the colonization of specific mutants[16]. In this experiment, HeLa cells were used in the adherence assay as the target cells. Before assays, HeLa cell suspension was spread gently onto a sterile glass slide (18 × 24 mm) and kept motionless at room temperature for 1 hr. Following supplementation with 10 mL of DMEM containing 10% FCS, the glass slide was incubated in a 5% CO_2-incubator at 37˚C 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, and then it was incubated at 37˚C for 1 hr in a humid chamber. Subsequently, the slides were washed 3 times and fixed with 10% formalin-0.01 M PBS (pH 7) for 30 min at room temperature. After being washed with PBS, the slides were stained by an immunofluorescence method with mouse anti-O139 serum prepared in our laboratory and then with fluorescein-conjugated horse-anti-mouse IgG (Vector, USA). The slides were then counter-stained with Even's blue, observed using an epi-fluorescence microscope (NIKON Eclipse E800, Japan), and the numbers of bacteria associated with the HeLa cells were counted for at least 5 fields with a total of 50-100 HeLa cells. The data presented in the study represented the mean of percentages of adhesion ± the standard error of the triplicate measurements. The values were calculated as

$$\% \text{ of adhesion} = \frac{\text{The number of adherent cells of experimental group/100 HeLa cells}}{\text{The numer of adherent cells of control group/100 HeLa cells}} \times 100$$

Inhibition of bacterial adhesion to HeLa cells with specific anti-O139 antibody at the indicated dilutions was also performed by pre-incubating the bacterial cells at room temperature for 1 hr, and then the adherence assay described above was carried out.

III. *Virulence Test*

The late-log phase bacterial cells were adjusted to concentrations of 10^7, 10^8, 10^9, 10^{10} and 10^{11} cells/mL with PBS. Before proceeding with this test, the 50% lethal dose (LD_{50}) of *V. cholerae* O139 cultured in control medium was first determined. Sixty mice (6-week-old; ICR strain) were divided into 5 test groups and 1 control group. Each mouse in the test groups was administered with 100 μL of bacterial suspensions to give doses of 10^6, 10^7, 10^8, 10^9 and 10^{10} cells by intraperitoneal injection, respectively. The control group was injected with an equal volume of sterile 0.01 M PBS. After injection, the number of dead mice was recorded daily. The survival rate was calculated using the formula: (the number of surviving mice/the total number of mice) × 100.

For detection of the virulence of *V. cholerae* O139 cultured under different salinity and pH conditions, mice were divided into 5 test groups and 1 control group. Each mouse in 4 of the 5 test groups was challenged with 100 μL of bacterial suspension (double dose of LD_{50}), where the bacteria were separately incubated in 0%, 0.5% and 5% NaCl TSB with pH 7 and 0.5% NaCl TSB with pH 5.5. The fifth test group was injected with *V. cholerae* O139 pre-incubated with specific anti-O139 antibody. In the experiment, 10 mice were used for each replicate and each experiment of bacterial challenge was performed in triplicate. In all the experiments, bacterial cells were collected from peritoneal cavities of dead mouse within 1 hr after death, and then identified to confirm that death was caused by *V. cholerae* O139.

IV. *Statistics*

All data from the experiments described above, including the adherence assay and the virulence test, were statistically analyzed using the ANOVA and the Duncan's multiple range test with a specified significance level of $P < 0.05$.

RESULTS AND DISSCUSION

I. *Growth and Adhesion of Vibrio Cholerae O139*

The growth of *Vibrio cholerae* O139 cultured in control medium (0.5% NaCl, pH 7.0) showed that the lag phase was from 0 to 2 hr after incubation, the log phase was 2-6 hr and the stationary phase was from the 6th hr. The bacterial growth was delayed when they were incubated in experimental media without NaCl or containing 5% NaCl. The culture time required to reach the late log phase for bacterial cells growing in 0% and 5% NaCl were about 8 hr and 14 hr after incubation began, respectively. In addition, the bacterial growth was influenced when it was cultured in medium with pH 5.5, and the culture time needed to reach late log phase was at 3 days. However, the bacterial growth in media with pH 6, 8 and 8.5 were similar to the growth in the control medium at pH 7. The results indicated that the growth of *V. cholerae* O139 was primarily influenced by salinity (0% NaCl and 5% NaCl) and acidity (pH 5.5), but not affected by alkaline conditions (pH 8 and 8.5).

To determine the optimum growth phase for the adhesive activity of *V. cholerae* O139, the adhesion was detected via the reaction of HeLa cells with bacterial cells from different growth phases. The results showed that the percentage of adhesion of bacterial cells from the late log phase was higher than that from the stationary phase (Figure 1). Therefore, in the later experiments, including adherence assays and virulence tests, late log-phase bacterial cells were used. In addition, the optimum reaction conditions of the adherence assay were identified as 37˚C for 60 min (Figure 1), and with a ratio of bacterial cells to HeLa cells of 20 to 1.

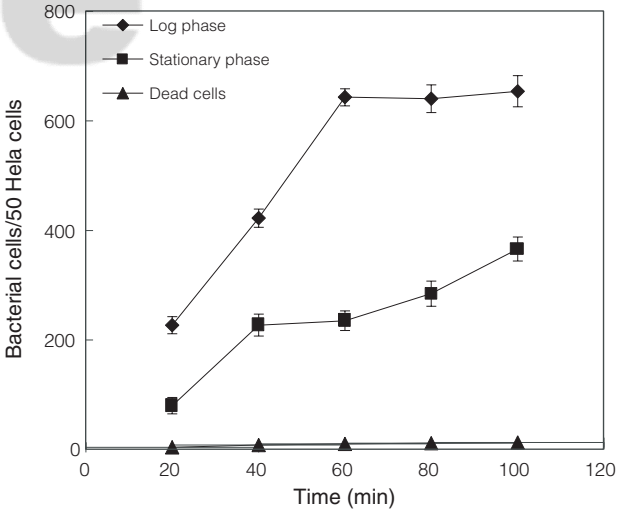

Figure 1. Adhesion of O139 strain to HeLa cells at different growth stages. In this experiment, bacterial cells were cultured in control medium (see material and methods) and according to the growth curve, bacterial cells from different growth stages were collected and prepared (see material and methods) before adherence assay. The dead cells which collected from late-log phase were prepared by treatment with 10% formaldehyde at 4˚C for 48 hr and used as non-specific adhesion control.

II. *Effect of Salinity and pH on Adhesion*

Bacterial adherence and colonization are the key steps that help enterotoxigenic and other enteropathogenic organisms to establish infection in the gut[27]. An earlier study showed that differences in temperature, salinity, ion concentrations and pH values, could affect the attachment of *V. cholerae* O1 and non-O1 to non-living substances, such as glass, nylon, cellulose and chitins etc., and that optimum attachment occurred at pH 2 and 1-1.5% NaCl[23]. Kumazawa *et al*.[28] also indicated that the attachment level of the *V. parahaemolyticus* to estuarine algae depended on the salinity and temperature of the water. In this study, adhesion of *V. cholerae* O139 to human cells (HeLa cells) was examined after bacterial cells were grown in media with different salinities and pH. The results showed that changes in either the salinity or acidity of culture media could reduce the adhesion of strain O139 to HeLa cells. When compared the level of adhesion to that of bacterial cells cultured in the control medium with 0.5% NaCl and pH 7, the percentage of adhesion was reduced significantly when the bacterial cells were incubated in media either containing high salt concentrations (2.5%, 3%, 4% and 5% NaCl) or without salt (0% NaCl) (Figure 2A). Of these, the most significant reduction in percentage of adhesion was detected when the bacterial cells were cultured in either 0% NaCl (21.12 ± 0.65%) ($P = 0.0007$) or 5% NaCl (15.28 ± 0.89%) ($P = 0.0008$). Furthermore, the acidic media with pH 5.5 and pH 6.0 were also found to decrease the adhesion of *V. cholerae* O139 to HeLa cells (Figure 2B); the percentages were 82.31 ± 4.17% ($P = 0.006$) and 90.72 ± 3.83% ($P = 0.016$), respectively. These results indicated that the effect of salinity on adhesion was stronger than the effect of pH value, and that the optimum adhesion of strain O139 to HeLa cells occurred in the control media with 0.5% NaCl and pH 7 (Figure 2). Besides, it was also noted that *V. cholerae* O139 was not able to grow, and if fact died, in media with pH below 5.5 (data not shown).

II. *Adhesion of Bacteria from Different Subcultures*

Adhesion is related to pathogenicity, since it is the first step in bacterial colonization and invasion of the host. Generally, the pathogenicity of many pathogens, such as *V. cholerae*, can be reduced when they are continuously subcultured in artificial media[29]. In this experiment, the adhesion of *V. cholerae* O139 cultured in the control medium showed no significant difference among the first, the fifth and the tenth subcultures ($P = 0.182$). The same phenomena were also observed when bacterial cells were grown in media containing 0% NaCl, 5% NaCl or pH 5.5 (Figure 3). However, when the bacterial cells from the tenth subculture grown in the experimental medium were sub-cultured in the control medium, the level of adhesion is

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similar to that of the bacteria continuously cultured in the control medium (Figure 3). These results suggest that the adhesion of strain O139 is not influenced after the serial subculture and that expression of adhesion is dependent on the growth conditions, such as salinity and pH.

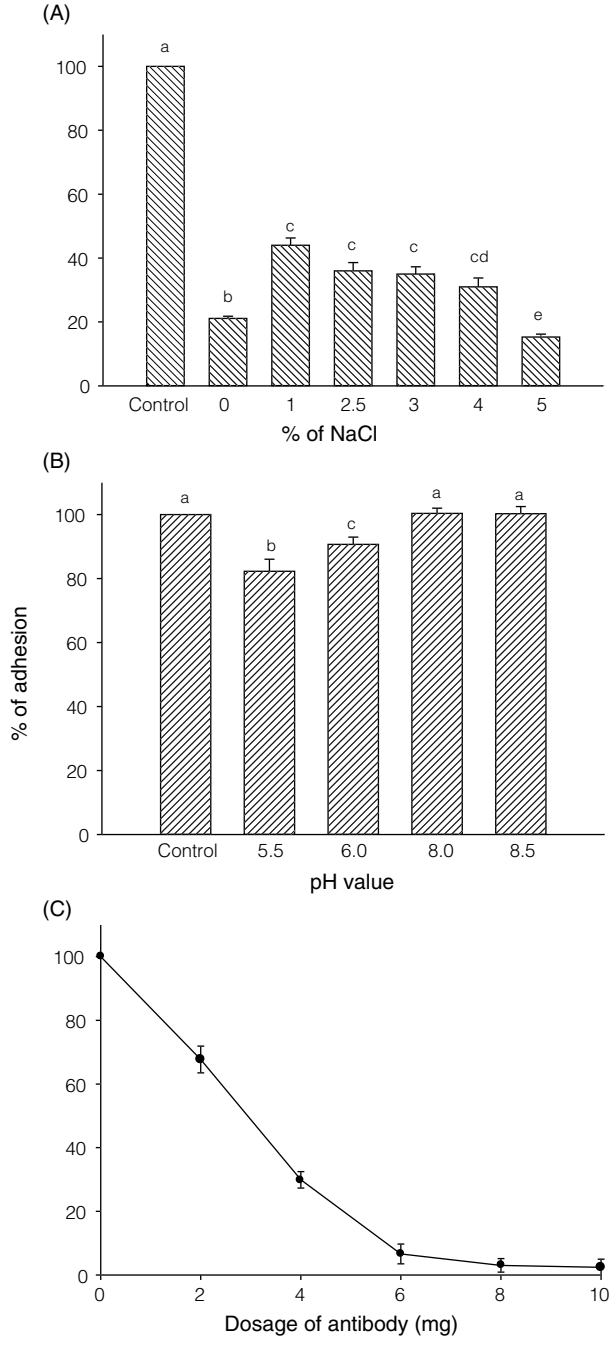

Figure 2. Adhesion of *Vibrio cholerae* O139 cultured in media with different conditions to HeLa cells. (A), bacterial cells were separately cultured in media (pH 7) containing different concentrations of sodium chloride. (B), bacterial cells were separately cultured in media (0.5% NaCl) with different pH values. (C), bacterial cells from control medium were neutralized by different dosages of specific anti-O139 antibody. Data are means and standard errors of 3 determinations performed in duplicate. There was significant difference ($P < 0.05$) between the values without the same lowercase letter above bars. Letter a correspond to the control which bacterial cells were grown in control medium with 0.5% NaCl and pH 7.

III. *Effect of Salinity and pH on Virulence*

To determine whether the virulence of *V. cholerae* O139 is affected when the level of adhesion changes, the survival rate of mice following challenge with the test strain were examined. The mice were challenged with different dosages of bacterial cells cultured in the control medium and 50% of a lethal dose (LD_{50}) was calculated to be 2×10^9 cells/mouse (Figure 4A). After mice were challenged with strain O139 at a concentration of $2 \times LD_{50}$, the survival rate of mice challenged with the bacterial cells grown in control medium was 20%, and death occurred 6 hr

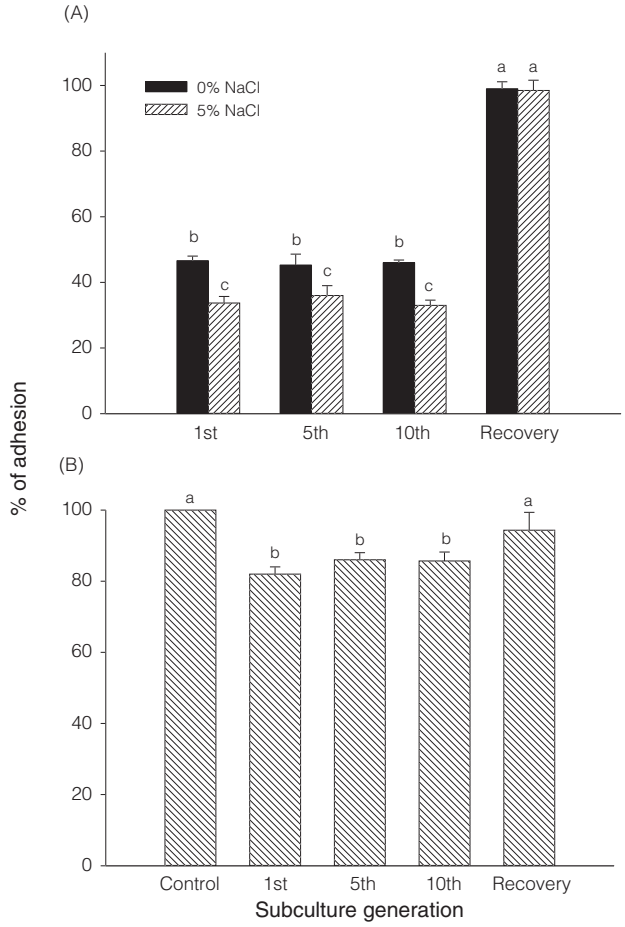

Figure 3. Adhesion of *Vibrio cholerae* O139 from different subculture generations cultured in media with different conditions to Hela cells. (A), bacterial cells were separately from different generations cultured in media without NaCl or containing 5% NaCl. (B), bacterial cells were separately prepared from different generations cultured in media with pH 5.5. Recovery, bacterial cells from the 10th generation cultured in media with 0% NaCl, 5% NaCl or pH 5.5 were separately re-inoculated into control medium with 0.5% NaCl and pH 7. Control, bacterial cells were cultured in control medium with 0.5% NaCl and pH 7. Data are means and standard errors of 3 determinations performed in duplicate. There was significant difference ($P < 0.05$) between the values without the same lowercase letter above bars. Letter a correspond to the control which bacterial cells were grown in control medium with 0.5% NaCl and pH 7.

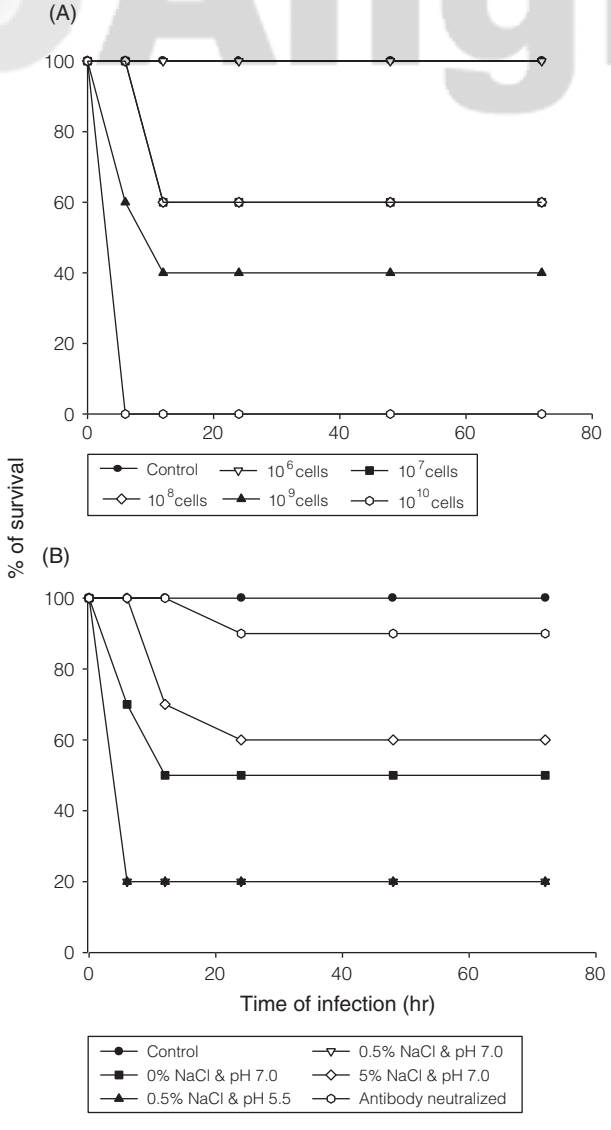

Figure 4. The survival rate of mice challenged with *Vibrio cholerae* O139 by intraperitoneal injection. (A), mice divided to six groups were separately challenged with different doses of bacterial suspension to determine the 50% lethal dose (LD_{50}) of *V. cholerae* O139 incubated in control medium with 0.5 NaCl and pH 7. (B), mice from four of five groups were separately challenged with bacterial cells cultured in media with 0.5% NaCl and pH 7 (▽), 0% NaCl and pH 7 (■), 5% NaCl and pH 7 (◇), and 0.5%NaCl and pH5.5 (▲), respectively. Mice in the fifth group were challenged with bacterial cells neutralized by specific anti-O139 antibody (○). Control, mice were challenged with 0.01 M PBS (pH 7.0).

after challenge (Figure 4B). The same phenomenon occurred in the mice challenged with bacteria grown in medium with pH 5.5. When challenged with bacterial cells from either 0% NaCl or 5% NaCl cultures, 50% and 60% of mice survived, and death occurred 6 and 12 hr after challenge, respectively (Figure 4B). Furthermore, in the study, the adhesion and virulence of *V. cholerae* O139 neutralized with O139-specific antibody were also examined. The bacterial adhesion was found to decrease and almost completely inhibited when the dosage of antibody was 6 μg (Figure 2C); also, 80% of mice survived after challenge with bacterial cells neutralized with 6 μg of specific antibody (Figure 4B).

Previous studies have reported that *V. cholerae* O1 strains were unlikely to lose their toxigenicity in aquatic environments as a result of the effects of various physicochemical conditions[3,4,5]. However, it has been determined that serogroup conversion of *V. cholerae* non-O1 to O1 could occur under different environmental conditions, such as temperature and salinity[32]. In addition, mutants of *V. cholerae* O139 with lower level of adherence and colonization were attenuated in virulence[30]. For whatever the effect of physicochemical conditions on toxigenicity, the results from both the virulence tests and the studies on the effect of changes in salinity and pH values had on adhesion (Figure 2) suggest that the level of adhesion can be varied according to different growth conditions, and that its expression may be able to further affect the pathogenicity of *V. cholerae* O139.

In conclusion, changes of growth conditions, including both salinity and pH, can affect both the expression of adhesion and virulence of *V. cholerae* O139. The study again demonstrates that bacterial adhesion is related to its pathogenicity; the blockage of adhesion therefore should be an essential process in preventing disease caused by bacterial infection. Animal experiments have shown that vaccines developed against *V. cholerae* O1 will not be effective against *V. cholerae* O139 and it is proposed that vaccines against *V. cholerae* O139 should be developed[31]. Therefore, vaccines developed from O139-specific adhesins may represent one of the possible strategies in disease prevention. In addition, the finding that the adhesion and pathogenicity could be recovered after *V. cholerae* O139 was regrown under the optimum condition may be vital in considering proper food storage.

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