Acta Medica et Biologica
Vol. 51, No.4, 149-156, 2003

Occurrence of a *Vibrio vulnificus* Infection in Niigata in 2003

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Received December 26 2003; accepted January 9 2004

Summary. **A *Vibrio vulnificus* infection occurred in Niigata in 2003. The patient was a 76-year-old male who had a medical history of diabetes, and was a heavy consumer of alcohol. He did not eat raw fish or shellfish before development of the symptoms, and the source of infection was unknown. The patient reported a disturbance in consciousness, and mild swelling of the lower leg was observed. The same *V. vulnificus* strains, as evidenced by pulsed-field gel electrophoresis, were isolated from venous blood and puncture fluid of the lower leg. White blood cells and platelets were decreased. The patient was treated with imipenem, but succumbed. The *V. vulnificus* strains were highly susceptible to the 3rd-generation cephems, carbapenems, tetracyclines, and fluoroquinolones. With respect to the tetracyclines and macrolides, the strains were most susceptible to minocycline and azithromycin, respectively. The strains were moderately resistant to the 1st- and the 2nd-generation cephems, kanamycin, streptomycin, roxithromycin, and fosfomycin. The *V. vulnificus* strains possessed a strong ability to adhere to and impair HCT-8 cells. In contrast to *V. cholerae* O1 or O139 and *V. parahaemolyticus*, the *V. vulnificus* strains had capsule-like wrinkles on the bacterial cell surface and occasionally exhibited a spiral-shaped body. These may be a cause, at least in part, of the invasive nature of the bacteria and fulminant development of the symptoms. Establishment of a rapid diagnosis is imperative.**

Key words— *Vibrio vulnificus*, infection case in Niigata, drug susceptibility, adherence, bacterial cell shape.

INTRODUCTION

Vibrio vulnificus is a gram-negative rod distributed in seawater at 15°C or higher as is *Vibrio parahaemolyticus*. In Japan, infection is more likely to occur between June and September when seawater temperature increases, and higher incidences occur in the western side of the Japan Sea[1-4]. *V. vulnificus* colonizes to sea fish and shellfish including shrimp or zooplanktons, and causes infection via oral ingestion of contaminated sea fish and shellfish or injury[5-9]. Many cases of infections by the ingestion of raw oysters have occurred in the United States, and it has been reported that *V. vulnificus* causes half of cases of *Vibrio* infection[5]. Infection can also occur via eels and freshwater fish[10].

V. vulnificus is the most pathogenic *Vibrio* species, and the infection causes the necrosis of soft tissues of the extremities, sepsis, and shock, and may lead to death within a few days[5-9]. Infected patients are generally heavy consumers of alcohol or immunocompromised patients such as those with underlying liver diseases[5-9], but the cause remains unknown in some cases.

A case of infection occurred in Niigata in 2003. In this study, we performed ultramicromorphological analysis of the Niigata fulminant strain of *V. vulnificus* and compared the results with those of *V. cholerae* O1 and O139 or *V. parahaemolyticus*. We also investigated the drug susceptibility of the *V. vulnificus* strain.

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Abbreviations—CFU, colony forming unit; MIC, minimum inhibitory concentration.

Table 1. Laboratory findings of the *V. vulnificus*-infected patient

Arterial blood gas
pH 7.548, $PaCO_2$ 17.0 mmHg, PaO_2 75.7 mmHg, HCO_3 14.5 mEq/l, B.E. -4.6 mEq/l
Complete blood count
WBC 3,200 /mm^3, RBC 464×10^4 /mm^3, Hb 15.4 g/dl, Ht 46.5%, Plt. 12.3×10^4 /mm^3
Blood biochemistry
AST 119 IU/l, ALT 40 IU/l, ALP 320 IU/l, LDH 544 IU/l,
CPK 2784 IU/l, T. Bil 3.3 mg/dl, TP 5.8 g/dl, Na 125 mEq/l,
K 3.7 mEq/l, Cl 93 mEq/l, BUN 31.3 mg/dl, Cre 1.4 mg/dl,
CRP 24.99 mg/dl, HbA_1C 7.0%
Chest X-ray
In the left inferior lung, pleural effusion was observed.
Brain CT
In the bilateral occipital regions, low-density areas were observed. Around the bilateral cerebral ventricles, light low-density areas were observed.

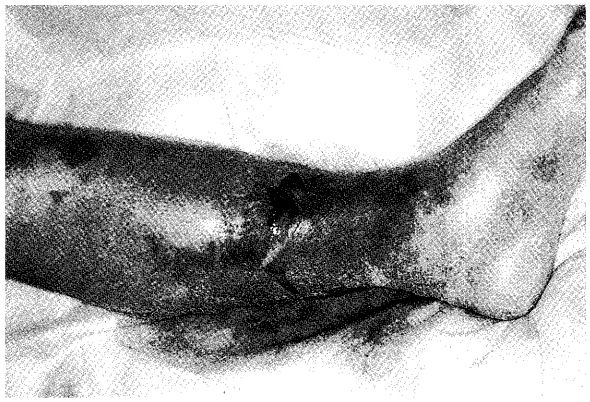

Fig. 1. The left lower leg of the *V. vulnificus*-infected patient showing necrosis of the soft tissues. The bilateral lower legs showed a slight swelling, and in particular, the left lower leg was dark red. *V. vulnificus* was isolated from the puncture aspirate collected from the left lower leg (shown in Figure), as well as from the venous blood.

METHODS

Case

Patient: A 76-year-old unemployed male.

Complaint: Consciousness disorder.

Medical history: At the age of 56 years, the patient developed diabetes mellitus. In addition, fatty liver and atrial fibrillation were indicated, but he refused treatment, and had discontinued consultations since 74 years of age. His family reported that the patient had a 1-year history of dark-skinned bilateral lower legs. The patient typically drank a large volume of alcohol daily.

Present illness: On August 3, 2003, systemic activities became slow in the evening, and the patient could not move. On August 4, delirium developed at noon, and the patient was transferred to the Emergency and Critical Care Medical Center, Niigata City General Hospital in the evening. The patient did not eat any raw marine products prior to the onset of symptoms.

Findings on initial consultation: Body temperature, 38.0°C; blood pressure, 105/70 mmHg; pulse, 150/min, irregular. The consciousness level was Japan Coma Scale (JCS) 20. The bilateral lower legs showed slight swelling, and in particular, the left lower leg was dark red. Neither purpura nor blood blister formation was observed. The consciousness level was reduced, and the presence or absence of pain was unclear.

Laboratory findings: The data are summarized in Table 1. The leukocyte and platelet counts were slightly decreased to 3,200/mm^3 and 123,000/mm^3, respectively. The creatine phosphokinase (CPK) level was increased to 2,786 IU/l. The C reactive protein (CRP) level was markedly increased to 24.99 mg/dl.

Bacteriologic examination: Prior to administration of an antibiotic, venous blood and puncture aspirate collected from the left lower leg were submitted for bacterial culture. The day after sample collection, gram-negative bacillus was detected, and *V. vulnificus* (strains NV1B and NV1L) was identified on August 6.

Differential diagnosis: Fever, tachycardia, the

increase in CRP, and decreases in the leukocyte and platelet counts suggested sepsis, and the clinical features of the lower legs suggested cellulites caused by bacteremia. Concerning causative bacteria, gram-negative bacillus was strongly suggested rather than *Streptococcus pyogenes* (causative gram-positive cocci of severe invasive streptococcal infections or group A streptococcal pharyngitis); however, the possibility of *V. vulnificus* was not considered. The increase in CPK suggested rhabdomyolysis.

Diagnosis: *V. vulnificus* was isolated from the venous blood and puncture aspirate collected from the left lower leg, and the patient was diagnosed as necrotizing soft tissue infection with *V. vulnificus*.

Treatment and course: After specimens for bacterial examination were collected, administration of imipenem/cilastatin (IPM/CS) (0.5 g/every 8 h) was started. Fever persisted, and dark red plaques with blood blisters appeared in the bilateral lower legs on August 6 (Fig. 1). We planned operative exploration and debridement, but informed consent could not be obtained from his family, and this could not be performed. On August 17, the patient died. There was no autopsy.

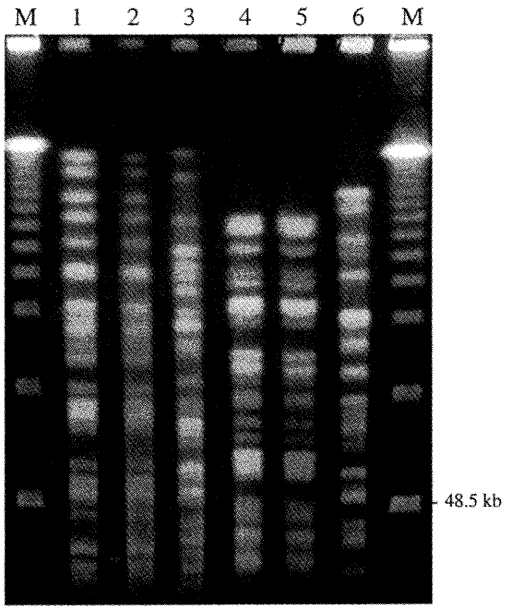

Fig. 2. DNA analysis of the *V. vulnificus* strains isolated from the venous blood and the puncture fluid of the lower leg of the patient. *V. vulnificus* DNA was analyzed by pulsed-field gel electrophoresis. Lanes: M, molecular size standards (lambda ladder); 1 and 4, strain NV1B isolated from venous blood; 2 and 5, strain NV1L isolated from the puncture fluid of the lower leg; 3 and 6, a *V. vulnificus* strain isolated previously in another area of Japan. DNA was digested with *Not*I in samples of lanes 1 to 3, and with *Sfi*I in in samples of lanes 4 to 6.

Media and bacterial growth

For bacterial growth, we used Luria-Bertani (LB) broth (Difco Laboratories, Detroit. Mich., USA) as a liquid medium which was inoculated and incubated at 37°C for 12-18 h with agitation. LB agar (Difco) and 5% sheep blood agar (Becton Dickinson, Tokyo) were used as solid media.

Pulsed-field gel electrophoresis

Total bacterial DNA was purified and subjected to pulsed-field gel electrophoresis, as previously described[11]. Briefly, bacterial DNA was digested with *Not*I or *Sfi*I, electrophoresed in 1.0% agarose (containing 50 μM thiourea) with the molecular size standards-lambda ladder (Bio-Rad, Hercules, CA, USA), and stained with ethidium bromide.

Antimicrobial agents

The antimicrobial agents were a gift from their manufacturers. They included: ampicillin (Meiji Seika, Tokyo) and piperacillin (Taisho Toyama Pharmaceutical Co., Tokyo) for penicillins; cefazolin (Fujisawa Pharmaceutical Co.), cefotiam (Takeda Chemical Industries, Osaka), cefmetazole (Sankyo Co., Tokyo), ceftazidime (Glaxosmithkline K. K., Tokyo), cefotaxime (Sigma, St. Louis, MO, USA), ceftriaxone, (Chugai Pharmaceutical Co., Tokyo), cefoperazone (Taisho Toyama Pharmaceutical Co.), cefpirome (Shionogi & Co., Osaka), and cefepime (Bristol-Myers Squibb Co., Tokyo) for cefems; imipenem (Banyu Pharmaceutical Co., Tokyo), panipenem (Sankyo Co.), meropenem (Sumitomo Pharmaceuticals Co., Osaka), and biapenem (Meiji Seika) for carbapenems; gentamicin (Schering-Plough K. K., Osaka), kanamycin (Meiji Seika), and streptomycin (Meiji Seika) for aminoglycosides; tetracycline (Wyeth K. K., Tokyo), doxycycline (Pfizer Pharmaceuticals), and minocycline (Wyeth K. K.) for tetracyclines; roxithromycin (Aventis Pharma, Tokyo), clarithromycin (Taisho Toyama Pharmaceutical Co., Tokyo), and azithromycin (Pfizer Pharmaceuticals Inc., Tokyo) for macrolides; ciprofloxacin (Bayer Yakuhin, Osaka) and levofloxacin (Daiichi Pharmaceutical Co., Tokyo) for quinolones; rifampicin (Daiichi Pharmaceutical Co.) and fosfomycin (Meiji Seika).

Table 2. MICs of antimicrobial agents for the *V. vulnificus* strains

Antimicrobial agent	MIC (μg/ml)
Penicilins	
Ampicillin	1
Piperacillin	0.5
Cephems	
(1st-generation)	
Cefazolin	8
(2nd-generation)	
Cefotiam	4
(3rd-generation)	
Cefmetazole	8
Ceftazidime	0.25
Cefotaxime	0.06
Ceftriaxone	0.015
Cefoperazone	0.5
(4th-generation)	
Cefpirome	0.5
Cefepime	1
Carbapenems	
Imipenem	0.06
Panipenem	0.06
Meropenem	0.008
Biapenem	0.015
Aminoglycosides	
Gentamicin	2
Kanamycin	8
Streptomycin	16
Tetracyclines	
Tetracycline	0.12
Doxycycline	0.06
Minocycline	0.03
Macrolides	
Roxithromycin	8
Clarithromycin	2
Azithromycin	0.12
Quinolones	
Ciprofloxacin	0.015
Levofloxacin	0.015
Others	
Rifampicin	0.12
Fosfomycin	16
Fosfomycin + G6P[a)]	8
Fosfomycin + G6P[b)]	4

a) MICs determined in the presence of 5 μg/ml of glucose-6-phosphate.
b) MICs determined in the presence of 50 μg/ml of glucose-6-phosphate.

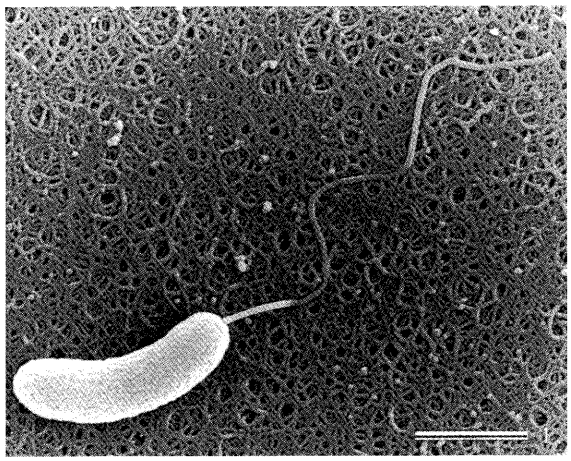

Fig. 3. Scanning electron micrograph showing *V. vulnificus* grown on blood agar plates. Comma-shaped *V. vulnificus*, possessing a capsular wrinkle-like structure and a polar monotrichous flagellum, is seen. The structure of strains NV1B and NV1L was essentially the same. Bar=1 μm.

Reagents

D-Glucose-6-phosphate disodium salt was purchased from Oriental Yeast Co. (Tokyo).

Susceptibility testing

Susceptibility testing of bacterial strains was done by the agar dilution method with Mueller-Hinton agar according to standard procedures[12,13]. The final concentrations of antimicrobial agents were from 0.002 to 128 μg/ml. The test bacteria were grown for 12 to 18 h at 35°C with agitation in LB broth, and diluted to approximately 10^6 CFU/ml. Aliquots of the bacterial suspension (approximately 10^4 CFU of bacteria per spot) were inoculated on the surface of antimicrobial agent-containing agar plates. Incubation was for 20 h at 35°C. The MIC was determined as previously described[12,13]. *E. coli* NIHJ JC-2 was used as a reference strain for quality control[13]. When the susceptibility to fosfomycin was tested, Mueller-Hinton agar supplemented with glucose-6-phosphate (at a concentration of 5 or 50 μg/ml) was also used, in addition to Mueller-Hinton agar alone[14].

Adherence to tissue culture cells

The adherence of *V. vulnificus* to the human intestinal cells (HCT-8 cells) were examined by a method described previously[15,16]. HCT-8 cells were grown on a plastic coverslip (diameter 13.5 mm, Sumitomo

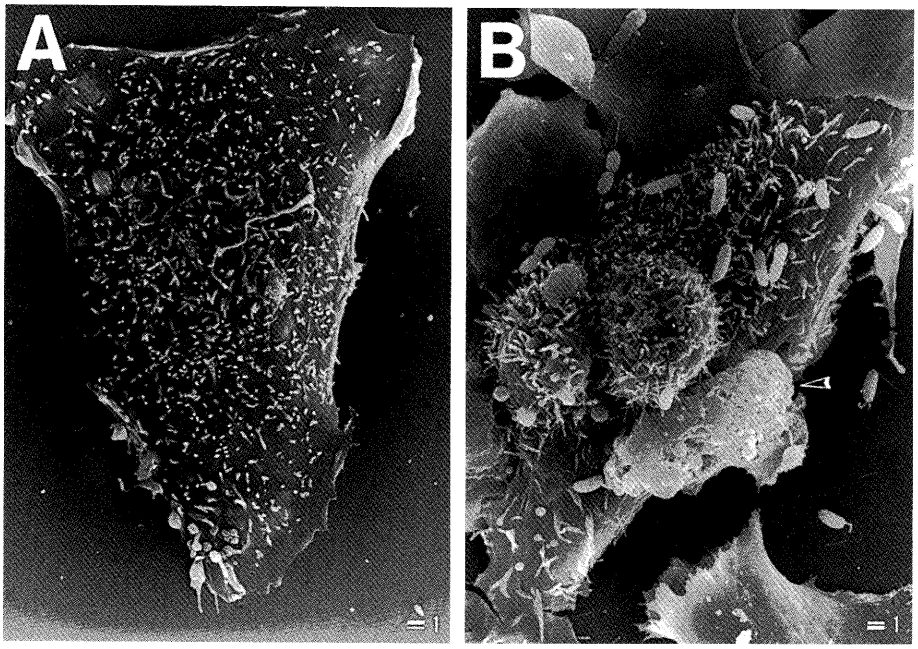

Fig. 4. Scanning electron micrographs showing adherence of *V. vulnificus* to HCT-8 cells. **A.** uninfected HCT-8 cells; **B.** HCT-8 cells infected with strain NV1B. A very similar adherence was also observed with strain NV1L. An arrowhead points to cell lesions caused by the *V. vulnificus* infection. Bars=1 μm.

Bakelite, Tokyo) at ~ 50% confluence in Eagle MEM (Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal bovine serum (FBS). The cells on a plastic coverslip in one ml of MEM supplemented with 6% FBS were incubated with 5 μl of bacterial cultures for 1 to 2 h at 37°C; in the case of 2 h incubation, the culture medium was changed 1 h after the initial incubation. The cells and bacteria on the plastic coverslips were washed with phosphate-buffered saline (PBS, pH 7.4).

Scanning electron microscopy

The washed plastic coverslips were fixed with 2.5% (vol/vol) glutaraldehyde in PBS (pH 7.4) for 2 h at room temperature and subsequently postfixed in 1% (wt/vol) osmium tetroxide for 1 h at 4°C. The samples were then dehydrated in acetone, critical-point dried, and coated with gold-palladium. The samples were finally analyzed by scanning electron microscopy[15,16].

RESULTS

DNA analysis of the *V. vulnificus* strains

The two *V. vulnificus* strains (NV1B and NV1L) isolated from the venous blood and puncture fluid of

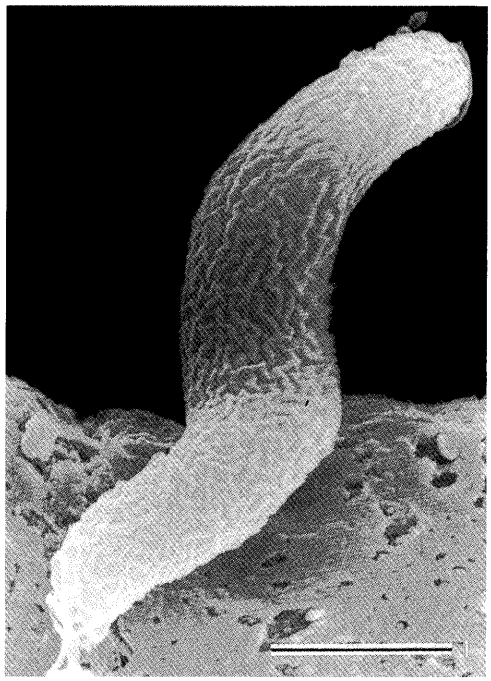

Fig. 5. Scanning electron micrograph showing adherent *V. vulnificus* on HCT-8 cells. Spiral-shaped *V. vulnificus,* possessing a capsular wrinkle-like structure on the bacterial cell surface, is seen. Bar=1 μm.

the lower leg of the *V. vulnificus*-infected patient were indistinguishable from each other when bacterial DNAs were digested with *Not*I and *Sfi*I and analyzed by pulsed-field gel electrophoresis (Fig. 2).

In vitro susceptibility to antimicrobial agents

The MICs of the antimicrobial agents against the *V. vulnificus* strains are summarized in Table 2. The MICs were essentially the same in each of the two strains. The strains were highly susceptible to many of the antimicrobial agents, especially to some of the 3rd-generation cephems, carbapenems, some of tetracyclines, and fluoroquinolones (MICs, ≤ 0.06 μg/ml). Among the antimicrobial agents tested, meropenem showed the greatest activity (MICs, 0.008 μg/ml). Among the tetracyclines, minocycline showed the highest activity (MICs, 0.03 μg/ml), and among the macrolides, azithromycin showed the highest activity (MICs, 0.12 μg/ml).

The strains were moderately resistant to the 1st- and the 2nd-generation cephems, kanamycin, streptomycin, and roxithromycin (MICs, ≥ 4 μg/ml). The strains were also moderately resistant to fosfomycin (MICs, 16 μg/ml) although the addition of glucose-6-phosphate to fosfomycin lowered the MIC values, when compared with fosfomycin alone.

Electron microscopic analysis of the *V. vulnificus* strains

V. vulnificus had a comma-shaped cell body, was enclosed in a capsular wrinkle-like structure, and had a polar monotrichous flagellum (Fig. 3).

When *V. vulnificus* was added to the HCT-8 culture, a marked adherence of *V. vulnificus* to the HCT-8 cells was observed (Fig. 4). A strong cytotoxic effect was also observed (Fig. 4B).

Occasionally, spiral-shaped *V. vulnificus* adhering to the HCT-8 cells was observed (Fig. 5). The capsule-like wrinkles were also obvious on the bacterial cell surface.

DISCUSSION

V. vulnificus causes infection via the oral ingestion of contaminated sea fish and shellfish, or via injury. Infection can also occur via eels and freshwater fish[5–9]. Infected patients are generally men[17], and heavy consumers of alcohol or immunocompromised patients such as those with underlying liver diseases[5–9]. The infection subsequently causes the necrosis of soft tissues of the extremities, sepsis, and shock, and may lead to death within a few days[5–9]. In Japan, more incidences occur on the Western side of the Japan Sea[1–3].

V. vulnificus infections also occur occasionally in Niigata. A previous report in Niigata was made in 2000[2]. The patient was a 60-year-old male who had ingested raw oysters before the infection, and ultimately died. Although *V. vulnificus* was isolated from his blood culture, the Niigata strain of *V. vulnificus* was not available for this study.

In the present *V. vulnificus* infection case in Niigata, the patient was a 76-year-old male who had a medical history of diabetes, and was a heavy consumer of alcohol. He did not eat raw fish or shellfish before the development of the symptoms, and the source of infection was unknown. The patient reported a disturbance in consciousness, and a mild swelling of the lower leg was observed. The same *V. vulnificus* strains, as evidenced by pulsed-field gel electrophoresis, were isolated from the venous blood and puncture fluid of the lower leg. In this patient, white blood cells and platelets were decreased.

The *V. vulnificus* strains were highly susceptible to carbapenems. The patient was treated with imipenem (IPM/CS), but succumbed, suggesting that the administration of antimicrobial agents during the early phase after infection is important. The *V. vulnificus* strains were also highly susceptible to the 3rd-generation cephems, tetracyclines, and fluoroquinolones. With respect to the macrolides, the strains were most susceptible to azithromycin.

The MICs of fosfomycin to the *V. vulnificus* strains were at a moderate level and were lowered by adding glucose-6-phoaphate (an inducer of the hexose phosphate transport [UhpT] system)[14]. There is a possibility that fosfomycin is transported into the bacterial cells mainly by the UhpT system in *V. vulnificus*.

In this study, we performed an ultramicromorphological analysis of the cellular structure of the Niigata fulminant strain of *V. vulnificus*. On comparison with other *Vibrio* species (*V. cholerae* O1 or O139[18,19], and *V. parahaemolyticus*[20]), polar monotrichous flagella and a comma-like curving bacterial body are common features. In contrast, capsule-like wrinkles seen on the bacterial cell surface, or spiral-shaped bacteria (which resembled *Helicobacter pylori*[21]) were not observed in *V. cholerae* O1 or O139 and in *V. parahaemolyticus*. These may be specific to *V. vulnificus*, and may be a cause, at least in part, of the fulminant development of the symptoms.

It has been reported that *V. vulnificus* capsules confer phagocytosis resistance and serum resistance on its bacteria[22–27]. *V. vulnificus* also exhibits a strong hemolytical activity by releasing multiple

proteases[28-30]. However, little is known about the early infection mechanism of *V. vulnificus*, particularly the adherence mechanism as well as the cellular invasion mechanism. In this study, we demonstrated that the *V. vulnificus* strains possess a strong ability to adhere to and impair HCT-8 cells. The strong cytotoxic effect may be due to multiple *V. vulnificus* proteases.

V. vulnificus inhabits seawater as *V. parahaemolyticus*, having no chance to contact with antimicrobial agents, and thus, no serious drug-resistance has been noted[31] (this study). Accordingly, antibiotic therapy is expected to exert a sufficient effect during the early phase of infection. However, many cases of *V. vulnificus* infection have resulted in death, most likely due to a failure to discover it during the early phase of infection. Establishment of a rapid diagnosis is urgently needed.

Acknowledgments. This work was supported by an International Cooperation Grant, Japan.

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