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A Detection Method for *Vibrio vulnificus* Using Monoclonal Antibodies

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Monoclonal antibodies were prepared in order to develop an assay method for *Vibrio vulnificus*. Sixteen mouse hybridoma cell lines were established by immunization of whole cell antigen to BALB/c mice, fusion with SP2/O myeloma cells, and cloning. Most of them secreted IgM λ antibodies. A sandwich enzyme-linked immunosorbent assay was developed with rabbit anti-*V. vulnificus* polyclonal antibodies as capture antibody, an IgM monoclonal antibody as detector antibody, and goat anti-mouse IgM-alkaline phosphatase conjugate as developer antibody. The range of detection was 10^4 to 10^7 *V. vulnificus* cells per microplate well. When four related *Vibrio* species were tested for cross-reactions, *V. parahaemolyticus* showed 3.5% reactivity and *V. carchariae*, *V. fluvialis*, and *V. furnisii* showed negligible (<1%) cross-reactivity.

Key words: *Vibrio vulnificus*, monoclonal antibody, sandwich ELISA.

Vibrio vulnificus is a human pathogen which has been implicated in both primary septicemia and wound infections (1, 20). The organism is indigenious to estuarine environments (5, 6, 11, 12), and its infection generally results from ingestion of raw seafood or exposure of wounds to seawater contaminated with the bacterium. People with suppressed immune responses or those with elevated serum iron levels by certain underlying chronic diseases are especially vulnerable to the infection (7). Primary septicemia infections are characterized by a rapid progression to secondary cutaneous lesions and necrotic ulcers of the extremities (13). The first sign of illness often occur less than 24 hours after ingestion and death may follow within 1 to 2 days, with mortality rate exceeding 50% (7). The mortality rate of wound infections is reported to be approximately 20% (13).

The sea-food industry is threatened because of the public health concerns over the rapid progress and high fatality of *V. vulnificus* infection, especially in the summer when the incidence of infection increases (8, 14). Since standard identification scheme for *V. vulnificus*, utilizing cultural and biochemical procedures, takes many days (2), development of a rapid detection method for this pathogen is becoming increasingly important. There has been much research toward developing rapid and specific detection methods for *V. vulnificus* in clinical and environmental samples (2, 9, 10, 17, 18). Immunoassay utilizing monoclonal antibody could be an ideal method for the detction of *V. vulnificus* due to its high specificity, rapidity and simplicity.

In the present study, reagent monoclonal antibodies to *V. vulnificus* were produced and a sandwich ELISA procedure using one of the prepared antibodies was developed to detect *V. vulnificus*.

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Materials and Methods

Bacterial culture

V. vulnificus ATCC 27562 was obtained from the American Type Culture Collection, Rockville, Md. and *V. fluvialis* (ATCC 33809), *V. furnissii* (ATCC 35016), *V. parahaemolyticus* (ATCC 33844) and *V. carchariae* (NCIMB 2717) were obtained from the Korean Collection for Type Cultures, Daejon, Korea.

Stock cultures were grown on heart infusion agar (Difco, Detroit, Mich.) slants at 30°C. Long-term storage of Vibrio species was achieved by adding sterile glycerol, to a final concentration of 25% (vol/vol), to log-phase cultures and then freezing each in 1.5 ml ampules at -70°C. Cell numbers were enumerated by either plate count on heart infusion agar plate or turbidity at 602 nm.

Preparation of whole cell antigen

V. vulnificus ATCC 27562 was cultured in heart infusion broth in a 30°C shaking water bath for 24

hours. Then the cells were collected by centrifugation at 10,000×g for 20 min. Cells were resuspended in phosphate-buffered saline (PBS, pH 7.3) and treated with 1% formaldehyde for 30 min at room temperature. The treated cells were washed three times with PBS, resuspended in PBS to approximately 10^{10} cells per ml, and stored at 4°C until used.

Preparation of polyclonal antibody

New Zealand White rabbits were immunized subcutaneously with 10^9 whole cell antigens in Freund's complete adjuvant (Sigma, St. Louis, Mo.). At 3 week intervals, the rabbits were boosted twice subcutaneously with the same amounts of antigen in Freund's incomplete adjuvant (Sigma). On the seventh day after the 3rd injection, rabbit blood was collected by heart puncture.

Rabbit immune serum was fractionated by 45% ammonium sulfate saturation. After dialysis against 10 mM Tris-HCl, pH 8.6, the antibody sample (370 mg protein) was applied on a DEAE Sephacel column (3.0×6 cm). The column was eluted initially with 10 mM Tris-Cl, pH 8.6 and then with a linear NaCl gradient (0 to 0.5 M) in the same buffer. Two ml fractions were collected. Absorbance at 280 nm and anti-*V. vulnificus* antibody concentration of each fraction were determined.

Preparation of monoclonal antibody

Whole cell antigens (10^8) suspended (1:1) in Freund's complete adjuvant were injected intraperitoneally into 5-week-old female BALB/c mice. After three weeks, the mice were injected with the same amount of whole cell antigen in Freund's incomplete adjuvant. On the seventh day after the 2nd immunization, antibody titers of the mice sera were tested. Three weeks after the 2nd immunization, whole cell antigen was injected intravenously into a high titer mouse. On the third day after the final injection, fusion was carried out with mouse spleen cells and myeloma cells.

Fusion was carried out by using the protocol of Hamilton and Davis (4) with some modifications. The spleen of immunized mouse was removed under sterile conditions, placed in Dulbecco's Modified Eagle (DME, GibcoBRL, Grand Island, NY) medium, cut into pieces, and sieved through a sterile metal mesh to obtain a fine cell suspension. The non-immunoglobulin-secreting Sp2/O-Ag14 cells (2×10^7) were mixed with 10^8 spleen cells. The cell mixture was centrifuged at 150×g for 10 min, and the supernatant discarded. A 1 ml sterilized 50% (vol/vol) polyethylene glycol 1450 (Sigma) in DME medium was prepared on the day of fusion. It was slowly added to the cell mixuture with gentile shaking for 1 min and the cell pellet was dispersed slowly for an additional 2 min at 37°C. The polyethylene glycol was diluted by slow addition of 30 ml of DME medium over a 15 min period. The cells were collected by centrifugation at 150×g for 5 min and resuspended in 100 ml of prewarmed post-fusion medium containing 15% fetal bovine serum (HyClone, Logan, Utah), 10% hybridoma cloning supplement (Boehringer Mannheim, Mannheim, Germany), 0.5% Nutridoma-SP (Boehringer Mannheim) and 0.6% lipopolysaccharide (Sigma) in DME medium. The cells were dispensed in 150 µl aliquots into 96-well microplates. They were incubated at 37°C with 5% CO_2. After 24 hours, 75 µl of hypoxanthine-aminopterin-thymidine medium (Sigma) was added to each well. After 4 days, medium was replaced with hypoxanthine-thymidine (HT) medium and the plates were observed for growth of hybridoma cells. The cells were fed every 3 day. Supernatants in hybridoma growing wells were screened by ELISA for anti-*V. vulnficus* antibodies.

Cloning was carried out at a cell density of 5 cells per ml in HT medium by the limiting dilution method (3), resulting in approximately 0.8 cells per well.

Eight-week-old female BALB/c mice were injected with 0.5 ml of pristane (Sigma). After 7 days, the mice were injected intraperitoneally with 10^7 hybridoma cells. Production of ascites was monitored daily starting 5 days after the injection of hybridoma cells. Ascitc fluid was collected with 18-gauge needle, centrifuged at 150×g for 10 min, and stored in the presence of 0.02% (w/vol) sodium azide at 4°C.

T1E4 monoclonal antibody was partially purified from ascites fluid for use in the sandwich ELISA. After fractionating with 45% ammonium sulfate saturation of the T1E4 ascites fluid, the antibody sample (180 mg protein in 3 ml) was applied on a Sepharose 6B column (1.5×90 cm). The column was eluted with PBS.

ELISA for anti-*V. vulnificus* antibodies

Assay of anti-*V. vulnificus* antibodies in culture supernatant was carried out by the following procedure. ELISA plates were coated with 100 µl of *V. vulnificus* whole cell antigen (2×10^8 cells/ml, A602=0.2) at 4°C overnight. The plates were blocked with 250 µl of blocking solution (1% bovine serum albumin [BSA, Sigma], 0.05% Tween 20 in PBS) at room temperature for 90 min and were washed three times with a wash solution of 0.1% Tween 20 in PBS. One hundred µl of hybridoma supernants

were then added to the plates and incubated for 90 min. After 3 washes, 100 µl of 1:2,000 diluted goat anti-mouse polyvalent immunoglobulins-alkaline phosphatase conjugate (Sigma A-0162) in blocking solution was added to each plate wells. After 90 min of incubation and washing, 100 µl of p-nitrophenyl phosphate substrate (NPP, Sigma) in diethanolamine buffer (915 mM diethanolamine, 0.24 mM $MgCl_2$, pH 9.8) was added to each well. After 30 min, the optical densities were read at 405 nm on a microplate reader (Metertek, Taipei, Taiwan). Mouse immune serum and culture medium were used as positive and negative controls, respectively.

For the determination of anti-*V. vulnificus* antibodies in purified samples, the same procedure was applied except for the use of working buffers as negative controls.

In the assay of anti-*V. vulnificus* antibodies in rabbit serum, goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma A-3811) was used as enzyme conjugate.

Isotyping of MAbs

The isotype of each monoclonal antibody was determined by the use of a mouse-hybridoma subtyping kit (Boehringer Mannheim) and comfirmed by Ouchterlony double diffusion with isotype specific goat anti-mouse immunoglobulins (Sigma ISO-2).

Sandwich ELISA for *V. vulnificus*

A sandwich ELISA procedure was developed for the detection of *V. vulnificus*. ELISA plates were coated with 100 µl of rabbit polyclonal antibodies (10 µg protein/ml) in PBS at 4°C overnight. Then the plates were incubated with 250 µl of blocking solution (1% BSA, 0.05% Tween 20 in PBS) in each well for 60 min. After washing three times with wash solution (0.1% Tween 20 in PBS), appropriate dilutions of *V. vulnificus* cells were added to the plate wells and incubated for 60 min. After washing, 100 µl of T1E4 monoclonal antibody (0.16 µg protein/ml) was added to each well and incubated for 60 min. The plates were washed again and 100 µl of goat anti-mouse IgM-alkaline phosphatase conjugate (Sigma A-9688), diluted 1:1,000 in blocking solution, was added to each well. After a 60 min incubation, unreacted enzyme conjugate was removed from the wells by washing. Finally, 100 µl of NPP substrate solution was added to each well and the plates were incubated for 30 min. Optical densities were read at 405 nm. All the incubations were carried out at room temperature unless otherwise specified.

Cross-reactions among several *Vibrio* species were determined. *Various* numbers of *V. parahaemolyticus, V. carchariae, V. fluvialis*, and *V. furnisii* were added to the plates and compared with the signal of *V. vulnificus* in sandwich ELISA.

Results and Discussion

Preparation of monoclonal and polyclonal antibodies to *V. vulnificus*

Our procedure for the hybridoma cell production was modified from that of Hamilton and Davis (4). It was very successful. One splenocyte-myeloma fusion produced about 1,000 hybridoma-growing wells. The growth of hybridoma cells were observed in 4 to 5 days after fusion. The hybridoma cloning supplement in post-fusion medium supported the growth of hybridoma cells without the help of feeder cells and the lipopolysaccharide appeared to encourage rapid cell growth (data not shown). About 30% of wells with growing hybridomas were found to produce antibodies reacting with *V. vulnificus* ATCC 27562, the immunogen. Positive hybridomas were further screened by cross-reactivity with non-*V. vulnificus* species. Cells showing high cross-reactivity were discarded. And 16 hybridoma cell lines were cloned by limiting dilution. Most of the established cell lines produced IgM, λ antibodies (Table 1). They were injected into peritoneal cavities of BALB/c mice and ascites fluid were collected. Monoclonal antibody T 1E4 was partially purified from ascites fluid by ammonium sulfate precipitation and gel filtration with Sepharose 6B column (Fig. 1).

Rabbit immune serum was prepared by three

Table 1. Isotypes of monoclonal antibodies

Antibody name	Isotype	
	Heavy chain	Light chain
P3A3	IgG1	Kappa
3T2E6	IgG2b	Kappa
H3H5	IgM	Lambda
T1E5	IgM	Lambda
3T1C4	IgM	Lambda
3T2D1	IgM	Lambda
H1C10	IgM	Lambda
H1G3	IgM	Lambda
H3F10	IgM	Lambda
3T3B5	IgM	Lambda
H3E1	IgM	Lambda
P2F1	IgM	Lambda
T2B5	IgM	Lambda
H3G7	IgM	Lambda
T2C3	IgM	Lambda
T1E4	IgM	Lambda

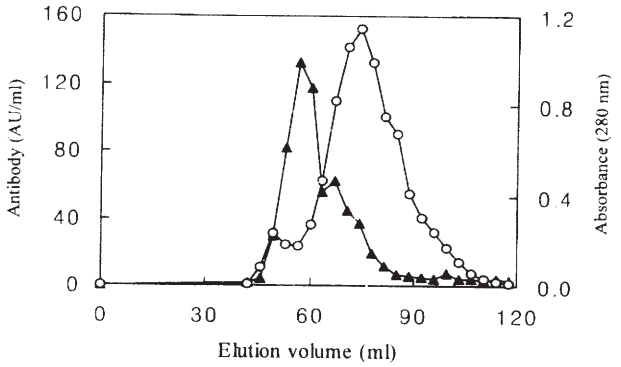

Fig. 1. Gel filtration of T1E4 monoclonal antibody. Ammonium sulfate cut of ascites fluid was processed through Sepharose 6B column. Symbols: O, absorbance at 280 nm; ▲, concentration of anti-*V. vulnificus* antibody (arbitrary units/ml).

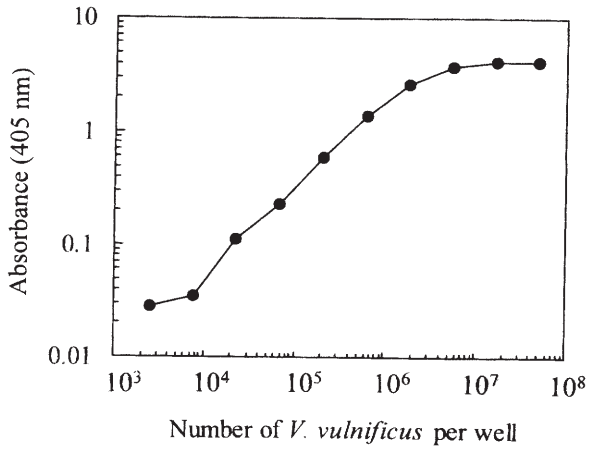

Fig. 2. Detection range of *V. vulnificus* by sandwich ELISA. Mean values of four readings are taken.

sequential immunizations with *V. vulnificus* whole cell antigen. After the third immunization, the antibody titer was detectable at a dilution of 1:10,000. Partially purified polyclonal antibodies were obtained from rabbit serum by ammonium sulfate precipitation and passage through DEAE-Sephacel column

Sandwich ELISA

A sandwich ELISA procedure was developed for the detection of *V. vulnificus*. Rabbit anti-*V. vulnificus* polyclonal antibodies, T1E4 IgM monoclonal antibody, and goat anti-mouse IgM-alkaline phosphatase conjugate were used as capture antibody, detector antibody, and developer antibody, respectively. Working concentration of each antibody solution in the sandwich ELISA was adjusted to be saturating by titration of each antibody solution over wide concentration range. Incubation times for all steps were standardized to 1 hr in consideration of signal quality and time efficiency. The limit of detection was 10^4 *V. vulnificus* ATCC 27562 cells, with saturation achieved at 10^7 cells (Fig. 2).

Cross-reactions among *Vibrio* species

Cross-reactions among related *Vibrio* species were tested with the sandwich ELISA. When 10^7 cells of related *Vibrio* strains were added to coated microplate wells and compared with *V. vulnificus* cells in the sandwich ELISA, *V. parahaemolyticus* showed 3.5% cross-reactivity while *V. carchariae, V. fluvialis,* and *V. furnisii* showed negligible (<1%) cross-reactivity (Table 2). This low cross-reactivity was a prerequsite for establishing a method for detecting *V. vulnificus*.

This sandwich ELISA technique to identify *V. vulnificus* has many advantages over the lengthy and labor-intensive standard cultural and biochemical assays. It does not involve culture time nor sophisticated equipments. With coated microplates, the procedure can be finished in 4.5 hours. The sensitivity of this assay could be enhanced if better hybridomas secreting high affinity antibodies were produced. Generally IgG antibodies have higher affinity than IgM antibodies (4). We are currently trying to produce high affinity anti-*V. vulnificus* IgG-secreting hybridoma cells. Other procedures to improve assay sensitivity could include using enzyme cycling reaction (15, 16) or chemiluminescent sub-

Table 2. cross-reactivity among *Vibrio* species

Strain	Cell number per well	Absorbance (405 nm)	Crossreactivity (%)[a]
V. vulnificus	10^7	1.5	100
	10^6	0.96	100
	10^5	0.21	100
	10^4	0.036	100
V. parahaemolyticus	10^7	0.63	3.5
	10^6	0.15	5.0
	10^5	0.060	10
	10^4	–	–
V. carchariae	10^7	0.077	0.17
	10^6	–	–
	10^5	–	–
	10^4	–	–
V. fluvialis	10^7	0.20	0.90
	10^6	–	–
	10^5	–	–
	10^4	–	–
V. furnisii	10^7	0.087	0.19
	10^6	–	–
	10^5	–	–
	10^4	–	–

– undetectable.
[a]Cross-reactivity was the ratio of estimated cell number on *V. vulnificus* standards to actual cell number.

strate (19) in the developing reaction of the ELISA. Also direct chemical coupling of alkaline phosphatase to detector antibody could increase the assay sensitivity, in addition to shortening the assay time by omitting the developer antibody step in the ELISA procedure. To increase the specificity of the sandwich ELISA, use of monoclonal antibodies as capture antibody instead of polyclonal antibodies could be attempted. But using monoclonal antibodies as capture antibody was unsuccessful in our trial due to low reactivities of our monoclonal antibodies.

The extremely rapid progress and high mortality of *V. vulnificus* septicemia emphasizes the need to detect contaminated food before consumption and to identify rapidly the infecting agent in septicemia patients. This sandwich ELISA procedure can be utilized as a simple detection method for *V. vulnificus*.

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