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A Selective Medium and a Specific Probe for Detection of *Vibrio vulnificus*

MARTA CERDÀ-CUÉLLAR,* JOAN JOFRE, AND ANICET R. BLANCH

Departament de Microbiologia, Facultat de Biologia, Universitat de Barcelona, 08028 Barcelona, Spain

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A selective medium (VVM) and a specific 16S rRNA gene (rDNA) probe (V3VV) for the detection of *Vibrio vulnificus* were developed. The medium contains D-(+)-cellobiose as the main carbon source and electrolytes ($MgCl_2$-$6H_2O$ and KCl), which stimulate bacterial growth. Polymyxin B, colistin, and moderate alkalinity and salinity provide selectivity properties. *V. vulnificus* grows on VVM as flat, bright yellow colonies. Other *Vibrio* species tested either did not grow or showed green-bluish colonies, with the exception of *V. campbelli*, *V. carchariae*, and *V. navarrensis*. There is a higher colony count on VVM agar than on cellobiose-colistin agar or on modified cellobiose-polymyxin B-colistin agar. The specific probe was evaluated by colony hybridization and dot blot hybridization with PCR-amplified 16S rDNA using collection strains and environmental isolates. No strain studied other than *V. vulnificus* showed positive hybridization with this oligonucleotide. The combined use of VVM agar and the V3VV probe provided the recovery of *V. vulnificus* from mixed bacterial suspensions and spiked mussels.

Vibrio vulnificus is a ubiquitous bacterium found in estuarine and marine environments. It comprises two biotypes: biotype 1 strains are pathogenic in humans, while biotype 2 strains are virulent in eels and opportunistic in humans, although they have been thought to be pathogenic only for eels until recently (2). It has been isolated from a wide variety of marine organisms, such as oysters, crabs, fish, mussels, shellfish, and plankton (10, 17, 18, 36), and from water and sediment (34). It is well known that raw-shellfish consumption increases the risk of human vibrio diseases. Particularly, *V. vulnificus* can cause fulminant and severe systemic human illness after the consumption of infected raw oysters (30). Mortality rates of up to 60% have been reported in such infections (18). It is remarkably virulent in individuals who are immunocompromised or have liver dysfunction, which results in increased levels of iron in serum (18, 28). Thus, its occurrence in aquatic environments is of concern to shellfish industries and public health agencies.

Because a great number and variety of indigenous bacteria are present in the environment together with some pathogenic *Vibrio* species, the isolation of *V. vulnificus* is usually performed by enrichment in alkaline peptone water (APW) or in APW supplemented with polymyxin B. However, this enrichment also increases the number of other bacteria, which requires further inoculation of the enriched sample on selective media (10, 16) such as *V. vulnificus* agar (7), sodium dodecyl sulfate (SDS)-polymyxin B-sucrose (SPS) agar (21), cellobiose-polymyxin B-colistin (CPC) agar (23), *V. vulnificus* enumeration agar (24), modified CPC (mCPC) agar (33), and cellobiose-colistin (CC) agar (16). The recovery of *V. vulnificus* was higher with CPC agar than with thiosulfate-citrate-bile salts-sucrose (TCBS), *V. vulnificus* enumeration, or SDS-polymyxin-sucrose agar (20, 23, 29, 31, 32). Later, CPC agar was modified (mCPC) by reducing the concentration of colistin (33), which improved the recovery and isolation of *V. vulnificus* from environmental sources. Recently, a new modification of CPC agar has been described (16).

To identify presumptive *V. vulnificus* recovered from these media, probes based on the cytolysin gene of *V. vulnificus* have been developed (11, 25, 37). The possible loss or rearrangement of nonessential genes, such as the cytotoxin-hemolysin gene, can lead to a false-negative result (12). Another method for molecular detection of *V. vulnificus* based on the 23S rRNA gene (rDNA) using nested PCR has been developed (4). However, certain molecular techniques cannot be used for the routine monitoring of environmental samples because of PCR inhibition (37). In this study, a new selective medium (VVM agar), which improves the recovery of *V. vulnificus*, and a specific probe (V3VV) have been established. The medium contains electrolytes that improve the recovery of *V. vulnificus*. The probe based on 16S rRNA sequences was evaluated by dot blot hybridization with PCR-amplified 16S rDNA and then by colony hybridization.

Thirty-eight collection strains, mainly *Vibrio* strains, were first used to evaluate the VVM medium and the V3VV probe (Table 1). Moreover, 232 *Vibrio* strains from several environmental origins were used to assess the selectivity of the new medium and the specificity of the probe (Table 2). These strains are available from the LMG collection (Laboratory of Microbiology, Ghent, Belgium). Sixteen *V. vulnificus* strains, from culture collections, of clinical and environmental origins, were used in the plating efficiency experiments. Sixty-four *V. vulnificus* strains of clinical and environmental origins (13 and 51 strains, respectively) were used for confirmation of the species specificity studies with the V3VV probe. *V. cholerae* CECT 658, *V. mimicus* LMG7896^T, and *V. vulnificus* NCIMB 2046^T were used to determine the threshold of detection when mixed bacterial suspensions were analyzed.

Selective VVM agar. The potential components of the medium were evaluated according to the differential metabolic features of the genus *Vibrio* and *V. vulnificus* (1, 15). After several compositions and growth conditions were assayed, the selective medium chosen had the following composition: D-(+)-cellobiose, 15 g; NaCl, 10 g, yeast extract, 4 g; $MgCl_2 \cdot 6H_2O$, 4 g; KCl, 4 g; cresol red, 40 mg; bromothymol blue, 40 mg;

* Corresponding author. Mailing address: Departament de Microbiologia, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain. Phone: 34 93 402 14 89. Fax: 34 93 411 05 92. E-mail: cerda@porthos.bio.ub.es.

TABLE 1. List of the collection strains used to evaluate the selectivity of VVM and the specificity of the V3VV probe

Species	strain[a]	VVM[c]	V3VV[d]
Aeromonas hydrophila	CECT 398	−	−
Edwarsiella ictaluri	F87-88[b]	−	−
Pseudomonas aeruginosa	CECT 116	G+	−
Vibrio aestuarianus	LMG 7909^T	−	−
Vibrio alginolyticus	LMG 4408^T	−	−
Vibrio anguillarum	ATCC 43305	G+	−
Vibrio campbelli	LMG 11216^T	Y+	−
Vibrio carchariae	LMG 7890^T	Y+	−
Vibrio cholerae	CECT 658	G+	−
Vibrio cincinnatiensis	LMG 7891^T	−	−
Vibrio costicola	CCM 2811	−	−
Vibrio damsella	LMG 7892^T	−	−
Vibrio diazotrophicus	LMG 7893^T	−	−
Vibrio fluvialis	LMG 7894^T	−	−
Vibrio furnissii	LMG 7910^T	−	−
Vibrio gazogenes	NCIMB 2250^T	−	−
Vibrio harveyi	LMG 4404^T	−	−
Vibrio mediterranei	LMG 11258^T	G+	−
Vibrio metschnikovii	LMG 11664^T	−	−
Vibrio mimicus	LMG 7896^T	G+	−
Vibrio mytili	CECT 632	−	−
Vibrio natriegens	LMG 10935^T	−	−
Vibrio navarrensis	LMG 15976^T	Y+	−
Vibrio nereis	LMG 3895^T	−	−
Vibrio ordalii	ATCC 33509^T	−	−
Vibrio parahaemolyticus	ATCC 27969, NCIMB 2850, ATCC 17803, ATCC 33844	−	−
Vibrio pelagius	ATCC 3897^T	−	−
Vibrio proteolyticus	LMG 3772^T	G+	−
Vibrio scophthalmi	CECT 4638^T	−	−
Vibrio splendidus	LMG 4042^T	−	−
Vibrio tubiashii	LMG 10936^T	−	−
Vibrio vulnificus	CECT 898, CECT 897, NCIMB 2046^T	Y+	+
Yersinia ruckeri	CECT 955	−	−

[a] ATCC, American Type Culture Collection (United States); CECT, Colección Española de Cultivos Tipo (Spain); CCM, Czech Collection of Microorganisms (Czech Republic); LMG, Laboratorium voor Microbiologie (Belgium); NCIMB, National Collection of Industrial and Marine Bacteria (United Kingdom).
[b] National Fisheries Research Laboratory (United States).
[c] Growth on VVM (−, no growth; G, green; Y, yellow).
[d] Hybridization using the V3VV probe.

polymyxin B, 10^5 U/liter; colistin methanesulfonate, 10^5 U/liter; agar, 15 g; distilled water, 1,000 ml. The compounds were dissolved with stirring and boiling. The pH was adjusted to 8.5 with 5 M NaOH after cooling to 50°C. VVM does not require autoclaving. The uninoculated VVM plates are violet-blue. The presumptive identification of *V. vulnificus* is based on the fermentation of D-(+)-cellobiose, which determines the color of the colonies. The colonies are bright yellow with a yellow diffusion halo. Overnight cultures of each of the 38 collection strains and the 232 *Vibrio* spp. were plated on nonselective Trypticase soy agar supplemented with 1.5% NaCl (TSA2). Cell suspensions (McFarland standard no. 3) of these cultures were made in sterile phosphate-buffered saline complemented with 1.5% NaCl at pH 7.2 (marine PBS). The specificity of the medium was studied by inoculation of 10 μl of each cell suspension onto a plate of VVM agar. Inoculated plates were incubated at 37°C and examined after 24 and 48 h of incubation. As a control, 10 μl of the same suspension was spotted onto TSA2. Plates were incubated for 24 h at 37°C or at 25°C in the case of *Vibrio* spp. which do not grow at 37°C (*V. costicola*, *V. logei*, *V. ordalii*, and *V. splendidus*).

In VVM agar, *V. vulnificus* was easily distinguishable from other *Vibrio* strains and other gram-negative bacteria. All of the culture collection strains of *V. vulnificus* tested showed bright yellow colonies with a yellow diffusion halo on VVM agar because of the fermentation of D-(+)-cellobiose (Table 1). Most of the culture collection strains tested either did not grow or showed green-bluish colonies on VVM agar. Three type strains also displayed bright yellow colonies on the medium: *V. campbellii*, *V. carchariae*, and *V. navarrensis* (Table 1). When the environmental strains were assayed, all of the *V. vulnificus* strains tested presented bright yellow colonies with a yellow diffusion halo on VVM agar. Most of the environmental strains either did not grow or gave green-bluish colonies on VVM agar (Table 2). The use of D-(+)-cellobiose as the main carbon source, the antibiotics polymyxin B and colistin, and moderate alkalinity and salinity provides the medium with the selectivity and differential properties needed to detect *V. vulnificus*. Taxonomic studies (15) reported that over 90% of the strains of *V. vulnificus* ferment D-(+)-cellobiose and are resistant to colistin and polymyxin B. These properties are not common among members of the family *Vibrionaceae*. However, there are a few *Vibrio* species that ferment D-(+)-cellobiose, are resistant to colistin and polymyxin B, and grow at 37°C: *V. aestuarianus*, *V. alginolyticus*, *V. anguillarum*, *V. campbellii*, *V. carchariae*, *V. harveyi*, and *V. navarrensis* (1, 15). Our results are consistent with these observations (Table 1 and 2). The pH of VVM agar was adjusted to 8.5, which has been reported to be optimum for the growth of *Vibrio* spp. (14).

Specific 16S rDNA probe. Full sequences of the 16S rDNAs of 47 *Vibrio* strains deposited in the EMBL genomic database were compared. Five of them belonged to *V. vulnificus*. Multiple alignment and visualization of homologies were performed by the methods of Needleman and Wunsch (26) and Devereux et al. (13), respectively. An oligonucleotide of 24 nucleotides (5′-GTC TGC CAG TTT CAA ATG CAG TTC-3′) located between positions 618 and 641 of the sequence of

TABLE 2. Results obtained with environmental isolates used to evaluate the selective medium VVM and the specificity of the V3VV probe

Species	No. of strains	VVM[a]	V3VV[b]
Vibrio aestuarianus	5	1	0
Vibrio alginolyticus	24	9	0
Vibrio anguillarum	67	3	0
Vibrio cincinnatiensis	1	0	0
Vibrio costicola	2	0	0
Vibrio cholerae	2	0	0
Vibrio damsella	5	0	0
Vibrio diazotrophicus	4	0	0
Vibrio fluvialis	8	0	0
Vibrio furnissii	2	0	0
Vibrio harveyi	32	12	0
Vibrio logei	3	0	0
Vibrio mediterranei	5	3	0
Vibrio metschnikovii	6	0	0
Vibrio mimicus	7	0	0
Vibrio mytili	2	0	0
Vibrio natriegens	4	0	0
Vibrio ordalii	11	0	0
Vibrio parahaemolyticus	7	0	0
Vibrio pelagius	1	0	0
Vibrio proteolyticus	3	0	0
Vibrio splendidus	12	0	0
Vibrio vulnificus	16	16	16
Plesiomonas shigelloides	3	0	0

[a] Number of strains, grown on VVM, showing yellow colonies.
[b] Number of strains which showed positive hybridization with the V3VV probe.

the 16S rDNA of *Escherichia coli* (8) was defined for use as a probe (V3VV). The specificity of V3VV was initially evaluated by comparing its sequences with the sequences of the EMBL database using the FASTA software (13). The oligonucleotide was synthesized and labeled at the 5′ end with digoxigenin (Boehringer, Mannheim, Germany). The specificity was evaluated by DNA-DNA hybridization using culture collection strains (Table 1). To that end, the 16S rDNAs of the 38 collection strains were first amplified. A colony from an overnight culture on TSA2 at 37°C was used to extract the DNA template using the Instagene Matrix Kit (Bio-Rad, Hercules, Calif.). PCR was performed using the AmpliTaq DNA polymerase kit (The Perkin-Elmer Corp., Norwalk, Conn.) in accordance with the instructions of the manufacturer. The PCR program was 1 cycle of 95°C for 0.5 min; 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2.5 min; and 1 cycle of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min. An aliquot of 200 μl of a 1:10 dilution in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) of the amplified DNA was blotted onto nylon membranes (Hybond N; Amersham, Amersham, United Kingdom), using Minifold-I equipment (Schleicher & Schuell, Dassel, Germany) and cross-linked. After the establishment of the conditions of stringency, the hybridization was performed at 68°C as described by Martínez-Picado et al. (22), with minor modifications. The washing steps after hybridization were performed using 2× SSC–0.1% SDS at 68°C and 0.5× SSC–0.1% SDS at room temperature. Detection of probe chemiluminescence was performed with CSPD (Boehringer) as the substrate for alkaline phosphatase. Later, specificity was also tested by colony hybridization using collection and environmental strains (Table 2). To perform colony hybridization, bacteria were grown on nylon membranes (Hybond N^+; Amersham) deposited onto TSA2 plates and incubated overnight at 37°C. Membranes were processed for hybridization as described above and by Martínez-Picado et al. (22).

In order to study the ecology of *V. vulnificus* and to evaluate the public health threat it constitutes, several biochemical and molecular methods have been developed. PCR tools for the detection of *V. vulnificus* have been used for environmental samples (4, 5, 6, 9). The presence of PCR inhibitors in such samples can hinder the amplification of the target molecules. Other molecular methods, such as hybridization with probes (plCVD702 and VVAP) directed against the cytolysin gene (25, 37), are sensitive and specific for the detection of strains carrying this gene. The *V. vulnificus* strains that do not have this gene cannot be detected by hybridization with these probes. On the other hand, hybridization based on cytolysin probes may give signal strength variations due to loss or rearrangement of the gene (12). All of the *V. vulnificus* strains tested hybridized with the probe V3VV (Tables 1 and 2). No strain studied, other than *V. vulnificus* (of environmental, clinical, and culture collection origins), showed yellow colonies on VVM and positive hybridization with this oligonucleotide.

Comparison of VVM and other selective agars. The efficiency of recovery on VVM agar was determined by comparing the colony counts of *V. vulnificus* on this medium with those on TSA2 and TCBS. Moreover, recovery on VVM agar was also compared with that on other selective media: mCPC and CC agars. TCBS (Oxoid) agar was prepared in accordance with the instructions of the manufacturer. The composition and preparation of the CC and mCPC agars have been described elsewhere (16, 33). Overnight cultures of each of the 16 *V. vulnificus* strains tested were prepared by growing them in Trypticase soy broth supplemented with 1.5% NaCl at 37°C. Tenfold dilutions of the overnight cultures in marine PBS were prepared. An aliquot of 10 μl of each dilution was inoculated in triplicate onto CC, mCPC, VVM, TCBS, and TSA2, and the spots were allowed to be absorbed. TSA2, TCBS, and VVM plates were incubated at 37°C, and mCPC and CC plates were incubated at 40°C as previously recommended (16, 33). Colonies were counted at 24 h and confirmed at 48 h. Efficiency of recovery was calculated by determining plating efficiency as described by Høi et al. (16). This term is defined as the percentage of CFU that can be recovered on a selective medium compared to the CFU encountered on a corresponding reference medium. Plating efficiencies on the different selective media were compared by using one-way analysis of variance with least significant differences between means using Student's *t* test (Statgraphics Statistical Graphics System; Manugistics, Inc. and Statistical Graphica Corporation; Rockville, Md.).

TABLE 3. Plating efficiencies for *V. vulnificus* strain on three selective media with respect to TSA2 and TCBS

Reference medium	No. of strains	Mean plating efficiency (%) on selective agar medium ± SD		
		VVM	mCPC	CC
TSA2	16	89 ± 41.07	37 ± 34.85	61 ± 38.71
TCBS	16	85 ± 45.38	36 ± 35.79	62 ± 41.02

VVM agar yielded, with respect to TSA2, a mean plating efficiency of *V. vulnificus* of 89%, while the mCPC and CC agars showed plating efficiencies of 37 and 67%, respectively (Table 3). Variations in plating efficiency among the 16 *V. vulnificus* strains tested caused elevated standard deviations. VVM agar showed significantly higher plating efficiency than mCPC or CC agar with respect to TSA2 ($P < 0.05$). It is worth pointing out that CC agar contains less antibiotic than VVM agar. The higher recovery on VVM agar could be due to $MgCl_2 \cdot 6H_2O$ and KCl, which have been described as stimulation growth factors for pathogenic *Vibrio* spp. (14). No differences were observed at 24 or 48 h in any of the tested media. No significant differences in plating efficiency with respect to TCBS were observed between the VVM and CC agars. However, significantly higher plating efficiency ($P < 0.01$) was obtained with respect to TCBS agar between the VVM and mCPC agars (Table 3). Although VVM agar seems to be more stressful than TCBS agar, it allows much clearer differentiation of *V. vulnificus*. TCBS agar was originally developed for the isolation of *Vibrio* spp. that are pathogenic in humans. It has been widely recommended for the isolation of *V. vulnificus* from clinical samples, but it has also been frequently used as the primary isolation medium in ecological studies. However, several studies have reported brand-to-brand variations in the growth of pathogenic *Vibrio* spp., such as *V. vulnificus*, on TCBS, as well as considerable variations in the recovery rate (27, 35).

Recovery of *V. vulnificus* from mixed bacterial populations. In order to determine the efficiency of recovery and the sensitivity of the medium, *V. vulnificus* from mixed bacterial cell suspensions was detected. Cultures of *V. vulnificus* NCIMB 2046^T *V. cholerae* CECT 658, and *V. mimicus* LMG 7896^T grown overnight in Trypticase soy broth–1.5% NaCl at 37°C were prepared. Sets of 10-fold dilutions of each culture were obtained in marine PBS. Several cell proportions (1:10, 1:100, and 1:1,000) of *V. vulnificus* and *V. cholerae* and of *V. vulnificus* and *V. mimicus* were assayed. Aliquots of 100 μl of each mixed suspension were inoculated in duplicate on VVM agar and TSA2. Inoculated plates were incubated at 37°C for 24 h. Colony counts on both media were performed. Yellow (*V.*

TABLE 4. Recovery of *V. vulnificus* in spiked mussel samples[a]

Medium	A	B	C	D	E
TSA2	5.35×10^5	6.00×10^5	4.00×10^5	5.3×10^5	8.00×10^5
VVM	2.15×10^4 (1.12×10^4)	1.11×10^5 (8.75×10^4)	1.04×10^5 (8.45×10^4)	3.10×10^4 (3.00×10^4)	5.60×10^4 (4.85×10^4)
V3VV	0.00[b]	3.9×10^4	ND	6.00×10^2	$<10^2$

[a] The estimated numbers (CFU per milliliter) of *V. vulnificus* used to spike mussel homogenate aliquots were as follows: A, 0.00; B, $5.27 \cdot 10^4$; C, $527 \cdot 10^3$; D, $5.27 \cdot 10^2$; E, $5.27 \cdot 10^1$. TSA2, total counts on TSA2; VVM, total counts on VVM (partial counts [yellow colonies] are in parentheses; V3VV, counts of colonies hybridizing with the *V. vulnificus*-specific probe (V3VV) from VVM agar plates. ND, no data available.

[b] No positive colony hybridization observed.

vulnificus) and blue (*V. cholerae* or *V. mimicus*) colonies on VVM agar were counted.

The colonies of *V. vulnificus* (flat, yellow colonies with a yellow halo) were easily differentiated from *V. cholerae* and *V. mimicus* on VVM agar, which showed round, blue-green colonies when the detection of *V. vulnificus* in mixed bacterial suspensions was studied. It was possible to visualize 1 colony of *V. vulnificus* among 10^3 colonies of *V. cholerae*. A similar situation took place when *V. vulnificus* and *V. mimicus* were mixed. This level of detection is difficult to surpass because of the limitations of the visualization of colonies growing on a plate.

Recovery of *V. vulnificus* from spiked mussels. Since the occurrence of virulent strains of *V. vulnificus* has been related, in some cases, to shellfish consumption, the threshold of detection of this species on VVM agar was tested with mussel samples. Mussels were spiked with a pure culture of *V. vulnificus* NCIMB 2046^T with a known concentration (3.16×10^5 CFU/ml). The mussels were collected in the Delta de l'Ebre (Spanish Mediterranean coast) and processed as follows. They were aseptically shucked with an autoclaved oyster knife to obtain 50 g of mussel meat, which was homogenized in a sterile blender. The homogenate of mussels was diluted in 50 ml of marine PBS and stirred for 20 min. The resulting suspension was filtered through nylon gauze with a 54-μm pore size to remove thick particles. The filtered suspension was then distributed in 5 aliquots of 5 ml each. An overnight culture of *V. vulnificus* NCIMB 2046^T was prepared in TSB2 at 37°C, and 10-fold dilutions in marine PBS were prepared to obtain concentrations of 10^5 to 10^2 CFU of bacteria per ml. Four aliquots of the filtered suspension of mussels were spiked with 1 ml of each dilution of the *V. vulnificus* culture. One, the negative control, was not spiked. Thereafter, 10-fold dilutions of the spiked and control suspensions in marine PBS were prepared. An aliquot of 100 μl of each dilution was plated in duplicate on TSA2 and VVM agar plates, which were incubated at 37°C for 24 h. Total and presumptive *V. vulnificus* (yellow) colony counts were performed. Replica plating was carried out on nylon membranes (Hybond N^+; Amersham) in order to confirm the detection of *V. vulnificus* colonies on VVM agar. Later, colony hybridization with the specific digoxigenin-labeled V3VV probe was performed at 68°C as described above. The locations of probe-positive signals on autoradiograms were compared with the positions of yellow colonies on VVM agar.

Yellow colonies were observed on the plates from the control sample, where no *V. vulnificus* was added. However, none of them hybridized with the V3VV probe. Consequently, they were not *V. vulnificus*. The final concentrations of *V. vulnificus* in the different aliquots of the spiked homogenate ranged from $5.27 \cdot 10^4$ to $5.27 \cdot 10^1$ CFU/ml. A slightly higher concentration of yellow colonies was observed on VVM agar than the estimated number of cells added to each aliquot. The counts of the pathogen were as expected when the enumeration of *V. vulnificus* cells was confirmed by colony hybridization with the probe (Table 4). There were differences between the total counts on TSA2 agar and those on VVM agar. Slightly higher counts on TSA2 agar might be due to the stressing effect of the selective medium.

In a recent study (3), CPC agar showed higher detection and recovery of *V. vulnificus* from marine samples than PCR approaches. However, the percentage of confirmed *V. vulnificus* cells on CPC agar was quite low. On the other hand, CC agar shows higher recoveries and is less stressful than CPC agar for *V. vulnificus*, probably because of the absence of polymyxin B in CC agar (16). Here, we show that VVM agar gives higher recovery than CC agar. It is interesting that VVM has the same proportion of polymyxin B and colistin methanesulfonate as mCPC agar and gives better recoveries than CC agar. The electrolytes ($MgCl_2 \cdot 6H_2O$ and KCl), which stimulate bacterial growth, or the different main source of nitrogen on VVM could explain such differences. Thus, VVM agar could be used as a selective medium in the standard protocol for the isolation of *V. vulnificus*. Such a protocol is used for the environmental monitoring of *V. vulnificus*. This is performed in two steps: first, an enrichment step in APW supplemented with polymyxin B or colistin (16) or in a recently described selective broth (19); second, inoculation of the enriched sample on selective agar and usually confirmation by specific immunoassays or DNA hybridizations. In this study, VVM agar and the V3VV probe showed their usefulness for differential detection of *V. vulnificus* in mixed bacterial suspensions and spiked mussel samples. Their use in the second step of the standard protocol used to detect *V. vulnificus* is feasible. However, further studies with natural environmental samples should be performed to evaluate if the combined use of VVM agar and the specific probe V3VV could improve the standard protocol for the environmental monitoring of *V. vulnificus* in shellfish and estuarine waters.

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