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Enumeration of *Vibrio vulnificus* in Natural Samples by Colony Blot Hybridization

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Abstract Colony blot hybridization using a VVHP DNA probe derived from the sequence of the hemolysin gene, *vvhA*, was specific in identifying all *V. vulnificus* strains, thereby, eliminating the need for any additional phenotypic identification. The colony blot hybridization procedure revealed a sensitivity and broad applicability sufficient for the direct enumeration of *V. vulnificus* in various natural samples, without the use of enrichment or culturing on selective media. *V. vulnificus* was detected in all natural samples collected during August and May at concentrations ranging from 2.1×10^1 to 4.0×10^3 organisms per ml. However, during November and February, when the mean temperatures of the seawater were 12°C and 5°C, respectively, *V. vulnificus* was not detected in any natural samples.

Key words: *Vibrio vulnificus*, enumeration, colony blot hybridization

The pathogenic marine bacterium *Vibrio vulnificus* can be isolated from seawater and estuarine environments [13, 20, 24, 31]. It also occurs in raw seafoods, including oysters, arkshells, small octopi, and fish, harvested from coastal waters [7, 14, 15, 32]. *V. vulnificus* has been identified as the causative agent for food-born diseases, such as gastroenteritis, and life-threatening septicemia in immuno-compromised individuals. It has also been reported that wound infections have resulted from exposure to seawater or from the handling of shellfish contaminated with the organism. Mortality from septicemia is quite high (exceeding 50%), and death may occur rapidly within 1 to 2 days after the first signs of illness [2, 19, 29]. Therefore, the rapid identification of *V. vulnificus* in seafoods is essential to reduce the potentially fatal effects of widespread consumption of seafoods contaminated with the organism.

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The current techniques for identifying *V. vulnificus* from environmental samples are enrichment with, and/or plating on, specific selective media. Using selective media has been known to yield a decreased detection sensitivity of a specific natural population due to the antibiotics or chemical compounds added to suppress the growth of background microorganisms. Another limitation is that the enrichment causes competition with more vigorously growing organisms in a mixed population, and may result in the overgrowth of unwanted bacteria, and thus alter the population structure of the microbial community. Therefore, it renders the detection of sparsely distributed microorganisms to be difficult [25, 28], and to develop a method that does not involve conventional laboratory culturing on selective media would be advantageous for detecting such microorganisms. The detection method developed should be sensitive and specific enough to identify low numbers of *V. vulnificus* against a large background of other prokaryotic and eukaryotic cells in natural samples.

Several detection and enumeration methods based on the application of molecular biological techniques have been developed for monitoring specific microorganisms in environmental samples [4]. Among them, the polymerase chain reaction (PCR), which has the ability to amplify unique DNA sequences, can provide a very specific, explicit, and rapid detection and identification of microbial species in food and dairy products [4, 26]. The broad application of PCR procedures for very specific and sensitive identification of *V. vulnificus* in diverse seafoods or natural samples has already been demonstrated [1, 3, 6, 10, 14, 15]. However, PCR procedures are not usually appropriate for an enumeration or quantitative analysis of a bacterial population in natural samples.

In comparison to the substantial body of literatures cited above for the identification of *V. vulnificus*, only a few studies on the development of procedures for their enumeration from seafoods or natural samples have been reported [9,

32, 33]. Most nucleic acid hybridization procedures have been developed and optimized for the detection of *V. vulnificus* in oysters by employing an oligonucleotide or DNA probe, which can hybridize to the DNA sequence unique to the pathogen [32, 33]. However, broader application of the procedure for the enumeration of *V. vulnificus* in other seafoods has not been demonstrated. Besides oysters, small octopi (*Octopus variabilis*), thin-shelled surf clams (*Ruditapes philippinarum*), and blue crabs (*Portunus trituberculatus*) are also popular in Korea and Japan [11, 12], and are often consumed raw or undercooked. As a result, an increasing number of outbreaks of fatal septicemia due to the consumption of these seafoods have been reported in Korea (J. H. Rhee, personal communication). Accordingly, developing a procedure with an improved general suitability is needed for extensive use as a diagnostic tool to enumerate *V. vulnificus* in different types of samples.

Information on the accurate population size of *V. vulnificus* in raw seafoods is important to predict the population structure and/or population activity of the pathogen present at the stage of consumption. In the production of seafoods, this information will provide valuable guidelines for designing the processing and the quality control strategies to eliminate the pathogen. The present study introduces a colony blot hybridization method for the rapid identification and enumeration of *V. vulnificus* in natural samples, including various raw seafoods, without any enrichment or cultivation on selective media.

MATERIALS AND METHODS

Bacterial Strains

A total of 20 strains of *Vibrio* spp. used to assess the specificity of the VVHP DNA probe, and their origins, are listed in Table 1. Among the *Vibrio* strains, *V. vulnificus* ATCC29307 was used to determine the sensitivity of the DNA probe. Halophilic *Vibrio* species were grown on Luria-Bertani (LB) plates or in LB broth containing 2% NaCl (LBS) at 30°C, and other strains were grown on LB plates or in LB broth (0.5% salt).

Table 1. Designations, sources, and relevant characteristics of *V. vulnificus* isolates analyzed by colony blot hybridization.

Designation	Species and Strain		Reference or Source	Hybridization with *vvhA*[a]
A1	*Vibrio vulnificus*	ATCC[b]29307		+
A2	*Vibrio vulnificus*	ATCC27562		+
A3	*Vibrio vulnificus*	CDC[c] C7184		+
A4	*Vibrio vulnificus*	CDC H3308		+
A5	*Vibrio vulnificus*	CNUH94-3	Clinical isolate from Chonnam National University Hospital	+
B1	*Vibrio vulnificus*	CNUH94-8	Clinical isolate from Chonnam National University Hospital	+
B2	*Vibrio vulnificus*	CNUH95-1	Clinical isolate from Chonnam National University Hospital	+
B3	*Vibrio vulnificus*	WK2	Clinical isolate from Wonkwang University Hospital	+
B4	*Vibrio vulnificus*	WK3	Clinical isolate from Wonkwang University Hospital	+
B5	*Vibrio vulnificus*	WK6	Clinical isolate from Wonkwang University Hospital	+
C1	*Vibrio vulnificus*	WK15	Clinical isolate from Wonkwang University Hospital	+
C2	*Vibrio vulnificus*	WK18	Clinical isolate from Wonkwang University Hospital	+
C3	*Vibrio vulnificus*	CS9133	Seawater isolate	+
C4	*Vibrio vulnificus*	V-15	Seawater isolate	+
C5	*Vibrio vulnificus*	V-16	Seawater isolate	+
D1	*Vibrio alginolyticus*	ATCC17749		-
D2	*Vibrio cholerae Inaba*	ATCC9459		-
D3	*Vibrio cholerae*	ATCC14033		-
D4	*Vibrio furnissii*	ATCC35016		-
D5	*Vibrio parahaemolyticus*	ATCC27519		-

[a]Determined from colony blot hybridization analysis using ^{32}P-labeled DNA probe as indicated.
[b]American type culture collections.
[c]Center for disease control and prevention.

Enzyme and Chemicals

The *Taq* polymerase and deoxynucleotide triphosphates (dNTPs) were purchased from Bioneer Co., Seoul, Korea, and were used as suggested by the supplier. The DNA-modifying enzymes were used as recommended by the supplier (Promega, Madison, WI, U.S.A.). The reagents for the media were purchased from Difco (Detroit, MI, U.S.A.) and the chemicals were from Sigma (St. Louis, MO, U.S.A.) at the highest purity available.

PCR Amplification and Labeling of DNA Probe

Two *V. vulnificus*-specific primers were synthesized by the Bioneer Co. (Seoul, Korea) to amplify the internal sequence of the *vvhA* region encoding the cytotoxin-hemolysin of *V. vulnificus*. The two 20-base primers, Choi-1 (5'-GACTAT-CGCATCAACAACCG-3', sense primer) and Choi-2 (5'-AGGTAGCGAGTATTACTGCC-3', antisense primer) were located within the open reading frame and were expected to generate a 704-base pair (bp)-long DNA fragment using a PCR. The PCR amplifications were carried out in a DNA thermal cycler (Perkin Elmer, GeneAmp, PCR System 2400, Norwalk, CT) using the Choi-1 and Choi-2 oligonucleotide primers and *Taq* DNA polymerase. The PCR reaction mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM of each dNTPs, 1.0 μM of each primer, and 2 units of *Taq* polymerase per 100 μl. The genomic DNA of *V. vulnificus* ATCC29307 was isolated as outlined by Sambrook *et al.* [27], and about 100 ng of extracted genomic DNA was added as the template DNA. Thirty cycles of amplification of the target sequence in the template DNA were conducted with an initial denaturation at 94°C for 2 min, and a post-amplification extension at 72°C for 10 min. Each cycle consisted of 1 min at 94°C, 1 min at 60°C, and 3 min at 72°C. The PCR-amplified DNA was confirmed using gel electrophoresis [27].

The DNA was labeled with [α-^{32}P]dCTP using random priming techniques [8] according to the manufacturer's specifications (Prime-a-gene labeling system, Promega, Madison, WI, U.S.A.). Any unincorporated free [α-^{32}P]dCTP was removed by column filtration (Sephadex G-50, Pharmacia, Piscataway, NJ, U.S.A.) and the resulting labeled DNA was used as the probe, and named as VVHP.

Determination of Specificity and Sensitivity of VVHP DNA Probe

To determine the specificity of the VVHP probe, each colony of *Vibrios* on LBS plates was lifted and transferred to nitrocellulose membranes (Schleicher and Schull, Keene, NH, U.S.A.). The cells were lysed, the liberated DNA was then denatured, neutralized, and washed using methods as described previously [27], with a slight modification. Briefly, the membrane was transferred and incubated on 3MM paper filters (Whatman, Maidstone, U.K.) saturated with a series of solutions for 5 min at room temperature for each step; 10% SDS (sodium dodecyl sulfate) for lysis of the cells, 0.5 N NaOH plus 1.5 M NaCl for denaturation, 0.5 M Tris-HCl (pH 7.4) plus 1.5 M NaCl for neutralization, and 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for washing. The DNA was fixed to the membrane by UV cross-linking for 10 sec at 0.120 Joules (Vilber Laurmat BLX-254, France) and hybridized with the VVHP DNA probe. The prehybridization and hybridization solutions consisted of 6× SSC, 5× Denhardt's reagent [27], 0.5% (w/v) SDS, and 100 mg/ml of denatured, fragmented salmon sperm DNA (Pharmacia, Piscataway, NJ, U.S.A.). The prehybridization and hybridization were carried out for 2 h and 16 h, respectively, at 65°C. After hybridization, the membranes were washed at room temperature for 2 h in 0.1× SSC-0.4% SDS and then at 68°C for 1 h in the same solution. The blot was exposed using a phosporimage analyzer (Fuji Photo Film Co. LTD, BAS1500 model, Tokyo, Japan).

For the sensitivity determination, an exponentially growing *V. vulnificus* ATCC29307 culture was serially diluted in sterilized artificial seawater (ASW) [18] while each dilution ranging from 10^3 to 10^7 colony forming units (CFU)/ml was loaded on a nitrocellulose membrane with a Bio-Dot microfiltration apparatus (Bio-Rad, Hercules, CA, U.S.A.). After filtration, the cells remaining on the surface of the membrane were hybridized using the same procedure as described above. The concentrations of *V. vulnificus* cells in each dilution were determined by counting the CFU, and aliquots (100 μl) of the serially diluted *V. vulnificus* ATCC29307 cultures were plated on LBS plates and incubated at 30°C for 24 h.

Collection and Preparation of Natural Samples

Four natural seafoods such as oysters, blue crabs, small octopi, and thin-shelled surf clams, as well as sedimented seawater samples, were obtained in February, May, August, and November 1998, from a single site on the southwestern coast of Korea. Individual samples in plastic bottles were placed in ice, and transported to the laboratory within 2 h of collection.

On arrival, 1 g of each seafood sample was added to 9 ml of sterilized ASW at 5°C, and homogenized. The microbial cells from the seawater were diluted by adding 1 ml to 9 ml of ASW. One gram of sediment was used to separate the microbial cells by suspending it in 9 ml of sterilized ASW and vortexing vigorously.

Enumeration using Viable Counts and Colony Blot Hybridization

The samples prepared as above were 10-fold serially diluted in sterilized ASW. The diluted samples (100 μl) were then spread on nonselective LBS plates, incubated at 30°C for 24 h, and the colonies that appeared were counted to determine the number of CFU of total culturable heterotrophic bacteria. For colony blot hybridization, the colonies on the

LBS plates were lifted and transferred to nitrocellulose membranes (Schleicher and Schull, Keene, NH, U.S.A.), then the hybridizations were processed as indicated above to determine the specificity and sensitivity. The probe-positive signals (dark blots) that appeared on the autoradiogram were counted to determine the concentrations of *V. vulnificus* in the natural samples; the results are reported as the number per ml of undiluted sample (ca. equivalent to 1 g of seafood and sediment). The mean number of CFU or blots was determined from duplicate counts and three trials for all experiments.

RESULTS AND DISCUSSION

Specificity of VVHP DNA Probe

V. vulnificus produces a cytotoxin-hemolysin. Although the protein does not appear to be a major determinant for pathogenicity, it has been implicated as a virulence factor for this organism [34, 35]. A 3.4-kilobase (kb) fragment of *V. vulnificus* DNA that encodes cytotoxin-hemolysin has been cloned and the structural gene for the protein, *vvhA*, has been sequenced [35, 36]. Although the *vvhA* gene has been shown to be unique to this organism by DNA hybridization, there are regions where the nucleotide sequences of the *vvhA* gene and the *hly* gene encoding the hemolysin of *V. cholerae* show significant homologies [36]. Therefore, a 704-bp fragment within the region of *vvhA* where the nucleotide sequences mismatched with the *hly* gene in *V. cholerae* was targeted for use as the DNA probe (VVHP) and amplified by a PCR.

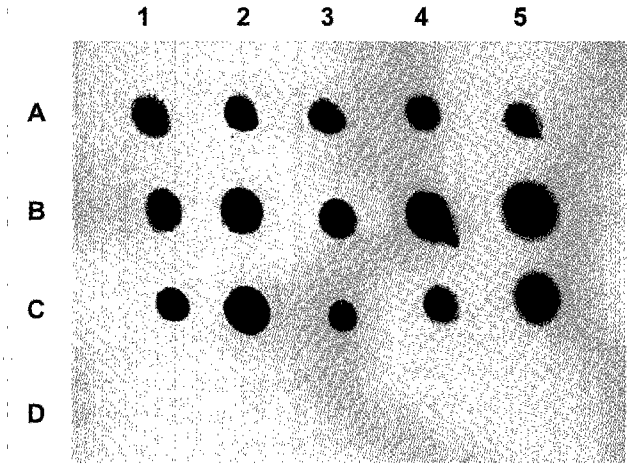

Fig. 1. Colony blot hybridization of *Vibrio* spp. with ^{32}P-labeled *vvhA* DNA probe.
Each blot represents *V. vulnificus* type cultures (A1-A4), clinical isolates (A5, B1-B5, and C1-C2), or environmental isolates (C3-C5), as indicated in Table 1. For Lane D, *V. parahaemolyticus* ATCC27519, *V. alginolyticus* ATCC17749, *V. cholerae* ATCC9459, *V. cholerae* ATCC14033, and *V. furnissii* ATCC35016 (blots D1 to D5, respectively) were blotted as negative controls.

V. vulnificus is frequently isolated together with other *Vibrio* species, such as *V. parahaemolyticus*, *V. cholerae*, *V. furnissii*, and *V. alginolyticus*. The specificity of the VVHP DNA probe was tested by performing a colony blot hybridization of the strains listed in Table 1. The characteristically positive signals for all *V. vulnificus* tested were generated with VVHP. However, none of the strains from other *Vibrio* species hybridized with the probe under the same stringent conditions (Fig. 1). The results summarized in Fig. 1 indicate that colony blot hybridization using VVHP can provide a very specific means to identify all *V. vulnificus*, including both environmental and clinical isolates. Since our techniques using selective media cannot provide an optimal differentiation of *V. vulnificus* from other halophilic *Vibrio* species, such specificity is especially beneficial for the development of a detection method. Consequently, it was also suggested that the colony blot hybridization technique using the VVHP DNA probe may have a potential to be a useful tool for the direct enumeration of *V. vulnificus* naturally present in environmental samples, without the need for any subsequent phenotypic identification.

Sensitivity for Detection of *V. vulnificus*

To assess the sensitivity of a direct colony blot hybridization assay using the VVHP DNA probe, 10-fold serial dilutions of *V. vulnificus* ATCC29307 pure cultures were tested. From the dots loaded with approximately 10^5 CFU or more, the targeted sequence was hybridized successfully by the VVHP DNA probe and characteristic blots were observed on the autoradiogram (Fig. 2). However, those samples seeded by *V. vulnificus* at a level of 10^4 CFU or less and analyzed by the procedure described above, did not show any positive signals.

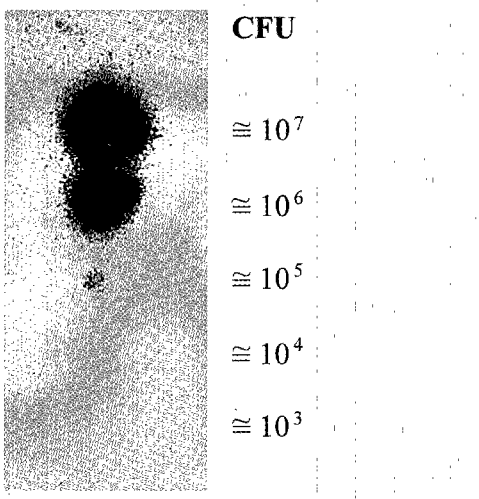

Fig. 2. Analysis of the limit of *V. vulnificus* detection using dot blot hybridization with ^{32}P-labeled *vvhA* DNA.
The numbers of *V. vulnificus* used for each blot were determined by counting the colony-forming units (CFU), and are indicated to the right.

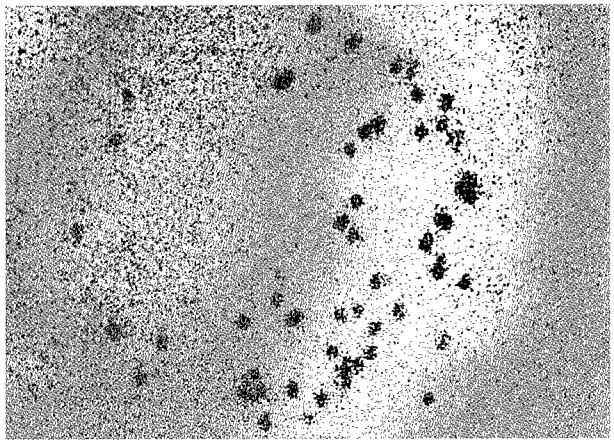

Fig. 3. Example of colony blot hybridization of *V. vulnificus* from raw oysters.
The LBS plate was inoculated with 100 µl of a serial dilution of oyster homogenates and hybridized with the VVHP ^{32}P-DNA probe.

Although the exact number of *V. vulnificus* cells in a colony after transfer to nitrocellulose membranes was not known, it has been assumed the pathogen exists at the concentrations ranging from 10^7-10^9 cells in one colony appearing on LBS plates incubated for 24 h (J. H. Lee, unpublished data). The sensitivity of the procedure seems to be quite high and practical for detecting *V. vulnificus* from an individual colony appearing on LBS plates, as far as when the transfer rate ranged between 0.01% and 1%. It is also proposed that a higher detection sensitivity of *V. vulnificus* could be obtained by optimizing the transfer and/or hybridization procedure.

General Suitability of Colony Blot Hybridization and Enumeration of *V. vulnificus* in Various Natural Samples

To demonstrate the general suitability of the colony blot hybridization optimized under experimental conditions using pure cultures of *V. vulnificus*, field trials for the direct detection of *V. vulnificus* in natural samples were also carried out. Using the VVHP DNA probe, *V. vulnificus* populations were readily detectable in raw seafoods, such as oysters, blue crabs, small octopi, and thin-shelled surf clams as well as in seawater and sediments collected in August 1998, without the use of any selective media or enrichment culturing, as shown in Fig. 3 and Table 2. The enumeration of *V. vulnificus* in raw seafoods revealed the presence of the pathogen at the concentrations ranging from 2.3×10^2 to 4.0×10^3 organisms per ml of sample (Table 2). Positive colony blot hybridization signals were also found in the seawater and sediment samples, indicating that *V. vulnificus* was present in these samples. The level of *V. vulnificus* populations in these samples ranged from 2.1×10^1 to 2.4×10^2 organisms per ml (Table 2). Obviously, the colony blot hybridization procedure appears to be suitable for enumerating the pathogen in various natural samples while maintaining the highest specificity.

Table 2. Enumeration of total heterotrophic bacteria and *V. vulnificus* in different natural samples obtained in summer.

Sample	CFU/ml[a]	
	Total[b]	*V. vulnificus*[c]
Seawater	5.7×10^2	2.1×10^1
Sediment	4.2×10^3	2.4×10^2
Oyster	4.5×10^4	4.0×10^3
Blue crab	5.6×10^4	2.4×10^3
Small octopus	2.9×10^3	3.6×10^2
Thin-shelled surf clam	1.7×10^4	2.3×10^2

[a]Expressed as numbers of bacteria per milliliter of seafood homogenate corresponding to approximately 1 g of seafood meat.
[b]Counted as colony-forming units.
[c]Determined by hybridization with VVHP DNA probe. See Materials and Methods for details.

The CFU were counted on nonselective LBS plates directly from the natural samples, and used as the number of total heterotrophic bacteria. In the natural samples, the heterotrophic populations ranged from 5.7×10^2 to 5.6×10^4 organisms per ml of sample (Table 2). It is apparent that *V. vulnificus* corresponded to 1.4% to 12.4% of the number of total heterotrophic bacteria, and constituted a significant proportion of the total culturable heterotrophic bacterial population in the natural samples collected in the warmer month (August 1998), when the mean temperature of the water was 27.5°C. These results agree with previous reports that *V. vulnificus* is ubiquitous and commonly found in temperate coastal environments [20, 24, 31, 32]. This ubiquitous presence of *V. vulnificus* at substantial levels in various marine animals and environments implies that there are no specific associations between the bacteria and particular organs of marine animals. It has been recently observed that *V. vulnificus* is one of the dominant species comprising the culturable heterotrophic bacterial populations in natural samples collected from Chesapeake Bay, U.S.A. [32].

Seasonal Incidence of *Vibrio vulnificus* in Various Natural Samples

Samples, including oysters, thin-shelled surf clams, water, and sediments were obtained every four months during 1998, and the total culturable heterotrophic bacteria and *V. vulnificus* were enumerated. During the warmer months, May through August, when the mean monthly temperatures were 18°C and 27.5°C, respectively, *V. vulnificus* was present between 2.1×10^1 to 4.0×10^3 CFU per ml of sample (Table 3). However, during the colder months, November through February, when the mean temperatures were 12°C and 5°C, respectively, *V. vulnificus* was not detectable in any samples. This decline in the numbers of *V. vulnificus* organisms indicates that the temperature of the environment can play a key role for the presence or viability of this

Table 3. Seasonal variation of total heterotrophic bacteria and *V. vulnificus* numbers in different natural samples collected from the southwestern coast of Korea.

Sample	CFU/ml[a]							
	August		November		February		May	
	Total[b]	*V. vulnificus*[c]	Total	*V. vulnificus*	Total	*V. vulnificus*	Total	*V. vulnificus*
Seawater	5.7×10^2	2.1×10^1	3.2×10^2	0	3.0×10^2	0	6.5×10^2	2.8×10^1
Sediment	4.2×10^3	2.4×10^2	4.0×10^2	0	3.2×10^2	0	2.1×10^3	3.5×10^2
Oyster	4.5×10^4	4.0×10^3	8.3×10^3	0	1.8×10^3	0	3.2×10^4	1.2×10^3
Thin-shelled surf clam	1.7×10^4	2.3×10^2	3.5×10^2	0	3.5×10^2	0	2.9×10^4	2.1×10^2

[a]Expressed as numbers of bacteria per ml of sample corresponding to approximately 1 g of seafood meat and sediment.
[b]Counted as colony-forming units.
[c]Determined by hybridization with VVHP DNA probe. See Materials and Methods for details.

pathogen, as previously reported [24]. In contrast to *V. vulnificus*, the number of culturable heterotrophic bacteria in the seawater did not vary significantly with temperature, and ranged from 3.0×10^2 to 4.5×10^4 CFU per ml of sample. Unlike the water samples, the numbers of culturable heterotrophic bacteria in the raw seafood and sediments during the colder season were relatively lower than during the warmer months. However, the degree of variation of about 10-fold, in the total heterotrophic bacteria relative to temperature, was not as dramatic as the variation of *V. vulnificus* observed in these samples.

It has been well established that the inability to recover *V. vulnificus* on solid media using standard culture techniques is not totally due to the cell death, but due to the entry of the cells into the viable but nonculturable (VBNC) state [21, 23]. *V. vulnificus* in the VBNC state can be induced by lowering the incubation temperature or by other natural environmental parameters [21, 23]. Although it has not been demonstrated that *V. vulnificus* populations are dead or in a VBNC state during colder months, entering a VBNC state in response to a decreasing temperature is one possible explanation for the drastic decrease of *V. vulnificus* in those samples collected in the colder months.

Recent outbreaks of food-borne diseases such as gastroenteritis and life-threatening septicemia, due to the consumption of raw seafood contaminated with *V. vulnificus*, can affect public health confidence and have a negative impact on the seafood (or fish-farming) industry. The quality assurance of *V. vulnificus*-free seafoods based on credible analytical methods is thus required for consumers and demanded by the industry itself. Accurate information on the population size of *V. vulnificus* in raw seafood is important for designing an effective process that can ensure the highest quality of pathogen-free seafoods. The enumeration of *V. vulnificus* in raw seafoods, by using ordinary culturing techniques, is difficult because of limitations inherent in the use of selective and/or enrichment media. Enrichment of *V. vulnificus* in natural samples in selective media, such as a colistin-polymixin B-cellobiose (CPC) agar, involves the use of colistin and polymixin to suppress the growth of background bacteria [16, 22]. However, these compounds also seem to be detrimental to the growth of *V. vulnificus*. The plating efficiency of *V. vulnificus* on a CPC agar appears to be about 10% of the plating efficiency on a nonselective brain heart infusion agar [22]. Obviously, direct hybridization with the VVHP DNA probe specific to *V. vulnificus* grown on a nonselective LBS medium, one of the most enriched media for the organism [14], will provide a more sensitive means for the enumeration of the pathogen without suppressing its ability to form colonies.

Furthermore, despite even the currently applied selective media that have been reported to be highly specific for this pathogen, further biochemical and/or serological confirmation are atill required for the final identification of *V. vulnificus* [16]. However, such biochemical tests for subsequent identification require the time-consuming subcultures of a large number of individual isolates to obtain statistically reliable results [17, 22]. Immunological methods using monoclonal antibodies have proven to be highly specific; however, subcultures of individual colonies from selective media are still required to provide an ample signal for detection [5, 30]. If the antigenic trait is not constitutive or the antigen exhibits a cross-reactivity to other species, an immunoassay will be difficult to apply. The cost and labor involved in this type of assessment can also be considered for practical use.

The results presented in this report are the first assessment of the populations of *V. vulnificus* in raw seafoods other than oysters, where the presence of *V. vulnificus* also represents a serious health risk for humans. Colony blot hybridization with the VVHP DNA probe was found to be specific and sensitive enough to directly enumerate *V. vulnificus* without any subculture of isolates for further phenotypic identification. Preincubation in an enrichment broth was not carried out, because it does not allow the quantitation of *V. vulnificus*, although this could increase the sensitivity in terms of presence or absence. Using the colony blot hybridization procedure, the time required for the identification and enumeration of *V. vulnificus* can be

substantially reduced by eliminating the need for enrichment culturing and additional biochemical tests. This improvement in speed is particularly beneficial for identification of *V. vulnificus* which can rapidly cause fatal infections.

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REFERENCES

1. Arias, C. R., E. Garay, and R. Aznar. 1995. Nested PCR method for rapid and sensitive detection of *Vibrio vulnificus* in fish, sediments, and water. *Appl. Environ. Microbiol.* **61:** 3476-3478.
2. Blake, P. A., R. E. Weaver, and D. G. Hollis. 1980. Diseases of humans (other than cholera) caused by Vibrios. *Annu. Rev. Microbiol.* **34:** 341-367.
3. Brauns, L., M. C. Hudson, and J. D. Oliver. 1991. Use of the polymerase chain reaction in detection of culturable and nonculturable *Vibrio vulnificus* cells. *Appl. Environ. Microbiol.* **57:** 2651-2655.
4. Bsat, N., M. Wiedmann, J. Czajka, F. Barany, M. Piani, and C. A. Batt. 1994. Food safety applications of nucleic acid-based assays. *J. Food Technol.* **6:** 142-145.
5. Chen, D., P. J. Hanna, K. Altman, A. Smith, P. Moon, and L. S. Hammond. 1992. Development of monoclonal antibodies that identify *Vibrio* species commonly isolated from infections of humans, fish, and shellfish. *Appl. Environ. Microbiol.* **58:** 3694-3700.
6. Coleman, S. S., D. M. Melanson, E. G. Biosca, and J. D. Oliver. 1996. Detection of *Vibrio vulnificus* biotypes 1 and 2 in eels and oyster by PCR amplification. *Appl. Environ. Microbiol.* **62:** 1378-1382.
7. DePaola, A., G. M. Capers, and D. Alexander. 1994. Densities of *Vibrio vulnificus* in the intestines of fish from the U. S. Gulf coast. *Appl. Environ. Microbiol.* **60:** 984-988.
8. Feinberg, A. P. and B. Vogelstein. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132:** 6-13.
9. Heidelberg, J. F., K. R. O'Neill, D. Jacobs, and R. R. Colwell. 1993. Enumeration of *Vibrio vulnificus* on membrane filters with a fluorescently labeled oligonucleotide probe specific for kingdom-level 16S rRNA sequence. *Appl. Environ. Microbiol.* **59:** 3474-3476.
10. Hill, W. E., S. P. Keasler, M. W. Trucksess, P. Feng, C. A. Kaysner, and K. A. Lampel. 1991. Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. *Appl. Environ. Microbiol.* **57:** 707-711.
11. Ichthyological Society of Japan. 1981. Dictionary of Japanese Fish Name and Their Foreign Equivalents. p. 884. Sanseido Co., LTD, Tokyo, Japan.
12. Je, J. G. 1989. Korean names of molluscs in Korea. *Korean J. Molacol.* Suppl. **1:** 1-91.
13. Kim, K. Y., H. C. Yang, M. L. Tamplin, and S. H. Choi. 1999. Intraspecific variation of environmental and clinical *Vibrio vulnificus* isolates as demonstrated by restriction endonuclease digestion profiles. *J. Microbiol. Biotechnol.* **9:** 78-83.
14. Lee, J. Y., J. B. Eun, and S. H. Choi. 1997. Improving detection of *Vibrio vulnificus* in *Octopus variabilis* by PCR. *J. Food Sci.* **62:** 179-182.
15. Lee, J. Y., Y. B. Bang, J. H. Rhee, and S. H. Choi. 1999. Two-stage nested PCR effectiveness for direct detection of *Vibrio vulnificus* in natural samples. *J. Food Sci.* **64:** 158-162.
16. Massad, G. and J. D. Oliver. 1987. New selective and differential medium for *Vibrio cholerae* and *Vibrio vulnificus*. *Appl. Environ. Microbiol.* **53:** 2262-2264.
17. Miceli, G. A., W. D. Watkins, and S. R. Rippey. 1993. Direct plating procedure for enumerating *Vibrio vulnificus* in oysters (*Crassostrea virginica*). *Appl. Environ. Microbiol.* **59:** 3519-3524.
18. Nealson, K. H. 1978. Isolation, identification, and manipulation of luminous bacteria. *Methods Enzymol.* **57:** 153-166.
19. Oliver, J. D. and J. B. Kaper. 1997. *Vibrio* species, pp. 228-264. *In* Doyle, M. P., Beuchat, L. R. and Montville, T. J. (eds.), *Food Microbiology Fundamentals and Frontiers.* ASM Press, Washington, D.C., U.S.A.
20. Oliver, J. D., R. A. Warner, and D. L. Cleland. 1983. Distribution of *Vibrio vulnificus* and other lactose-fermenting *Vibrios* in the marine environment. *Appl. Environ. Microbiol.* **45:** 985-998.
21. Oliver, J. D., L. Nilsson, and S. Kjelleberg. 1991. Formation of nonculturable *Vibrio vulnificus* cells and its relationship to the starvation state. *Appl. Environ. Microbiol.* **57:** 2640-2644.
22. Oliver, J. D., K. Guthrie, J. Preyer, A. Wright, L. M. Simpson, R. Siebeling, and J. G. Morris, Jr. 1992. Use of colistin-polymixin B-cellobiose agar for isolation of *Vibrio vulnificus* from the environment. *Appl. Environ. Microbiol.* **58:** 737-739.
23. Oliver, J. D., F. Hite, D. McDoulgad, M. L. Andon, and L. M. Simpson. 1995. Entry into, and resuscitation from, the viable but nonculturable state by *Vibrio vulnificus* in an estuarine environment. *Appl. Environ. Microbiol.* **61:** 2624-2630.
24. O'Neill, K. R., S. H. Jones, and D. J. Grimes. 1992. Seasonal incidence of *Vibrio vulnificus* in the Great Bay estuary of New Hampshire and Maine. *Appl. Environ. Microbiol.* **58:** 3257-3262.
25. Roszak, D. B. and R. R. Colwell. 1987. Survival strategies of bacteria in the natural environment. *Microbiol. Rev.* **51:** 365-379.
26. Saiki, R. K., D. H. Garland, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988.

Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239:** 487–491.
27. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual.* 2nd ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor. New York, U.S.A.
28. Stahl, D. A. and M. D. Kane. 1992. Methods of microbial identification, tracking and monitoring of function. *Curr. Opin. Biotechnol.* **3:** 244–252.
29. Tacket, C. O., F. Brenner, and P. A. Blake. 1984. Clinical features and an epidemiological study of *Vibrio vulnificus* infections. *J. Infect. Dis.* **149:** 558–561.
30. Tamplin, M. L., A. L. Martin, A. D. Ruple, D. W. Cook, and C. W. Kaspar. 1991. Enzyme immunoassay for identification of *Vibrio vulnificus* in seawater, sediment, and oysters. *Appl. Environ. Microbiol.* **57:** 1235–1240.
31. Tamplin, M. L., G. E. Rodrick, N. J. Blake, and T. Cuba. 1982. Isolation and characterization of *Vibrio vulnificus* from two Florida estuaries. *Appl. Environ. Microbiol.* **44:** 1466–1470.
32. Wright, A. C., R. T. Hill, J. A. Johnson, M. C. Roghman, R. R. Colwell, and J. G. Morris, Jr. 1996. Distribution of *Vibrio vulnificus* in the Chesapeake Bay. *Appl. Environ. Microbiol.* **62:** 717–724.
33. Wright, A. C., G. A. Miceli, W. L. Landry, J. B. Christy, W. D. Watkins, and J. G. Morris, Jr. 1993. Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. *Appl. Environ. Microbiol.* **59:** 541–546.
34. Wright, A. C. and J. G. Morris, Jr. 1991. The extracellular cytolysin of *Vibrio vulnificus*: Inactivation and relationship to virulence in mice. *Infect. Immun.* **59:** 192–197.
35. Wright, A. C., J. G. Morris, Jr., D. R. Maneval, Jr., K. Richardson, and J. B. Kaper. 1985. Cloning of the cytotoxin-hemolysin gene of *Vibrio vulnificus*. *Infect. Immun.* **50:** 922–924.
36. Yamamoto, K., A. C. Wright, J. B. Kaper, and J. G. Morris, Jr. 1990. The cytolysin gene of *Vibrio vulnificus*: Sequence and relationship to the *Vibrio cholera* El Tor hemolysin gene. *Infect. Immun.* **58:** 2706–2709.