Protocol for Specific Isolation of Virulent Strains of *Vibrio vulnificus* Serovar E (Biotype 2) from Environmental Samples

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The eel pathogen *Vibrio vulnificus* biotype 2 comprises at least three serovars, with serovar E being the only one involved in both epizootics of eel vibriosis and sporadic cases of human infections. The virulent strains of this serovar (VSE) have only been recovered from clinical (mainly eel tissue) sources. The main objective of this work was to design and validate a new protocol for VSE-specific isolation from environmental samples. The key element of the new protocol is the broth used for the first step (saline eel serum broth [SEB]), which contains eel serum as a nutritive and selective component. This approach takes advantage of the ability of VSE cells to grow in eel serum and thus to separate themselves from the pool of competitors. The growth yield in SEB after 8 h of incubation was 1,000 times higher for VSE strains than for their putative competitors (including biotype 1 strains of the species). The selective and differential agar *Vibrio vulnificus* medium (VVM) was selected from five selective media for the second step because it gave the highest plating efficiency not only for the VSE group but also for other *V. vulnificus* groups, including biotype 3. The entire protocol was validated by field studies, with alkaline peptone water plus VVM as a control. *V. vulnificus* was isolated by both protocols, but serovar E was only recovered by the new method described here. All selected serovar E isolates were identified as VSE since they were virulent for both eels and iron-overloaded mice and resisted the bactericidal action of eel and iron-overloaded human sera. In conclusion, this new protocol is a suitable method for the isolation of VSE strains from environmental samples and is recommended for epidemiological studies of the pathogenic serovar E.

Vibrio vulnificus is an aquatic bacterium from tropical and warm brackish waters with pathogenic potential for humans and aquatic animals (1, 8, 14, 17, 23, 38, 43, 45, 46, 47). The species is currently subdivided into three biotypes (14, 45), with biotype 2 being recognized worldwide as an eel pathogen. This biotype causes hemorrhagic septicemia (vibriosis) in cultured eels (8), and the first cases were recorded in Japan in the mid 1970s (38). From Japan, the disease arrived in Europe in the late 1980s and spread to eel farms in Mediterranean and Atlantic countries such as Spain, Sweden, The Netherlands, and Denmark (9, 10, 18, 23, 27, 28). Now the disease is the main cause of economic losses in brackish water eel culture in Europe. Biotype 2 strains are subdivided into at least three different O-antigenic serovars (11, 13, 23, 28). Serovar E corresponds to the original isolates that gave rise to the subdivision of the species into biotypes (45). This serovar is the only one that is related to sporadic human infections as well as epizootics or outbreaks with high mortality in eel farms (1, 9, 10, 11, 17, 47). Eels were the vehicle for transmission to humans in at least four clinical cases (zoonoses) (17, 47).

At present, the hazard that virulent serovar E (VSE) strains present to public health is underestimated. The main reason for this is that no VSE strain has been recovered from seafood, fish, or water during routine environmental sampling to monitor *V. vulnificus* (7, 9, 16, 30, 32). Current isolation procedures involve an enrichment step in alkaline peptone water (APW; 1% peptone, 1% NaCl [pH 8.6]), supplemented or not with antibiotics (19), followed by plating onto selective differential media such as thiosulfate-citrate-bile salts-sucrose (TCBS) agar, cellobiose-polymyxin-colistin (CPC) agar, and their derivatives, modified CPC (mCPC) agar, colistin-cellobiose (CC) agar, and *V. vulnificus* medium (VVM) (15, 29, 36, 40, 42, 44). According to several studies, the recovery of serovar E is difficult because these strains are present in very small numbers in the aquatic environment (7, 16, 30, 32). However, this contention is not supported by experimental data, as this serovar is not recovered even from eel farm water during epizootics or outbreaks, when high numbers of viable cells are released into the water (7, 16). Our hypothesis is that the isolation methodology fails because APW favors the growth of bacterial competitors (including biotype 1 strains) that can be deleterious for VSE cells and thus inhibit their isolation. This hypothesis is based on results obtained by Marco-Noales et al. (34) for survival experiments performed with water microcosms that were coinoculated with VSE strains and selected competitors. In those experiments, competition phenomena which were detrimental to the recovery of VSE strains from mixed populations were observed in both the presence and absence of nutrients (34). If our hypothesis is correct, then the relevance of this serovar from an epidemiological point of view is higher than previously thought.

The main objective of the present study was to develop a new two-step protocol for the specific isolation of VSE strains from asymptomatic eel carriers and water samples. The new protocol should favor the growth of VSE strains and inhibit that of putative competitors, especially those of biotype 1, which can be coisolated from aquatic and fish samples and which have been observed to induce VSE strains to enter into a viable but nonculturable state (34). Since the main recognized trait that distinguishes biotype 2 from biotype 1 strains is

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the ability of the former to grow in nonimmune eel serum (5), we designed a selective broth containing eel serum for the first isolation step. The efficacy of this new method was compared with that of the currently employed APW method, and the optimal incubation conditions were established and tested by use of a large collection of putative competitors belonging to *V. vulnificus* (biotypes 1 and 3) and other aquatic bacterial species. In parallel, several selective and differential media were compared for the ability to recover VSE strains, and the most efficient medium was selected. The efficacy of the entire protocol was tested by the use of natural environmental samples, including seawater, freshwater, eel tank water, and healthy eel tissues. The results obtained clearly indicate the suitability of the new protocol for successfully isolating VSE strains from natural mixed populations. Finally, the new serovar E isolates were characterized and compared with strains collected from clinical origins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 37 *V. vulnificus* strains of different biotypes, sources, and origins, as well as 23 strains of other species, were used for this study (Tables 1 and 2). The strains were maintained both as lyophilized stocks at room temperature (25°C) and as frozen stocks at −80°C in marine broth (Difco) plus 20% (vol/vol) glycerol. Strains were routinely grown in Trypticase soy broth (TSB) or on Trypticase soy agar (TSA) (Difco) supplemented with 0.5% (wt/vol) NaCl (TSB-1 and TSA-1, respectively) at 28°C for 24 h.

Efficiency of the new selective enrichment broth. (i) SEB. Pooled eel sera were obtained from eels as previously described (22) and then stored at −80°C. Phosphate-buffered saline (PBS; 0.02% KCl, 0.15% Na$_2$HPO$_4$, 0.20% KH$_2$PO$_4$) supplemented with NaCl to a final concentration of 1% (PBS-1), 2% (PBS-2), or 3% (PBS-3) was adjusted to the pH of eel blood (pH 8.3). Saline eel serum broth (SEB) was prepared by diluting eel serum 1:5 in PBS-1 (SEB-1), PBS-2 (SEB-2), or PBS-3 (SEB-3) immediately before use.

(ii) Bacteria. Sterile microcosms of artificial seawater (50) were prepared in screw-cap 30-ml glass tubes as previously described (33) and were inoculated with bacterial suspensions from TSA-1 plates in artificial seawater to a final concentration of 10^6 to 10^7 CFU per ml. All tubes were incubated in the dark in a static state at room temperature for at least 1 month to simulate the starvation conditions typical of natural environments. Microcosms were sampled weekly for cultivable counts by the drop plate method (26) using TSA-1 plates (33).

(iii) Growth curves. The effectiveness of SEB and APW in inhibiting the growth of competitors and enhancing the growth of VSE strains was tested with pure cultures (Tables 1 and 2). Three independent experiments were performed with each strain and combination of strains. For each experiment, the enrichment broth was inoculated with starved cells (10^2 to 10^3 CFU per ml) and incubated with shaking at 28°C for 10 h. Plate counts for single cultures were done with TSA-1 plates by the drop plate method (26), and samples were taken after 0, 1, 2, 3, 4, 6, 8, and 10 h of incubation. The average and standard deviation of bacterial counts per incubation time were calculated, and the significance of the differences between data was determined by variance analysis performed with SPSS, release 11.0. The most effective combination of broth and time of incubation was tested with all of the strains listed in Tables 1 and 2 and with mixed cultures (VSE strains plus one competitor). For this purpose, microcosms of these bacteria were prepared as described above and were used to inoculate the enrichment broth. Plate counts for single cultures were done with TSA-1 plates by the drop plate method (26), and for mixed cultures, plate counts were done with the differential medium MSWYE-BTB agar plus 1% mannitol (34) (on this medium, VSE strains develop green colonies while colonies of the selected competitors are yellow). Experiments were performed in duplicate. The final growth rate (GR) was calculated for each strain as the log of the final count divided by the initial count and was coded as follows: 0, GR ≤ 1; 1, 1 < GR ≤ 2; 2, 2 < GR ≤ 3; 3, 3 ≤ GR ≤ 4; or 4, 4 ≤ GR.

Efficiency of selective media. Five selective media (TCBS agar [Difco], CC agar, CPC agar, mCPC agar, and VVM) were tested for their efficiencies of recovery of *V. vulnificus* serovar E strains with respect to the general medium TSA-1. These selective media were prepared according to their original descriptions (15, 29, 36, 44). Selected *V. vulnificus* strains (Table 1) were grown over-

night in MSWYE at 28°C, and bacterial counting was done with the selective and general media by the drop plate method (26). Plates were incubated for 24 to 48 h at the following recommended temperatures: TSA-1 and TCBS agar, 28°C; VVM, 37°C (15); and CC, mCPC, and CPC agar, 40°C (29, 36, 44). To test the influence of the incubation temperature on VSE recovery, we also incubated CC, mCPC, and CPC agar plates at 37°C. The efficiency of recovery was expressed as a recovery rate (RR), which was calculated as the percentage of CFU recovered on each of the selective media compared to the CFU obtained on the corresponding TSA-1 plate and was expressed as the mean value ± the standard deviation. A Mann-Whitney nonparametric analysis of variance was performed to evaluate the statistical significance of differences in the efficiency values (SPSS for Windows, release 11.0). The most effective selective medium was tested for its effectiveness in inhibiting the growth of putative competitors. For this purpose, all of the strains listed in Table 2 were grown in MSWYE at 28°C for 18 h, and a loopful of each was streaked onto the selected agar medium. The plates were incubated at the recommended temperatures for 24 to 48 h and then examined for growth.

Field sampling. A total of eight samples of water from a Spanish coastal site (Delta del Ebro), a freshwater lake (Albufera Lake), and two eel farms, as well as nine samples of tissues taken from wild and cultured eels, were tested (Table 3). One of the farms had experienced recurrent outbreaks of vibriosis 2 years before (Table 3). In the case of water samples, 250-ml samples were collected in sterile flasks and filtered through sterilized 0.22-μm-pore-size membrane filters (Millipore). In the case of adult eels, samples of mucus, spleens, gills, and livers from four eels per sampling were taken aseptically, pooled, and homogenized in PBS-1. In the case of glass eels, the whole animals were homogenized in PBS-1. Filters and tissue homogenates were incubated with shaking in flasks containing 250 ml of APW or in tubes containing 25 ml of the selected SEB at 28°C for 18 and 8 h (see Results), respectively. Tenfold dilutions in PBS-1 of all enrichments were performed, and aliquots of 100 μl were streaked onto the selective medium selected in prior experiments. An average of 30 suspected colonies from each sampling were purified on TSA-1 for further studies.

Identification and characterization of environmental isolates. (i) Identification of species. For identification to the species level, colony hybridization with a *V. vulnificus*-specific alkaline phosphatase-labeled DNA probe directed against a cytolysin-hemolysin gene was used (51).

(ii) Serological identification and biochemical characterization. Confirmed *V. vulnificus* isolates were serologically identified by slide agglutination (4) with previously obtained rabbit anti-serovar E, anti-serovar A, anti-serovar O3, and anti-serovar O3/O4 polyclonal antibodies (4, 23; also our unpublished results). A total of nine randomly selected serovar E strains were seeded in API 20E and API 20NE strips (Biomerieux, Madrid, Spain), and the API profiles were compared with the API database (Apilab Plus, version 3.3.3; Biomerieux). Additionally, the following biochemical tests were performed: Simmons citrate test, Thornley's arginine dihydrolase test, and decarboxylation of lysine and ornithine (ODC) in Mueller base [Difco]) (12).

(iii) Serum resistance. The sensitivities of the selected *V. vulnificus* serovar E strains to eel and human sera were evaluated with bacteria grown on TSA-1 in microtiter plates (6). In each well, 50 μl of eel serum or iron-overloaded human serum (supplemented with 10 μM desferoxamine mesylate [Desferal; Sigma]) (3) was mixed with 50 μl of a suspension of bacteria (10^3 to 10^4 CFU/ml) in PBS-1. The assays were performed in triplicate, and samples were taken after 0, 1, 2, 3, and 4 h of incubation at room temperature. Viable counts were determined by drop plating on TSA-1.

(iv) Virulence assays. The potential virulence of the *V. vulnificus* serovar E isolates for humans and eels was tested by using BALB/c mice with an average weight of 20 g (5 to 6 weeks old) and juvenile European eels with an average weight of 10 g (elvers), respectively. Groups of six animals were used for each experiment, which lasted 1 week. Mice were pretreated for 2 h with Desferal (250 μg/g of body weight; iron-overloaded mice) because the virulence of *V. vulnificus* is markedly increased when the serum contains this iron chelator (a hydroxymate-type siderophore that promotes growth in serum) (3). Eels were maintained in aerated tanks containing 6 liters of saline water (1% NaCl) at 25°C (2). The *V. vulnificus* serovar E environmental isolates were grown on TSA-1 at 28°C for 24 h, and bacterial suspensions in PBS-1 were prepared. Approximately, 10^4 CFU/fish and 10^3 CFU/mouse were injected into elvers (0.1 ml per fish) and mice (0.2 ml per mouse). Appropriate controls for each experiment were also included (mice and elvers inoculated with PBS-1) (2, 3). Mice and elver mortalities were recorded daily and were only considered if bacteria were isolated in pure culture from internal organs.

TABLE 1. Origins biotypes and serovars of the reference *V. vulnificus* strains used for this study

Strain[a,m]	Origin	Source	Biotype-serovar[b]	Virulence in eels/virulence in mice[c]	GR[d] in SEB
CECT 529[T] (ATCC 27562)*	Human blood	USA	BT1	−/+	0
CECT 5164 (374)[e]	Human blood	USA	BT1	−/+	0
CECT 5167 (L-180)[e]	Human blood	Japan	BT1	−/+	0
CECT 5168 (CDC7184)**	Human blood	USA	BT1	−/+	0
CECT 5169 (H3308)[e]	Human blood	USA	BT1	−/+	0
ATCC 33816	Human blood	USA	BT1	−/+	0
94385[f]	Leg wound	Spain	BT1	−/ND	0
V4[g]	Human blood	Australia	BT1	−/ND	0
CECT 5165 (UNCC 890)[e]	Sea water	USA	BT1	−/ND	0
CECT 4608*,**	Eel farm water	Spain	BT1	−/−	0
JE[h]	Oyster	USA	BT1	−/ND	0
VV425[h]	Oyster	USA	BT1	−/ND	0
CG100[i]	Oyster	Taiwan	BT1	−/+	0
CG106[i]	Oyster	Taiwan	BT1	−/ND	0
CG110[i]	Seawater	Taiwan	BT1	−/ND	0
A2	Eel farm water	Spain	BT1	−/−	0
CECT 4601	Diseased eel	Spain	BT2-VSE/E	+/+	4
CECT 4604*,**	Diseased eel	Spain	BT2-VSE/E	+/+	4
CECT 4605	Diseased eel	Spain	BT2-VSE/E	+/+	4
CECT 4917	Diseased eel	Spain	BT2-VSE/E	+/+	4
CECT 4864	Diseased eel	Spain	BT2-VSE/E	+/+	4
CECT 4865	Diseased shrimp	Taiwan	BT2-VSE/E	+/ND	4
Ö122[j]	Diseased eel	Sweden	BT2-VSE/E	+/ND	4
CECT 5198	Diseased eel	Spain	BT2-VNSE/A	+/ND	4
CECT 5343	Diseased eel	Spain	BT2-VNSE/A	+/ND	3
CECT 5768	Diseased eel	Spain	BT2-VNSE/A	+/ND	1
95-8-6[k]	Diseased eel	Denmark	BT2-VNSE/O3	+/ND	1
95-8-7[k]	Diseased eel	Denmark	BT2-VNSE/O3	+/ND	1
95-8-161[k]	Diseased eel	Denmark	BT2-VNSE/O3:O4	+/ND	2
95-8-162[k]	Diseased eel	Denmark	BT2-VNSE/O3:O4	+/ND	4
97[l]	Human	Israel	BT3	−/ND	1
12[l]	Human	Israel	BT3	−/ND	0
11028[l]	Human	Israel	BT3	−/ND	1
58[l]	Human	Israel	BT3	−/ND	ND
1033[l]	Human	Israel	BT3	−/ND	2
32[l]	Human	Israel	BT3	−/ND	1
162[l]	Human	Israel	BT3	−/ND	0

[a] CECT, Spanish Type Culture Collection; ATCC, American Type Culture Collection; CDC, Centers for Disease Control and Prevention. T, type strain.

[b] BT1, biotype 1; BT2-VSE, biotype 2, virulent serovar E; BT2-VNSE, biotype 2, virulent, non-serovar E; BT3, biotype 3. Biotypes were determined on the basis of eel virulence and cellobiose fermentation. Biotype 2 serovars: A and E are according to Biosca et al. (13) and Fouz and Amaro (23); serovars O3 and O3/O4 are according to Martin and Siebeling (35).

[c] Data on virulence are from Biosca (9), Fouz and Amaro (23), and Amaro et al. (unpublished results). ND, not done.

[d] The final GR was calculated for each strain as the log (final count/initial count) and was coded as 0 (GR ≤ 1), 1 (1 < GR ≤ 2), 2 (2 < GR ≤ 3), 3 (3 ≤ GR ≤ 4), or 4 (4 ≤ GR).

[e] Supplied by J. D. Oliver, Department of Biology, University of North Carolina, Charlotte.

[f] Supplied by L. Torres, Department of Microbiology, Miguel Servet University Hospital, Zaragoza, Spain.

[g] Supplied by L. Gibson, Department of Cell and Molecular Biology, The University of Technology, Sydney, Australia.

[h] Supplied by M. L. Tamplin, USDA-ARS-ERRC, Wyndmoor, Pa.

[i] Supplied by L. Hor, Department of Microbiology and Immunology, National Cheng Kung University, Tainan, Taiwan, Republic of China.

[j] Supplied by T. Hongslo, Department of Fish, National Veterinary Institute, Uppsala, Sweden.

[k] Supplied by I. Dalsgaard and J. L. Larsen, Department of Veterinary Microbiology, Royal Veterinary and Agricultural University, Frederiksberg, Denmark.

[l] Biotype 3 strains were provided by N. Bisharat from Nuffield Department of Clinical Laboratory Science, University of Oxford, United Kingdom, through J. D. Oliver (University of North Carolina), J. C. Piffaretti (Istituto Cantonale di Microbiologia, Bellinzona, Switzerland), or S. Miyoshi (Faculty of Pharmaceutical Science, Okoyama University, Okoyama, Japan)

[m] *, strains selected for growth curves of single populations in selective enrichment broth; **, strains selected for growth curves of mixed populations in selective enrichment broth.

RESULTS

Efficiency of selective enrichment broths. Growth curves for starved cells of selected VSE, *V. vulnificus* biotype 1, and *Vibrio* sp. strains (Tables 1 and 2) in SEB are shown in Fig. 1. The *Vibrio* sp. strain was selected because it was coisolated with *V. vulnificus* biotype 1 on VVM from water of an eel tank in a routine control study. *V. vulnificus* biotype 1, VSE, and *Vibrio* sp. strains showed similar growth patterns in APW, without

significant differences between the curves (Fig. 1). In contrast, the growth of the VSE strain in SEB-1 and SEB-2 was significantly higher than that of the nontarget bacteria throughout the incubation period (Fig. 1). Significant differences were also observed with SEB-3, but only in the fourth hour of incubation (Fig. 1). The generation times (*g*) in SEB-1 and SEB-2 of the nontarget bacteria were very similar (about 1 h in SEB-1 and 0.7 h in SEB-2) and were longer than those of the target bacteria (about 0.4 h in both media) (Fig. 1). In contrast,

TABLE 2. Origins and virulence of reference strains other than *V. vulnificus* used for this study

Species	Strain[a,g]	Origin	Source	Virulence for eels[b]	GR in SEB[c]	Growth in VVM
V. harveyi	CECT 604	Seawater	Spain	−	0	−
	CECT 605	Marine plankton	Spain	ND	0	−
	CECT 606	Marine plankton	Spain	−	0	−
	CECT 607	Seawater	Spain	ND	4	−
	CECT 608	Seawater	Spain	−	0	−
		Diseased horse	Japan	−		
V. alginolyticus	CECT 521[T]	Mackerel			0	−
	CECT 600	Seawater	Spain	ND	0	−
	CECT 601	Seawater	Spain	ND	0	−
	CECT 603	Marine plankton	Spain	ND	0	−
	CECT 610	Marine plankton	Spain	ND	0	−
	RA3[d]	Diseased amberjack	Spain	ND	0	−
V. mediterranei	CECT 615	Marine plankton	Spain	−	0	+(G)[f]
	CECT 621[T]	Marine sediment	Spain	−	0	+(G)[f]
V. splendidus	CECT 528	Marine fish	Spain	−	0	−
	CECT 4204	Seawater	USA	−	0	−
V. parahaemolyticus	CECT 611	Seawater	Spain	−	0	−
	CECT 612	Seawater	Spain	ND	0	−
	CECT 613	Marine plankton	Spain	ND	0	−
Vibrio sp.	PD-4*	Eel tank water	Spain	ND	0	−
Edwardsiella tarda	CECT 886	Eel tank water	USA	+	0	−
Pseudomonas anguilliseptica	CECT 899[T]	Diseased eastern fish	Japan	+	0	−
Aeromonas hydrophila	E37[e]	Diseased eel	Spain	+	1	
A. jandaei	S345[e]	Healthy eel	Spain	ND	4	−

[a] CECT, Spanish Type Culture Collection. T, type strain.
[b] Data on virulence are from Biosca et al. (9) and Esteve et al. (20). ND, not done.
[c] The final GR was calculated for each strain as the log (final count/initial count) and was coded as 0 (GR $\leq$ 1), 1 (1 < GR $\leq$ 2), 2 (2 < GR $\leq$ 3), 3 (3 $\leq$ GR $\leq$ 4), or 4 (4 $\leq$ GR).
[d] Supplied by E. Alcaide, Universidad de Valencia, Valencia, Spain.
[e] Supplied by C. Esteve, Universidad de Valencia, Valencia, Spain.
[f] G, green colonies.
[g] *, strain selected for growth curves of single and mixed populations in selective enrichment broth.

growth rates of both target and nontarget bacteria were very similar in SEB-3 (about 0.75 h) (Fig. 1). The largest differences in the final GRs between the target and nontarget bacteria were found in SEB-1 after 8 h of incubation (3×10^7 to 2.5×10^9 versus 1×10^3 to 2×10^4). Thus, SEB-1 was coinoculated with one VSE strain plus one competitor (Tables 1 and 2) in proportions of 1:1. After 8 h of incubation, only green colonies could be counted in the experiments, regardless of the strain that was coinoculated with the se-

rovar E strain. Finally, SEB-1 was tested with biotype 2 strains of other serovars (Table 1), with biotypes 1 and 3 (Table 1), and with selected competitor species (Table 2). After 8 h of incubation, only four strains, two of biotype 2, one of *Vibrio harveyi*, and one of *Aeromonas jandaei*, achieved the same GR as the VSE isolates. Five biotype 2 strains and four biotype 3 isolates grew with GR values between 1 and 3, and the rest of the strains did not grow (Tables 1 and 2).

TABLE 3. Isolation of *V. vulnificus* serovar E from water and fish samples by the APW + VVM and SEB-1 + VVM procedures

Type of sample[a] (n)	Eel body wt (g)	Physicochemical parameters of water[b]		No. of positive samples for *V. vulnificus*/no. of *V. vulnificus* serovar E-positive samples (%)	
		T (°C)	Salinity (%)	APW+VVM	SEB+VVM
Water (8)					
Ebro delta (1)		25	2.5	1/0	1/1 (50)
Albufera Lake (1)		25	0.5	0/0	0/0
Freshwater eel farm (3)		25–28	0.1	3/0	3/0
Brackish-water eel farm (3)		25–28	1.5–1.7	3/0	3/2 (10/50)
Tissues (9)					
Wild glass eels (3)	0.1			0/0	0/0
Wild eels (2)	27.5			1/0	1/0
Cultured elvers (4)	10	25–28	0.1	2/0	2/1 (100)

[a] All samplings of water and eels from eel farms were performed in the absence of epizootics or outbreaks. The cultured elvers came from the freshwater eel farm, which had experienced epizootics of vibriosis 2 years before the sampling. Wild glass eels came from the Mediterranean Sea and adult eels came from Albufera Lake and were bought alive in a public market in Valencia (Spain).
[b] The temperature and salinity of tank water from which cultured eels were captured are also indicated.
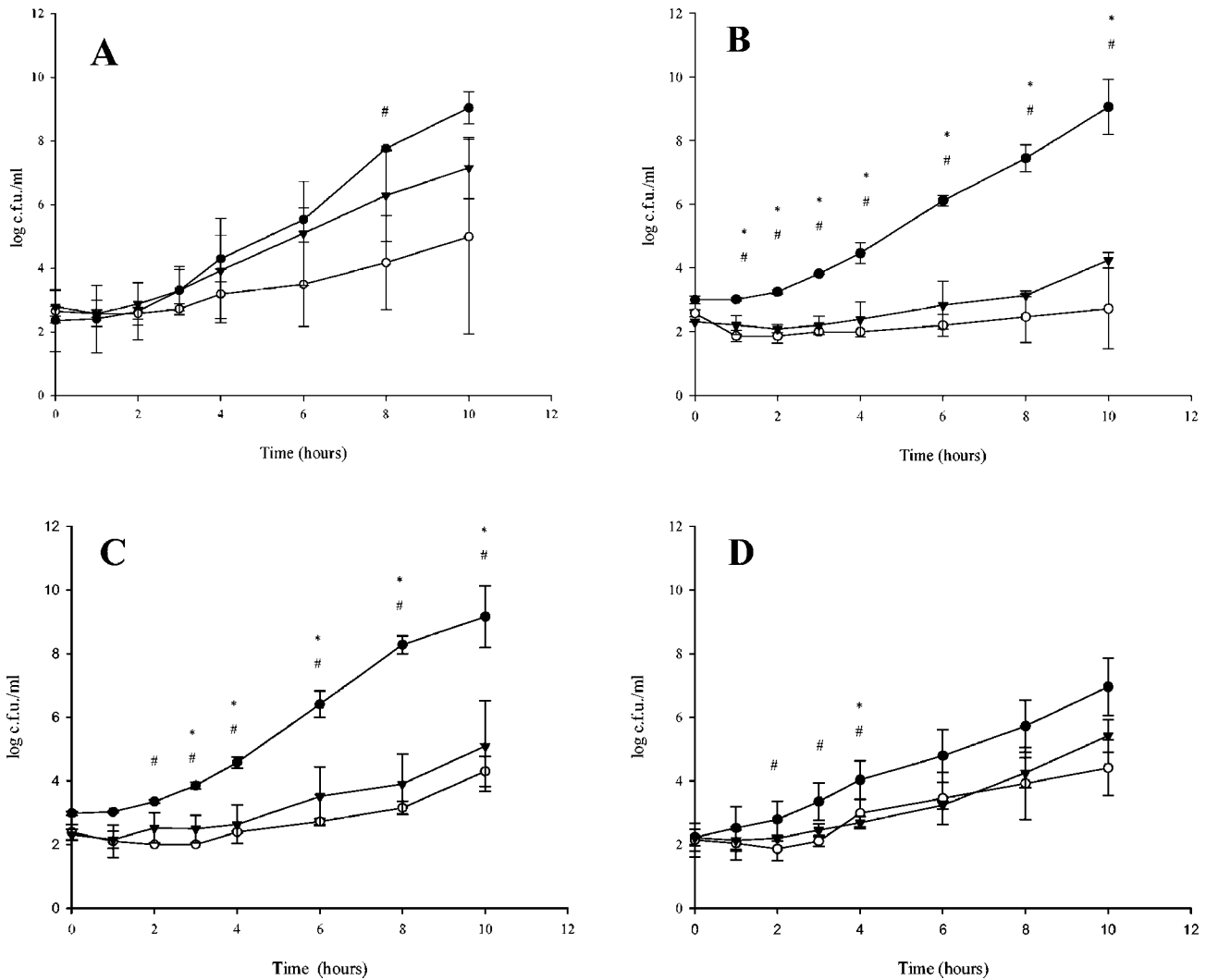
FIG. 1. Growth curves of *V. vulnificus* CECT 4604 (●), CECT 529^T (○), and *Vibrio* sp. strain PD-4 (▼) in APW (A), SEB-1 (B), SEB-2 (C), and SEB-3 (D). Each point represents the average ± standard deviation of values from at least three different experiments. Significant statistical differences (α = 0.05) between CECT 4604 and CECT 529^T (#) and between CECT 4604 and *Vibrio* sp. strain PD-4 (*) are indicated.

Efficiency of selective media. The strains of *V. vulnificus* were classified into four groups, as follows: BT1 (biotype 1), BT2-VSE (biotype 2, virulent serovar E), BT2-VNSE (biotype 2, virulent, non-serovar E), and BT3 (biotype 3) (Table 1). The recovery rates (RRs) obtained for each group and for each selective medium are shown in Fig. 2. Although high standard deviations within each group were observed, statistical analysis revealed significant differences between the groups and media. No significant differences in colony counts were registered regarding the incubation time (24 or 48 h) or, in the case of CPC, mCPC, and CC agars, the incubation temperature (40 or 37°C) ($P \leq 0.05$). In all cases, the lowest RRs were seen for CPC agar (from 0.02 to 1.75%), and the highest were seen for VVM (from 50.42 to 71.23%) (Fig. 2). BT2-VSE and BT3 strains did not grow well on mCPC agar and CC agar, which does not contain polymyxin B (29), and BT3 strains did not grow on TCBS agar either. In addition, statistical differences between groups regarding the RRs from several media were detected. Thus, (i) the BT1 group showed significantly higher
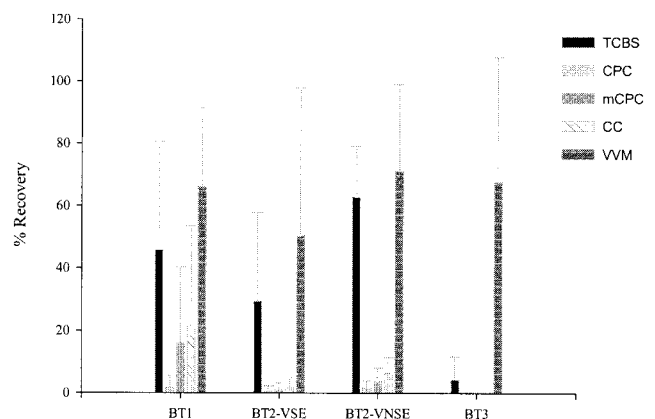

FIG. 2. Recovery rates (%) of the different groups of *V. vulnificus* on the selective media tested. Vertical bars represent standard deviations.

TABLE 4. Biochemical characteristics of selected *V. vulnificus* serovar E strains isolated from water and asymptomatic carriers

Strain	Source	API 20E profile	API 20NE profile	Result of conventional test			
				CIT	ADH	LDC	ODC
PD-8	Eel tank water	5006005[b]	5473745[d]	−	−	+	−
AnS1	Gills	5006005[b]	5472645[e]	+	−	+	+
C1	Liver	5006005[b]	5472745[f]	+	−	+	−
Riu-2	Seawater	5006005[b]	5472745[f]	+	−	+	−
PD-2-47	Tank water	5006005[b]	5472745[f]	+	−	+	+
PD-2-50	Tank water	5006005[b]	5472745[f]	+	−	+	+
PD-2-51	Tank water	5006005[b]	5472745[f]	+	−	+	+
PD-2-55	Tank water	5006005[b]	5472745[f]	+	−	+	−
PD-2-56	Tank water	5006005[c]	5472745[f]	+	−	+	+
CECT 4604[a]	Diseased eel	5006005[c]	5472745[f]	+	−	+	−

[a] VSE control strain.
[b] Identification according to the API data base: *V. vulnificus* (54.4%) or *Burkholderia cepacia* (45.3%).
[c] Identification according to the API data base: *B. cepacia* (89.3%) or *V. vulnificus* (10.4%).
[d] Identification according to the API data base: *Aeromonas hydrophila/caviae* (94.5%).
[e] Identification according to the API data base: *V. vulnificus* (75%), *V. cholerae* (17.4%), or *V. alginolyticus* (4.6%)
[f] Identification according to the API data base: *V. cholerae* (45%) or *A. hydrophila/caviae* (41.7%).

RRs on TCBS, CC, and mCPC agars than the BT3 group and on CC and mCPC agars than the BT2-VSE group; (ii) the BT2-VNSE group had significantly higher RRs on TCBS and CC agars than the BT2-VSE and BT3 groups; and (iii) the BT2-VSE group yielded significantly higher RRs on TCBS agar than the BT3 group. Since the highest RRs of VSE strains were achieved with VVM, this medium was selected for further experiments. On this medium, the three biotypes of *V. vulnificus* developed bright colonies of about 2 mm, with biotypes 1 and 2 being yellow with a yellow diffusion halo and biotype 3 being green without a halo. The effectiveness of VVM at inhibiting the growth of putative competitors was tested with the strains listed in Table 2. Only *Vibrio mediterraneii* strains were able to grow on VVM, giving green colonies with a similar morphology to those of biotype 3.

Field sampling. *V. vulnificus* was isolated from seven of eight water samples and from three of nine tissue samples by both isolation procedures (Table 3). Regarding water samples, *V. vulnificus* was isolated from seawater (2.5% salinity) and tank water from fresh- and brackish-water eel farms, but not from lake water (Table 3). In the case of eel tissue samples, *V. vulnificus* was recovered from both cultured and wild eels (Table 3). After enrichment in APW, >95% of the presumptive yellow colonies on VVM were identified as belonging to *V. vulnificus* by colony hybridization with the *V. vulnificus*-specific alkaline phosphatase-labeled DNA probe, but none of the isolates agglutinated with serovar E-specific antisera. After enrichment in SEB-1, 100% of the presumptive colonies were identified as *V. vulnificus*. Serovar E was recovered from three of seven *V. vulnificus*-positive (VV⁺) water samples (seawater and eel farm water) and from one of three VV⁺ tissue samples (cultured eels) (Table 3). The percentages of confirmed serovar E colonies ranged from 10 to 50% in the case of water samples to 100% in the case of eel samples (Table 3). About 20 randomly selected non-serovar E isolates from water and eel samples were tested with serovar A-, serovar O3-, and serovar O3/O4-specific antisera. Two isolates (10%) from tank water agglutinated with anti-serovar A sera, and the rest (50%) did not agglutinate with these sera or were autoagglutinating (40%).

Characterization of environmental VSE strains. Nine randomly selected serovar E isolates were further characterized. The origins of these strains and the results of the API 20E and NE systems as well as some additional conventional taxonomic tests are summarized in Table 4. Eight of these strains gave the same API 20E profile as the control clinical VSE strain, while one differed only in citrate utilization. According to the API database, the major profile corresponded to *V. vulnificus* with a probability of 54.4%, while the minor profile had a probability of 10.4% (Table 4). Because of previously reported discrepancies between conventional and commercial assays (12), several tests were reexamined. More than 80% of the isolates were positive for citrate by the conventional test, and >60% were positive for ODC, a phenotypic trait that had a negative result in the API 20E system. Regarding the API 20NE system, seven isolates gave the same profile as the control strain, which, according to the API database, did not correspond to *V. vulnificus* (Table 4). The other two strains showed slight differences, and only one was identified as *V. vulnificus* with a probability of 75% (Table 4).

All serovar E isolates could survive and grow in undiluted fresh eel and iron-overloaded human sera, giving bacterial yields similar to or even higher than those of the control strain (Fig. 3). In addition, all strains were virulent for eels and iron-overloaded mice, giving a mortality rate of >75% after the injection of 10^4 CFU/fish or 10^3 CFU/mouse. Eel and mouse mortality occurred before 48 h, and bacteria were recovered as pure cultures from internal organs. The infected eels showed redness on their bodies, particularly on the head and tail. Internally, the liver and kidneys appeared hemorrhagic, as did the muscle wall on occasion. No external pathological signs were observed for moribund mice except for occasional small ulcers on the tail.

DISCUSSION

For the present study, a two-step protocol for the isolation of VSE strains from asymptomatic carriers and environmental samples was developed and validated in the field. Selected
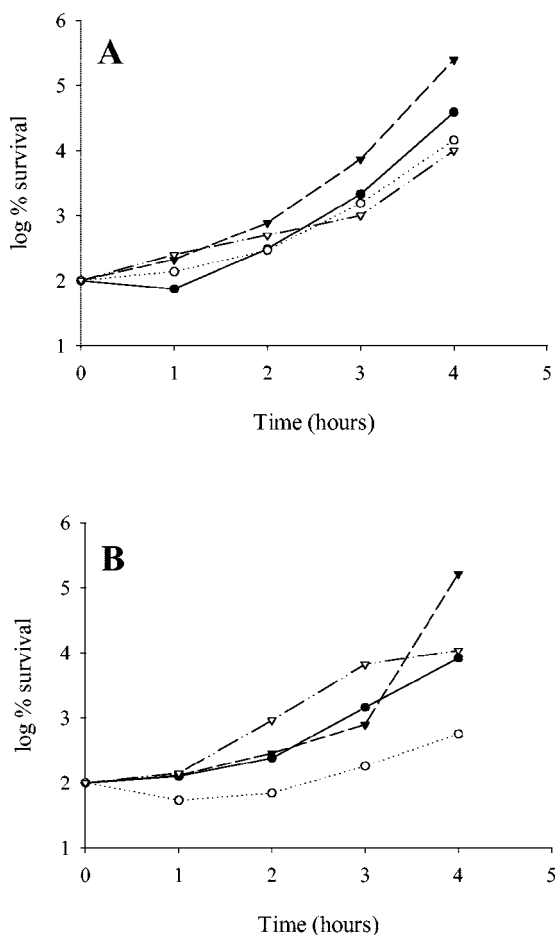
FIG. 3. Growth curves of environmental serovar E strains VSE PD-8 (●), AnS1 (○), and C1 (▼) in eel (A) and iron-overloaded human (B) sera with respect to the control strain CECT 4604 (▽). Each point represents the average of three different experiments.

target and nontarget bacteria grew equally well in the enrichment broth recommended by the U.S. Food and Drug Administration (FDA), which confirmed that APW is not adequate for the selection of VSE strains from a pool of *V. vulnificus* strains. Fresh eel serum diluted in alkaline PBS (SEB) was chosen because eel virulence in *V. vulnificus* is related to resistance to serum complement (5) as well as to the growth rate in eel serum (5, 21; also our unpublished results). Until now, animal sera had only been used after heat inactivation as a nutrient supplement for the growth of some fastidious pathogenic bacteria (31, 48). The present approach takes advantage of the ability of these bacteria to cause infection (grow in eel serum) as a means of selecting them from a pool of competitors. The results obtained with pure and mixed cultures confirmed that SEB acted as a culture broth for VSE strains, while it was bacteriostatic or bactericidal for competitors, including biotype 1 and 3 strains of this species. The optimal selective conditions were obtained by incubating bacteria in SEB-1 for 8 h at 28°C. Under these conditions, VSE cells could be selected, even from a pool of biotype 2 cells, on the basis of the higher growth rates of the VSE strains in eel serum.

With regard to the evaluation of the efficacies of the selec-

tive media, the results clearly demonstrated that CPC agar should not be used for VSE recovery (RR < 0.5%). The inability of VSE strains to grow in CPC agar had previously been reported by Macián et al. (32) and suggested by Høi et al. (29). Furthermore, this inability was generalized for all of the *V. vulnificus* groups, including the biotype 1 group (RR < 2%), which contrasts with data reported by other authors (15, 27). This discrepancy may be due to differences in the physiological state of the cells since they were previously grown in a low-nutrient medium (MSWYE) instead of a high-nutrient one (TSA and others) as usual. mCPC and CC agars, which contain less antibiotic, are recommended instead of CPC agar by many investigators, including the FDA, which recommends mCPC agar. Nevertheless, as shown in this study, these CPC derivatives are also inadequate for the recovery of VSE (RR below 1.5%), VNSE (RR below 6%), and biotype 3 (RR below 0.02%) strains, although the RR of biotype 1 strains increased significantly (around 20%). These results may explain by themselves why no biotype 2 and 3 strains have been isolated from the environment. The efficacies of recovery of all groups except one (biotype 3) increased significantly when TCBS agar was employed. This medium is widely used for the isolation of the genus *Vibrio* (7, 25, 37, 41, 49) and is the second medium recommended by the FDA for the isolation of *V. vulnificus*. However, its efficacy has been questioned by several authors (15, 29, 49), primarily due to the difficulty of reproducing results. In fact, we obtained RRs that were much higher than those reported by other laboratories (29). In addition, the RRs on TCBS agar of VSE strains were significantly lower than those of VNSE strains. This result may be related to the etiological characteristics of the diseases since VNSE strains colonize the intestines and mostly produce intestinal hemorrhages and feces with blood (23). Oddly enough, VSE strains were more similar to biotype 3 strains, and VNSE isolates were more similar to biotype 1 isolates, regardless of the strains' origins. This apparent subdivision of the eel pathogenic group supports the polyphyletic origin proposed by Gutacker et al. for the biotype 2 strains of the species (24). Fortunately, VVM significantly increased the efficacies of recovery of all groups, without differences between eel pathogenic subgroups. This medium contains electrolytes ($MgCl_2 \cdot 6 H_2O$ and KCl) that stimulate the growth of pathogenic vibrios (19). The binding of these cations to the bacterial membrane may alter its ionic potential and make the more sensitive strains (VSE and biotype 3) more resistant to polymyxins. VVM was tested with a wide collection of strains belonging to putative competitor species, and only *V. mediterranei* grew, developing green colonies. Nevertheless, since this species was inhibited by SEB-1, its presence in a sample should not cause problems.

In the second part of this study, the protocol was validated in the field, with APW plus VVM used as a control. Firstly, *V. vulnificus* was isolated by both protocols from water and healthy tissue samples. However, the percentage of positive isolations was considerably higher than that previously reported for other sites on the Mediterranean Sea with higher salinities (7, 32) and was similar to that found for other habitats with similar salinities (16, 29). Serovar E strains were only recovered after SEB-1 enrichment, which confirmed that the competition phenomena present in APW can be abolished by using this selective enrichment broth. In fact, 43 and 33.33% of

the samples that were positive for *V. vulnificus* from water and tissues, respectively, were also positive for serovar E recovery, which constituted between 10 and 100% of the *V. vulnificus* colonies, depending on the sample. In addition, after SEB enrichment, serovar A strains, together with other nontypeable strains, were also isolated. Interestingly, serovar E was not detected in wild eels. Although more samplings are needed, this result supports the hypothesis that wild European eels are not the natural host for this serovar, which underscores the highly virulent potential of this pathogen for nonimmunized eels (5, 9). All of the selected environmental isolates were clearly identified as VSE strains since they were virulent for eels and iron-overloaded mice and resisted the bactericidal action of eel and iron-overloaded human sera. The biochemical profiles of the VSE isolates were quite homogeneous and similar to that of the clinical control VSE strain included in this study. In contrast to the report of O'Hara et al. (39), we found that all VSE strains were negative for the indole test in the API 20E system, which is in accordance with previous studies (4, 10, 11, 12, 13, 38, 45). Some discrepancies in the results of Simmons citrate and ODC tests which have already been described (12) were detected, as most of the environmental isolates were positive for both characteristics in conventional tests but not in commercial ones. In addition, none of the isolates was correctly identified by the API 20E and NE systems, which shows the limited value of these systems for the identification of *V. vulnificus* unless these profiles are included in the API database.

In conclusion, a new two-step protocol for VSE-specific isolation from environmental samples has been developed and validated in the present work. The key element of this protocol is the enrichment broth used for the first step, which yielded differences in bacterial counts between VSE strains and competitors that were large enough to allow the isolation of this serovar from natural mixed populations after only 8 h of incubation. For the second step, the selective and differential agar VVM was selected because it gave the highest plating efficiencies, not only for the VSE group, but also for the rest of the *V. vulnificus* groups, including biotype 3. The entire protocol was validated in the field since VSE strains were isolated for the first time from seawater and eel farms in the absence of epizootics or outbreaks. Finally, the overall results demonstrate that serovar E strains are present in the aquatic environment and that only with adequate procedures of isolation can their true epidemiological relevance be revealed.

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