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Safety and vaccine efficacy of an attenuated Vibrio vulnificus strain with deletions in major cytotoxin genes

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One sentence summary: CMM781 having deletions in three genes encoding major virulence factors appears to be a safe and effective vaccine candidate that would provide significant protection against Vibrio vulnificus infection.

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ABSTRACT

Vibrio vulnificus is a human pathogen causing a rapidly progressing fatal septicemia. We have previously reported that a V. vulnificus large toxin RtxA1 causes programmed necrotic cell death through calcium-mediated mitochondrial dysfunction. Here we developed a live attenuated vaccine strain (CMM781) having deletions in three genes encoding major virulence factors: RTX cytotoxin (rtxA1), hemolysin/cytolysin (vvhA) and metalloprotease (vvpE) of a clinical isolate strain CMCP6. The CMM781 strain showed significant attenuation in cytotoxicity and mouse lethality. The safety of CMM781 was also confirmed by measuring the transepithelial electric resistance of Caco-2 cell monolayers. Intragastric immunization of mice with the live attenuated V. vulnificus strain resulted in induction of systemic and mucosal antibodies specific to the pathogen. Moreover, the vaccinated mice were protected from challenges with high doses of the virulent strain through various injection routes. These results suggest that CMM781 appears to be a safe and effective vaccine candidate that would provide significant protection against V. vulnificus infection.

Keywords: live attenuated vaccine; Vibrio vulnificus; RtxA1 toxin; cytotoxin

INTRODUCTION

Vibrio vulnificus, a halophilic estuarine bacterium causes a rapidly progressing fatal septicemia and is associated with a mortality rate of more than 50% within a few days (Tacket, Brenner and Blake 1984; Oliver 2005). Human vibrio illnesses are increasing worldwide, which may be associated with global warming and rise in the sea surface temperature (Vezzulli,
Colwell and Pruzzo 2013). Vibrio vulnificus infection has become one of the major socioeconomic burdens in many countries. In this regard, the World Health Organization published an official risk assessment technical report jointly with the Food and Agriculture Organization of the United Nations (World Health Organization 2005). In addition, numerous V. vulnificus isolates have been found to be resistant to antibiotics routinely prescribed for the infection (Radu et al. 1998; Baker-Austin et al. 2009). Many countries, including USA, Korea and Japan, have active surveillance systems for V. vulnificus infections and educate people not to consume raw seafood during the summer season when the pathogen flourishes in seawater. Vaccination has been an efficient way of preventing bacterial infections. In developing countries, vaccines containing killed Vibrio cholerae were very effective in reducing mortality and socioeconomic burdens (Qadri et al. 2015). Vibrio species are notorious for their heterogeneity in genome sequences and genomic plasticity due to frequent horizontal gene-transfer events (Antonova and Hammer 2015). In this regard, vaccines using safe whole cells, either live or killed, could be better than those manufactured with a limited number of defined antigens. The most outstanding characteristic of pathogenic V. vulnificus is acute cytotoxicity (Kim et al. 2008). Three major cytotoxin genes have been discovered in virulent V. vulnificus strains and many report their roles in the pathogenesis: they are rtxA1, vvhA and vvpE (Gray and Kreger 1985; Kohary and Kreger 1987; Kim et al. 2008; Jeong and Satchell 2012; Lee et al. 2013). RtxA1 plays a dominant role in the V. vulnificus cytotoxicity and causes programmed necrotic cell death through calcium-mediated mitochondrial dysfunction (Kim et al. 2008, 2013; Jeong and Satchell 2012; Ziola et al. 2014). Although V. vulnificus hemolysin VvhA exhibits minor cytotoxicity (Kim et al. 2008), it was reported to have some roles in vivo (Lee et al. 2004; Kim et al. 2010; Jeong and Satchell 2012). Metalloprotease VvpE was reported to be responsible for the pathology of V. vulnificus infection (Kim et al. 2007; Miyoshi et al. 2012). In this study, we constructed an attenuated V. vulnificus strain with deletions in those three cytotoxin genes and evaluated its safety and efficacy as a live attenuated vaccine strain.

MATERIALS AND METHODS

Construction of a V. vulnificus live attenuated vaccine strain

Vibrio vulnificus and Escherichia coli were maintained in 2.5% NaCl heart infusion (HI) and Luria–Bertani (LB) media (Becton, Dickinson and Company, Sparks, MD, USA), respectively. Vibrio vulnificus CMCP6 is a highly virulent clinical isolate (Kim et al. 2011) and the complete genome sequence was reported by our group (http://www.ncbi.nlm.nih.gov/genome/189?genome_assembly_id=165749). A triple gene mutant of V. vulnificus CMCP6 was constructed by deletions of vvpE, vvhA and rtxA1 genes encoding elastolytic protease, cytolytic hemolysin and RtxA1 toxin, respectively. For the mutant construction, a vvpE-deletion mutant of CMCP6 was constructed by allelic exchange with R6K origin suicide vector pCVD442, as previously reported (Shin et al. 2005). A vvhA deletion mutation in CMCP6 vvpE mutant was also constructed via allelic exchange using the suicide vector pDM4, as previously reported (Kim et al. 2008). Deletion of the GD domain corresponding to RtxA1 4583–5199 amino acids was conducted in a CMCP6 double mutant with the deletion in vvhA and vvpE via allelic exchange using the suicide vector pDM4, following the method of Kim et al. (2008). The V. vulnificus mutant strain with the deletion of rtxA1, vvhA and vvpE genes of CMCP6 was designated CMM781 (Table 1).

Cytotoxicity assay

To evaluate the safety of the CMM781 strain, cytotoxicity to HeLa cells (Korea Cell Line Bank, Seoul, Korea) was evaluated as described previously (Kim et al. 2008). HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (GIBCO Invitrogen, Carlsbad, CA, USA) in a 37 °C incubator with 5% CO2. HeLa cells (1 × 106 cells well−1) were seeded into 24-well cell-culture plates (Nalge Nunc International, Rochester, NY, USA) and incubated overnight. The HeLa cells were washed with serum-free DMEM and infected with V. vulnificus strains at a multiplicity of infection (MOI) of 20 in a 5% CO2 incubator at 37 °C. Lactate dehydrogenase released after contact with V. vulnificus was measured using the CytoTox96® non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA).

Staining of actin and the nuclei of HeLa cells infected with V. vulnificus strains

HeLa cells cultured in an eight-well glass-chambered coverslip (Nalge Nunc International) were infected with V. vulnificus strains at an MOI of 100 and 37 °C for 2 h. The actin and nuclei were stained using Alexa Fluor 594-conjugated phalloidin and 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Invitrogen), as described previously (Kim et al. 2008).

Hemolysis assay of red blood cells

Human red blood cells (RBCs) (1 × 107 (500 μl)−1) were incubated with V. vulnificus in phenol red-free DMEM (GIBCO Invitrogen) at an MOI of 1 and 37 °C for 60 min. The contact hemolysis was determined by measuring hemoglobin released in the supernatant at 540 nm after centrifugation at 13 000 r.p.m. for 1 min.

Table 1. Vibrio vulnificus strains used in this study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Characteristics</th>
<th>Source or reference</th>
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<tr>
<td>CMCP6</td>
<td>V. vulnificus, clinical isolate (GenBank no. AEO16795 and AEO16796)</td>
<td>Chonnam National University Hospital</td>
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<tr>
<td>CMCP6 vvpE−</td>
<td>CMCP6 with a deletion in 232–1176 bp of vvpE</td>
<td>This study</td>
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<tr>
<td>CMCP6 vvhA−</td>
<td>CMCP6 with a deletion in 111–890 bp of vvhA</td>
<td>This study</td>
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<tr>
<td>CMCP6 vvpE−/vhvA−</td>
<td>CMCP6 with double deletion in vvpE and vvhA genes</td>
<td>This study</td>
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<tr>
<td>CMCP6 rtxA1−</td>
<td>CMCP6 with a deletion in 13747–15597 bp of rtxA1</td>
<td>This study</td>
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<tr>
<td>CMM781</td>
<td>CMCP6 with triple deletion in rtxA1, vvhA and vvpE genes</td>
<td>This study</td>
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Measurement of transepithelial electric resistance in Caco-2 cell monolayer by Ussing chamber technique

Epithelial monolayers of Caco-2 cells were formed in the upper layer of a Transwell culture plate (Corning Costar, Cambridge, MA, USA) and V. vulnificus strains were infected at an MOI of 100 and 37 °C for 90 min. Transcell electric resistance was measured using an Ussing apparatus (World Precision Instruments, Sarasota, FL, USA) as described previously (Blanco and DiRita 2006).

Determination of the median lethal dose (LD50)

Specific pathogen-free 8-week-old, or 5-day-old, CD-1 mice were infected using the intraperitoneal (i.p.), intragastric (i.g.) or intranasal (i.n.) route. Five mice in each group were tested and the infected mice were observed for 48 h. The LD50 was calculated by the Reed and Muench method (Reed and Muench 1938). All animal procedures were conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Korean Food and Drug Administration. The protocol was approved by the Chonnam National University Committee on the Ethics of Animal Experiments. All efforts were made to treat the mice humanely.

Mice immunization and assay of immune responses by enzyme-linked immunosorbent assay

Five-week-old BALB/c mice were randomly divided into two groups. The V. vulnificus CMM781 strain was grown to late log phase in HI broth at 37 °C with vigorous aeration. For oral vaccination, the mice were fasted overnight and were subsequently immunized three times at 2 week intervals with the CMM781 mutant strain at 1 × 10², 2 × 10³ and 4 × 10³ colony-forming units (CFUs) in 400 μl of phosphate-buffered saline (PBS). The control mice were treated with PBS only. Seven days after the last immunization, blood samples were collected by cardiac puncture of mice anesthetized with Zoletil (Virbac Laboratories, Carros, France) and Rompun (Bayer Korea, Seoul, Korea). Saliva was collected from the mice after i.p. injection of 100 μg pilocarpine (Sigma, Seoul, Korea).

Antibody titers reactive to V. vulnificus whole cell lysate were quantified by enzyme-linked immunosorbent assay (ELISA). High-binding and flat-bottom 96-well ELISA plates (Corning Laboratories, Corning, NY, USA) were coated with V. vulnificus CMCP6 total cell lysate (50 μl well⁻¹ of 5 μg ml⁻¹) and incubated at 4 °C overnight. Subsequently, the plates were incubated with a blocking buffer (0.05% Tween 20, 1 mM EDTA, 0.5% bovine serum albumin in PBS) for 30 min, and 50 μl of serum or mucosal samples were applied as 2-fold dilution series in the blocking buffer. After washing with distilled water, 50 μl of anti-mouse immunoglobulin–horseradish peroxidase conjugates (Sigma) diluted with the blocking buffer were added to each well, and the plates were incubated for 2 h at room temperature. Color was developed with 50 μl of 3,3,5,5′-tetramethyl-benzidine solution (Sigma). The reaction was stopped by adding 50 μl of 1 N H₂SO₄ (Sigma). Absorbance was read by a microplate reader (Molecular Devices Corp., Menlo Park, CA, USA) at 450 nm.

Challenge of virulent wild-type strain and protection assay

For the oral challenge, mice were injected intramuscularly with iron dextran (25 mg per mouse, Sigma) 2 h prior to i.g. inoculation with wild-type CMCP6 at 50 × LD50 (2.3 × 10⁵ CFU in HI). For i.p. or i.n. challenges, CMCP6 strain at 20 × LD50 (10⁷ and 2 × 10⁶ CFUs, respectively) was injected into the mice.

Statistical analysis

Statistical differences were analyzed using Student’s t test. All experiments were repeated three times, and the result from a representative experiment is shown.

RESULTS

The V. vulnificus triple mutant was significantly less cytotoxic to HeLa cells and RBCs

The V. vulnificus clinical isolate CMCP6 kills almost all cells within 3 h after contact with HeLa cells at an MOI of 20 (Fig. 1A). In contrast, CMM781 showed significantly reduced cytotoxicity to HeLa cells even after overnight incubation (Fig. 1A).

To monitor morphologic changes, we stained actin and nuclei of HeLa cells infected with V. vulnificus strains. CMCP6 caused cell rounding and shrinkage of HeLa cells, whereas CMM781 did not cause any morphologic damages (Fig. 1B). These results indicate that CMM781 does not have any significant cytotoxicity to HeLa cells.

In V. vulnificus pathogenesis, hemolysis may be a cause of V. vulnificus toxicity in vivo. To test the safety of the vaccine strain, RBCs were incubated with V. vulnificus at an MOI of 1 for 1 h. Vibrio vulnificus CMCP6 caused contact hemolysis of RBCs (Fig. 1C). However, recognizable hemolysis was not observed when human RBCs were incubated with the CMM781 strain (Fig. 1C). This result suggests that CMM781 would not cause any significant harm to host cells.

CMM781 did not change the transepithelial electric resistance

Though major cytotoxin genes were mutated, there could have remained minor virulence factors that could negatively affect host cells. To be used as a mucosal vaccine, the live attenuated vaccine strain should not derange the epithelial integrity in mucosal compartments. Hence, we observed the transepithelial electric resistance of Caco-2 cell monolayers infected by the triple mutant using an Ussing chamber. The transepithelial electric resistance of the Caco-2 monolayer infected with wild-type CMCP6 for 90 min decreased to 50% of mock cells (Fig. 2). However, the transepithelial electric resistance of Caco-2 cells infected with CMM781 was similar to that of mock cells (Fig. 2). These results suggest that a vaccine strain V. vulnificus CMM781 will not significantly disturb the integrity of mucosal epithelia in vivo.

The triple mutation resulted in a significant increase in LD50 values in mice: the triple mutant had a very wide safety margin

The LD50 values of wild-type CMCP6 or the triple mutant CMM781 strain were measured through three routes: intraperitoneal, intragastric or intranasal infections. For intraperitoneal and intranasal infections, adult mice were used with or without iron overload. Since patients having higher serum-free iron levels are more vulnerable to the infection (Hor, Chang and Wang 1999), we wanted to see whether the induced immune responses were efficacious even in iron-overloaded conditions. For the intragastric LD50, suckling mice were used since they provided
Figure 1. Cytotoxicity of V. vulnificus strains to HeLa cells and RBCs. (A) Cytotoxicity assay. HeLa cells in 24-well cell-culture plates were infected with V. vulnificus strains at an MOI of 20 in a 5% CO$_2$ incubator at 37°C. Lactate dehydrogenase released in the culture supernatant was measured using the CytoTox96 non-radioactive cytotoxicity assay kit. (B) Staining of actin and nuclei. HeLa cells cultured in an eight-well glass chambered coverslip were infected with V. vulnificus strains at an MOI of 100 for 2 h. The actin and nuclei were stained using Alexa Fluor 594-conjugated phalloidin (red color) and DAPI (blue color). (C) Hemolysis assay. Human RBCs were incubated with V. vulnificus in phenol red-free DMEM at an MOI of 1 for 60 min. The contact hemolysis was determined by measuring hemoglobin released in the supernatant at 540 nm after centrifugation at 13 000 r.p.m. for 1 min.

Table 2. LD$_{50}$ values of V. vulnificus strains administered to mice.

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<th>Administration routes</th>
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<tr>
<td>Intraperitoneal</td>
<td>3.6 $\times$ 10$^5$ 5.5 $\times$ 10$^7$</td>
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<tr>
<td>Intraperitoneal (in iron-overloaded mice)</td>
<td>7.8 $\times$ 10$^6$ 7.0 $\times$ 10$^4$</td>
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<tr>
<td>Oral (in suckling mice)</td>
<td>7.5 $\times$ 10$^5$ 5.5 $\times$ 10$^8$</td>
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Figure 2. Measurement of the transepithelial electric resistance in Caco-2 cell monolayer by the Ussing chamber technique. Epithelial monolayers of Caco-2 cells were formed in the upper layer of a Transwell culture plate and the cells were infected with V. vulnificus strains at an MOI of 100 and 37°C for 90 min. Transcell electric resistance was measured by using an Ussing apparatus. wt, wild-type. ***P < 0.001; **P < 0.01 versus cells untreated with Vv.

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difficulties in breathing, bristled fur and decreased activity. LD$_{50}$ difference between the wild-type and CMM781 was most notable in the iron-overloaded intraperitoneal infection group, with an 8974-fold increase in the latter (Table 2). These results indicate that CMM781 could serve as a safe live attenuated vaccine candidate for the prevention of V. vulnificus infections.

Oral CMM781 immunization induced protective immune responses against V. vulnificus challenge infection

The mice were vaccinated three times with CMM781 through the intragastric route. The antibody titers against V. vulnificus whole cell lysate were determined by ELISA. Immunized mice developed significant IgG, IgG1 and IgG2a antibody responses in the serum (Fig. 3). We also assessed IgA production as the
parameter of active mucosal immune responses. Immunized mice developed significant IgA antibody responses in serum, saliva and feces (Fig. 3).

Finally, the mice immunized with CMM781 were dramatically prevented from developing a lethal infection by a virulent strain through oral, nasal or peritoneal challenges (Table 3).

**DISCUSSION**

There have been various attempts to develop *V. vulnificus* vaccines (Devi et al. 1995, 1996; Fouz et al. 2001; Esteve-Gassent, Fouz and Amaro 2004; Jung, Park and Heo 2005; Lee et al. 2014a, b; SongLin et al. 2015). In eels, killed cells were tried with substantial protection (Fouz et al. 2001; Esteve-Gassent, Fouz and Amaro 2004). Capsular polysaccharide antigen conferred significant protection against the same capsule-type infections (Devi et al. 1995). However, the practicality of capsular polysaccharide antigens of *V. vulnificus* has been seriously doubted given very heterologous carbotypes and very poor cross-protectivity across different capsular serotypes (Devi et al. 1996). Recently, our group reported that the C-terminal domain could be an efficacious subunit vaccine candidate (Lee et al. 2014a, b). Monoclonal antibodies against the C-terminal domain showed both protective and therapeutic efficacy, which suggests that neutralization of the RtxA1 effector domain could be protective. However, it is possible that the RtxA1 C-terminal polypeptide vaccine would not effectively suppress earlier stage infection since the RtxA1 toxin is induced after close contact with host cells. There are other virulence factors that could be targeted for vaccine development such as outer membrane protein OmpU and flagellins (Jung, Park and Heo 2005; Kim et al. 2014). The triple mutant expressed wild-type level OmpU and flagellin proteins suggesting intact expression of other possible vaccine targets (data not shown). In the present study, we developed a safely attenuated live vaccine candidate by deleting three major exotoxin genes (rtxA1, vvhA and vpeA) of *V. vulnificus*. We primarily checked the safety of the virulence attenuation constructed by the deletion of those three cytotoxin genes. The mutant strain manifested significantly increased LD₅₀ (at least 2 log scale) through various infection routes (intraperitoneal, oral and intranasal) and under an iron-overloaded condition. At safe dosages, the vaccine candidate induced significant protective immune responses in both mucosal and systemic compartments when it was orally vaccinated. The types of immune responses were further analyzed by measuring isotype-specific antibody titers. Both IgG1 and IgG2a antibody levels were significantly increased, suggesting that both the complement-mediated bacteriolysis and Fc receptor-mediated phagocytosis should have played important roles in the protection against the live bacterial challenge (Fig. 3). Taken together, the triple exotoxin mutant CMM781 may serve as a safe live attenuated vaccine against fatal *V. vulnificus* septicemia with high protective efficacy. Combination of this live attenuated vaccine with the RtxA1 C-terminal antigen may confer synergistic protective efficacy as was noted in the *V. cholerae* vaccination that combined killed bacteria and cholera toxin B subunit antigen (Resnick et al. 1980; Stewart-Tull, Lucas and Bleakley 2004). Since the cholera toxin is the most potent virulence factor responsible for the cholera pathogenesis, targeting both replicating bacteria by killed whole cells and a component of diarrheogenic virulence factor (cholera toxin B subunit) would synergistically potentiate the protective efficacy.

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**Conflict of interest.** None declared.

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