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Original Article

Destructive Intestinal Translocation of *Vibrio vulnificus* Determines Successful Oral Infection

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Vibrio vulnificus causes primary septicemia as a result of the consumption of contaminated seafood. The intestinal epithelial layer is the first host barrier encountered by *V. vulnificus* upon oral intake; however, epithelial translocation (invasion) of *V.vulnificus* has not been extensively studied. In this study, we investigated *in vivo* translocation of *V. vulnificus* using clinical (CMCP6) and environmental isolates (96-11-17M). And we analyzed physiological changes of intestinal epithelium concurrent with bacterial translocation by using polarized HCA-7 transwell culture system. The efficiency of epithelial translocation of 97-11-17M strains was significantly lower than that of pathogenic clinical isolate CMCP6 in a murine ligated ileal loop model. In an oral infection model, the survival rate was reciprocally related with efficacy of *in vivo* epithelial translocation. These results indicate that efficient translocation of *V. vulnificus* through intestinal epithelium is highly correlated with successful oral infection. We determined translocation of the bacteria from upper to lower chamber, changes of transepithelial electric resistance (TER) and cytotoxicity of the polarized HCA-7 cells to understand general features of *V. vulnificus* invasion. Bacterial translocation was accompanied by big decrease of TER (about 90%) and about 50% cytotoxicity of the epithelial cells. Taken together, these results indicate that *V. vulnificus* actively translocates the epithelium by destruction of epithelium and the efficiency of intestinal invasion by *V. vulnificus* is critical for successful oral infection. From this result, it is suggested that integrity of intestinal barrier is an important factor for susceptibility to oral infection of *V. vulnificus*.

Key Words: *Vibrio vulnificus*, Epithelial translocation (invasion), Oral infection, Pathogenesis

INTRODUCTION

Vibrio vulnificus is a halophilic estuarine bacterium that can cause fatal septicemia upon the consumption of contaminated seafood. For successful survival and pathogenesis within the host, orally ingested *V. vulnificus* must overcome multiple barriers and gain access to the bloodstream. Orally ingested *V. vulnificus* likely encounters extreme gastric conditions before arriving at the intestinal mucosa, where some portion of the bacteria invade intestinal barrier and eventually enter the bloodstream and finally induce

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septicemia. Healthy individuals have been reported to experience vomiting, diarrhea, and abdominal pain within 16 hours of the ingestion of contaminated seafood (1, 2). This report suggests that the first step of infection relevant to pathogenesis is the penetration of the intestinal barrier. During the establishment of infection, pathogenic bacteria employ several strategies to optimize their ability to survive and replicate within the host (3~6). The epithelial barrier provides first line of defense against an extensive array of microbiota and invading pathogens (7~12). Pathogenic microorganisms may therefore develop strategies to invade the epithelial barrier and gain access to the deeper tissue either via the breakdown of the epithelial barrier through the activation of host- and/or microbe-proteases or via transport by host immune cells, such as M cells or dendritic cells, as is suggested by the Trojan horse hypothesis (13~15) (16). The intestinal epithelium faces the lumen of the gastro-intestinal tract and acts as the boundary between host tissue and the environmental milieu. Highly polarized epithelial cells are tightly connected to their adjacent cells via apical junction complexes. These junctions are composed of trans-membrane proteins, cytoskeletal elements, and cytoplasmic scaffolding proteins (7, 11). The process of translocation (or invasion) across the epithelial barrier by *V. vulnificus* and relationship between integrity of intestinal barrier function and susceptibility of *V. vulnificus* oral infection have not been extensively studied.

In this study, we selected pathogenic clinical isolate and relatively nonpathogenic environmental isolates, of *V. vulnificus* to investigate whether *V. vulnificus* actively invades the epithelial barrier, and if so, whether efficacy of epithelial translocation is critical for successful oral infection. We present evidence that *V. vulnificus* actively invades the intestinal epithelial barrier in a destructive manner and the efficiency of epithelial translocation is critical for successful oral infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The *V. vulnificus* strains used in this study are listed in Table 1. *V. vulnificus* strains were grown in 2.5% NaCl-containing heart infusion (HI) medium (Becton Dickinson; Sparks, MD, USA).

In vivo invasion assay

To assess the invasion of the CMCP6 (17) and 96-11-17M (18) *in vivo*, *V. vulnificus* translocation from the intestine to the bloodstream was measured as previously described (19). To rule out interference of any dietary product with *V. vulnificus* in the intestine, eight-week-old specific pathogen-free (SPF) female outbred mice ICR (CD-1®) mice were starved for 16 hours and anesthetized with 50 mg/kg tiletamine HCl/zolazepam HCl (Zoletil 50®; Virbac Laboratories, France) and 10 mg/kg xylazine HCl (Rumpun®; Byer Korea, Korea) in phosphate buffered saline (PBS). The ileum was tied off in a 5-cm segment, and then, *V. vulnificus* cells (400 μl; 1.0×10^7 CFU/ml in PBS) were inoculated into the ligated segment. The blood samples were collected from the ocular plexus at appropriate time intervals, and viable bacterial cells were counted by plating on 2.5% NaCl HI agar plates.

In vivo growth of *V. vulnificus*

To compare the *in vivo* growth of CMCP6 and 96-11-17M, we performed dialysis tube implantation assays. CelluSep® H1 dialysis tubing (MWCO 12,000~14,000; Membrane Filtration Products, Inc. Texas, USA) was incubated with PBS overnight. The dialysis membrane was disinfected with 70% alcohol for 1 hour and washed three times with sterile PBS before use. Eight-week-old female Sprague Dawley (SD) rats (DBL. Co. Ltd, Daejeon, Korea) were anesthetized with 50 mg/kg tiletamine HCl/zolazepam HCl (Zoletil 50®; Virbac Laboratories, France) and 10 mg/kg xylazine HCl (Rumpun®) in PBS. Five-cm dialysis tube which contained 2 ml of 1×10^7 CFU/ml *V. vulnificus* cells were surgically implanted into the rat peritoneal cavity. The bacteria were harvested 2, 4, and 6 hours after implantation for bacterial viable cell counting on 2.5% NaCl HI agar plates.

Serum bactericidal activity

To determine the sensitivity of the *V. vulnificus* strains to serum bactericidal activity, 0.22 μm membrane-filtered human normal pooled sera (NPS) was used. Logarithmic-phase *V. vulnificus* strains were inoculated in 75% NPS in PBS at an initial concentration of 1×10^8 CFU/ml and incubated at 37℃. Viable *V. vulnificus* cells were counted on 2.5% NaCl HI agar plates after 30 and 60 min of incubation as previously described (20, 21).

Intragastric infection of *V. vulnificus*

Five-day-old infant CD-1® mice (Daehan Animal Co., Korea) were administered 10-fold serial dilutions of exponentially grown bacterial suspensions containing 0.1% Evans blue (Sigma-Aldrich Co., St. Louis, MO, USA) in PBS. The survival of infected mice was measured for 48 h, and the LD_{50} was calculated using the Reed and Muench method (22). All animal procedures were conducted in accordance with the guidelines of the Animal Care and Use Committee of Chonnam National University.

Cell culture and determination of epithelial integrity

HCA-7 cell line was cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum and 100 units/ml penicillin plus 100 μg/ml streptomycin at 37℃ with 5% CO_2, as described previously (23). Polarized monolayers were generated from cells cultured in complete medium in Transwell® filter chambers (8 μm pore size; CoStar, Cambridge, MA, USA). Cells were seeded at 3×10^5 in 0.2 ml of media into the upper chamber, and 1 ml of medium was added to the lower chamber. The medium was changed every two days. To determine the degree of polarization of the HCA-7 cells, the trans-epithelial electrical resistance (TER) was measured using a Millicell Electrical Resistance System-2 (Millipore, Bedford, MA, USA) as previously described (23). When the TER reached more than 600 ohms, the culture was considered to be properly polarized.

Epithelial translocation (invasion) of the *V. vulnificus* in polarized epithelial cell system

To measure the epithelial invasion of *V. vulnificus*, the polarized HCA-7 culture was washed with fresh DMEM media without serum and antibiotics, and bacterial cells at the exponential growth phase suspended in PBS were added to the apical surface on Transwell filters at a multiplicity of infection (MOI) of 5. Bacterial translocation from the upper chamber to the lower chamber was measured by twofold serial dilution and subsequent bacterial viable cell counting on 2.5% NaCl HI agar plates as previously described (20). The results are expressed as a percentage of the initial resistance.

Determination of LDH release from polarized epithelial cells

To determine the degree of cytotoxicity of the bacterial strains on the polarized HCA-7 cells, lactate dehydrogenase (LDH) release was measured in culture media collected from the upper and lower chambers using the CytoTox non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA).

Statistical analysis

The results are expressed as the mean ± SEM unless otherwise noted. Significant differences between two groups were analyzed using Student's *t*-test. The difference in survival rates between the experimental groups was tested by Kaplan-Meier analysis. P values < 0.05 were considered statistically significant. All experiments were repeated more than three times, and results from representative experiments are shown.

RESULTS AND DISCUSSION

***V. vulnificus* actively translocates from the intestinal epithelial barrier to blood stream in an ileal loop infection model**

V. vulnificus is a causative agent of fatal septicemia by the consumption of contaminated seafood. To successfully

Table 1. Bacterial strains used in this study

Strain	Characteristic	Source or Reference
CMCP6	Pathogenic clinical isolate Opaque colony Serum-resistant	Chonnam National University Hospital, Korea; (17)
96-11-17M	Environmental isolate from shrimp Opaque colony Serum-resistant	The Royal Veterinary & Agricultural University, Denmark; (18)

establish an infection, orally ingested *V. vulnificus* must penetrate and/or invade the intestinal epithelial barrier and reach deeper tissues. *V. vulnificus* has been isolated from the blood sample from septicemic patients, which is the most distinctive characteristic of *V. vulnificus* compared with other *Vibrio* species (24). However, the professional invasive mechanism of *V. vulnificus* is not fully understood. To assay invasion *in vivo*, we examined ligated murine ileal loops infected with *V. vulnificus*. In this study, we used pathogenic clinical isolate CMCP6 and environmental strain 96-11-17M (Table 1). The genomic sequence of CMCP6 was recently re-annotated and is regarded as the most complete and accurate of the published *V. vulnificus* genomes (17, 25). Because the growth and survival of *V. vulnificus* in the host is largely affected by sensitivity against serum bactericidal activity (2, 26, 27) which is correlated with *V. vulnificus* pathogenesis (28, 29), we ruled out serum-sensitive environmental strains for this purpose by intensive screening (data not shown). To determine invasiveness of *V. vulnificus*, viable cells in the bloodstream were counted at 3 and 6 h after infection into a ligated ileal loop. In CMCP6-infected mice, 1.2×10^4 CFU/ml and 1.7×10^6 CFU/ml *V. vulnificus* cells were detected at 3 h and 6 h, respectively (Fig. 1). When cell counts reached around 1×10^7/ml of blood, the majority of infected mice had succumbed to the infection. However, in mice infected with 96-11-17M, the translocation of *V. vulnificus* from the intestinal tract into the bloodstream was significantly decreased at 3 and 6 h after infection when compared with CMCP6 ($p = 0.015$ and $p = 0.0057$, respectively). To mount a successful infection *in vivo*, bacterial survival under *in vivo* conditions is preliquisite. Therefore, we compared *in vivo* survival of CMCP6 and 96-11-17M by measuring *in vivo* growth. In this study, to reproduce *in vivo* growth condition, we established the dialysis tube implantation model, as described in the Materials and Methods section, and determined the number of viable cells in the transplanted tubes. The bacterial count of the *V. vulnificus* strains in the *in vivo* growth condition showed no significant difference among groups (Table 2). The generation time for *in vivo* growth of CMCP6 and 96-11-17M were 22.41 ± 0.62 and 23.29 ± 1.44 ($p > 0.05$) in triplicate experiment. This result clearly indicates that since the *in vivo* growth rate showed no differences between CMCP6 and 96-11-17M (Table 2), lower number of viable cells in bloodstream of 96-11-17M (Fig. 1) was soley dependent on the low invasiveness of the strains.

Table 2. Generation time of *V. vulnificus* in dialysis tubing implantation model

Strains	Generation time (min)[a]	
	Mean ± SEM	*P* value
CMCP6	22.41 ± 0.62	$p > 0.05$
96-11-17M	23.29 ± 1.44	$p > 0.05$

[a]: Generation time of *V. vulnificus* was determined in dialysis tube implantation model.

Taken together, these results suggest that 1) *V. vulnificus* actively invades the intestinal epithelial barrier *in vivo*, 2) the clinical isolate CMCP6 is more invasive than the environmental isolate 96-11-17M, 3) *in vivo* growth is comparable in both strains, 4) since both strains are serum-resistant, invasiveness of the strain is critical factor for pathogenesis of the strains after bacterial administration in ligated ileal loop.

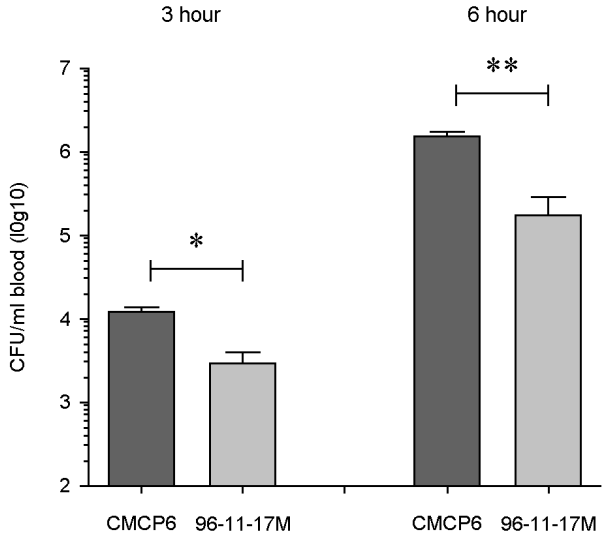

Figure 1. *In vivo* invasion by *V. vulnificus* strains in a ligated ileal loop infection model. *V. vulnificus* (4.0×10^6 CFU/400 μl) was inoculated into ligated ileal loops of mice under anesthesia. After 3 and 6 hours, blood samples were collected from the ocular plexus from the infected mice. Viable bacterial cells were counted by plating on 2.5% NaCl HI agar plates. Bacterial invasion from the ligated ileal loop into bloodstream was significantly decreased in 96-11-17M infected mice. The data represent the average values and SEM from three or four mice. *$p < 0.05$; **$p < 0.01$ compared with *V. vulnificus* CMCP6.

Table 3. Oral infection of *V. vulnificus* for LD_{50} determination

CFU administered[a]	CMCP6	96-11-17M
10^4/mouse	6/6[b]	6/6
10^5/mouse	6/6	6/6
10^6/mouse	6/6	6/6
10^7/mouse	3/6	6/6
10^8/mouse	0/6	4/6
10^9/mouse	0/6	3/6
LD_{50}[d]	8.9×10^5/mouse	ND[c]

[a]: intragastric administration
[b]: number of surviving mice/total number of mice
[c]: not determined
[d]: LD_{50} determined 48 hours after intra-gastric administration

Intestinal invasion of *V. vulnificus* is critical for successful oral infection

In addition to the *in vivo* invasion assay, we determined the LD_{50} for intragastric infection using a suckling murine infection model that is similar to the infection route that occurs during human primary *V. vulnificus* septicemia. The intragastric (ig) LD_{50} value for *V. vulnificus* CMCP6 was 8.9×10^5/mouse (Table 3). However, because a group of mice administered the highest dosage of 96-11-17M (1×10^9/mouse) showed a survival rate of 50%, we were unable to calculate ig LD_{50} for the 96-11-17M using the Reed and Muench method. And the time required for death for mice administered with 96-11-17M was significantly delayed ($p < 0.05$ at 10^9/mouse and $p < 0.01$ at 10^8/mouse by Kaplan-Meier survival analysis) (Fig. 2). Additionally, the clinical symptoms and signs were milder in the mice injected with the mutant strain. These results strongly suggest that transepithelial invasion is prerequisite for successful oral

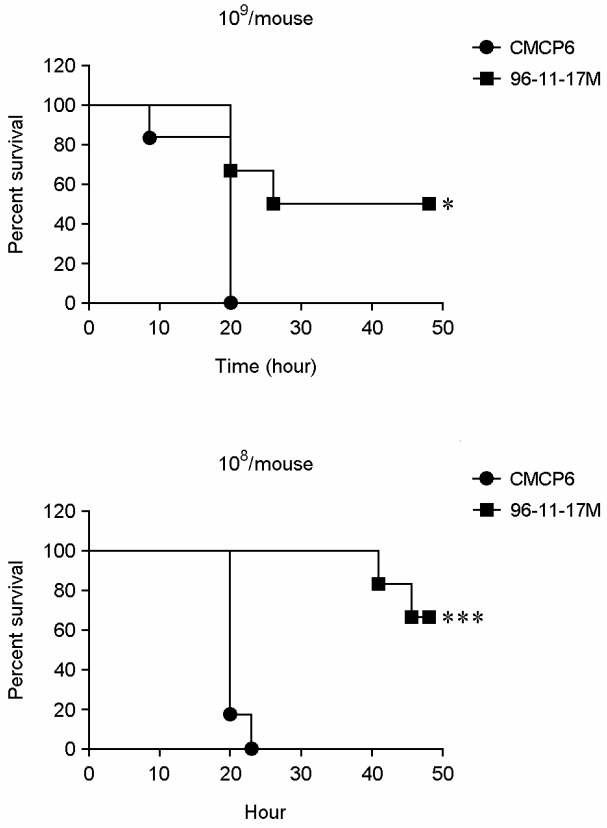

Figure 2. Survival curve for mice infected intragastrically with *V. vulnificus* strains at a dose of 1.0×10^9 cfu/mouse (A) or 1.0×10^8 cfu/mouse (B). The survival rate was significantly increased in the 96-11-17M-infected mice. The survival curve was constructed according to the Kaplan-Meier method, and statistical significance was determined by the log-rank test. *$p < 0.05$; **$p < 0.01$ compared with *V. vulnificus* CMCP6.

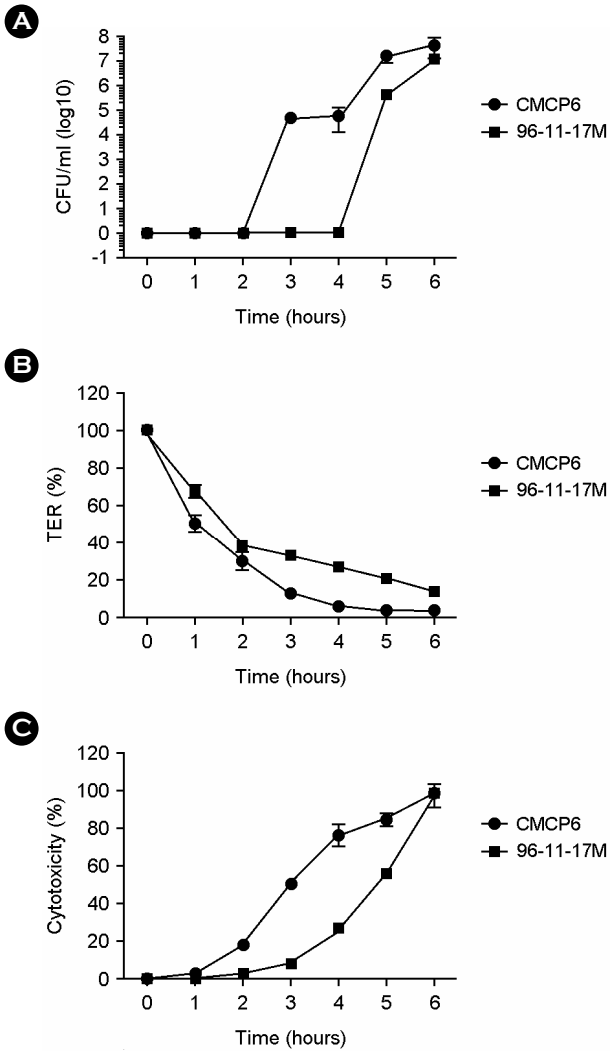

Figure 3. *In vitro* invasion by *V. vulnificus* CMCP6 and 96-11-17M in polarized HCA-7 cell monolayers. HCA-7 cells grown on transwell filters were apically infected with *V. vulnificus* CMCP6 or 96-11-17M. The bacterial epithelial translocation **(A)** was determined by measuring the number of bacterial cells that translocated from the upper chamber to the lower chamber of the transwell. The change in transepithelial electrical resistance (TER) **(B)** and LDH release in the upper (apical) and lower chamber (basolateral) **(C)** were determined. Viable bacterial cells were counted by plating on 2.5% NaCl HI agar plates.

infection by *V. vulnificus* and that efficacy of epithelial translocation (invasiveness) plays a critical role in efficient oral infection and full blown pathogenesis of *V. vulnificus*.

Vibrio vulnificus translocates across polarized intestinal epithelial monolayers in a destructive manner

To model the *in vitro* intestinal epithelial barrier, polarized HCA-7 cells were grown on transwell filters and apically infected with CMCP6 at an MOI of 5 under serum free conditions as previously described (19, 20). Higher MOIs accelerate the translocation of the bacteria (data not shown). *In vitro* invasiveness was determined by measuring the number of bacterial cells that translocated from the upper to the lower chamber of the transwell. As shown in Fig. 3A, after 3 hours of incubation 4.8×10^4 CFU/ml CMCP6 cells were detected in the lower chamber, and after 6 hours of incubation 4.4×10^7 CFU/ml were detected in the lower chamber. Transepithelial electrical resistance (TER) decreased after 1 hour for HCA-7 cells infected with CMCP6 (Fig. 3B). This result indicates that CMCP6 infection induces a gradual decrease in TER, and bacterial translocation was detected at a critical time point (3 h). Following repeated experiments, we consistently observed that when approximately 50% LDH release was detected from the upper chamber, the translocation of *V. vulnificus* had begun (Fig. 3C and data not shown). In contrast, the translocation of 96-11-17M cells was not detected until 5 h after infection. Similarly, TER decrease and LDH release were delayed compared with CMCP6 (Fig. 3B and 3C). The ZOT toxin has been shown to play a role in invasion of *V. cholera* (Vc-*zot*). However, a blast search of the *V. vulnificus* genome did not reveal a sequence homologous to Vc-*zot*. It is likely that at earlier time point of incubation, cytotoxins derived from the *V. vulnificus* are responsible for the cytotoxicity, however, at later time points (more than 5 h incubation) bacteria-derived LPS, the activation of host-derived proteases or environmental changes, such as the accumulation of organic acids play roles for destruction of intestinal barrier. These *in vitro* results indicate that 1) *V. vulnificus* actively translocate across the intestinal epithelial monolayer in the *in vitro* system without the involvement of M cells; 2) translocation of *V. vulnificus* shows destructive manner; 3) TER changes occur earlier than LDH release; 4) bacterial translocation of *V. vulnificus* is accompanied by

about 50% LDH release. However, we cannot rule out the possibility that 5) host-derived proteases are activated after 5 hours of incubation.

Because intestinal epithelial cells are overlaid with organized lymphoid organs, such as the gut-associated lymphoid organ (GALT) or isolated lymphoid follicles (ILFs) (30). Under steady-state conditions, microfold (M) cells are highly enriched in this organ (30, 31). Additionally, M-cell-mediated pathogen invasion is considered an important initial step in mucosal infections (31, 32). Although we cannot rule out the possibility that M-cells mediate the passage of *V. vulnificus* from the lumen into deeper tissues, our data suggest that *V. vulnificus* actively invades the intestinal mucosa in a destructive manner.

Taken together, we demonstrates that clinical isolates of *V. vulnificus* exhibit higher invasiveness than environmental isolates. From these results, we propose that only invasive *V. vulnificus* strains induce clinical manifestation through oral ingestion, while non-invasive strains rarely reach deeper tissues and cannot induce full infection. And we also suggest that *V. vulnificus* actively translocates/invades the intestinal epithelial barrier in a destructive manner and that transepithelial invasion is a critical step for successful oral infection with *V. vulnificus*. These results imply that weakness of the intestinal barrier may influence susceptibility to *V. vulnificus* primary septicemia.

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