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Species-Specific Detection of *Vibrio anguillarum* in Marine Aquaculture Environments by Selective Culture and DNA Hybridization

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Methods for specific detection of *Vibrio anguillarum* in complex microbial communities within diverse marine aquaculture environments were evaluated. A system for the detection of culturable cells based on the combined use of a selective medium and a nonradioactively labeled oligodeoxynucleotide complementary to 16S rRNA was developed. Four hundred fourteen bacterial cultures were evaluated in order to assess the specificity of the method. When both the selective medium and the specific probe gave positive results, the cultures were always identified as *V. anguillarum*. The selectivity for colony hybridization was 1 *V. anguillarum* cell in 10,000 total bacterial cells in environmental samples. The utility of the method was also compared with detection by dot blot hybridization of either raw DNA purified from environmental samples or PCR-amplified DNA of 16S rRNA genes, using universal eubacterial primers. The post-PCR hybridization was more sensitive (8×10^2 cells) than direct hybridization of the whole purified DNA (10^6 cells). However, the selective medium-probe combined method was as sensitive as post-PCR hybridization, albeit more specific.

Vibrio anguillarum is an opportunistic fish pathogen that is common to marine and estuarine environments. It has been identified as the main cause of vibriosis, a potentially fatal septicemia that affects fish and shellfish in marine aquaculture, with consequent economic losses (5, 9, 29).

The phenotypic determination and identification of *V. anguillarum* present certain difficulties, especially in marine environments where there are either very complex microbial communities or communities within which *V. anguillarum* is a minor component of a group of similar microorganisms, especially other *Vibrio* species.

Isolation of *V. anguillarum* generally involves plating on nutrient medium containing 1 to 2% NaCl and selective medium, mainly thiosulfate-citrate-bile salts-sucrose agar (TCBS) (8). However, the effectiveness of TCBS for *V. anguillarum* detection has been questioned (3, 10). Complete identification usually relies on a battery of biochemical (35) or serological (32) tests. These methods require the subculture of numerous single isolates, and phenotypic variability of strains could make identification questionable; routine processing is thus time-consuming and expensive, with poor or low levels of sensitivity.

Previously, we reported the use of both a selective-differential agar medium (VAM) (3) and a genetic probe of 24 nucleotides in length (VaV3) complementary to 16S rRNA (22) for the presumptive identification of *V. anguillarum*. In the present study, we evaluate a method for the detection of *V. anguillarum* in the environment that combines the use of the oligonucleotide probe VaV3 and VAM medium. The probe was used in hybridization methods, which involved both raw and PCR-amplified DNA of sample lysates. The combined method (medium plus probe) was applied to both environmental isolates phenotypically identified as *Vibrio* sp. and samples from different environments to compare sensitivity and specificity in natural bacterial populations. Samples included water, sediments, live food (rotifers and brine shrimps), whole fish larvae, and kidney and intestinal contents of fish. Environments included aquarium facilities where sea bream (*Sparus aurata*) were experimentally infected during the period of sampling, two hatcheries of turbot (*Scophthalmus maximus*) and sea bream, and a series of brackish lagoons containing wild fish, including several species reported to be susceptible to *V. anguillarum*.

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MATERIALS AND METHODS

Bacterial strains and media. A total of 414 bacterial cultures were tested. Ninety-three were culture collection strains (Table 1). The rest were *Vibrio* cultures studied in our laboratory, taken from aquatic samples, which were phenotypically classified by using 28 biochemical tests and a set of keys for the biochemical identification of environmental *Vibrio* species (1, 2).

Strains were grown in nonselective medium: either marine agar or Trypticase soy agar (TSA) supplemented with 0.5% NaCl (TSA1) for culturable heterotrophic bacteria. The selective media used were TCBS agar for presumptive vibrios (8) and VAM agar for presumptive *V. anguillarum* strains (3). All cultures were incubated at 25°C and examined at 24, 48, and 72 h.

Samples. Samples were taken from three environments of increasing complexity. First, the recovery of *V. anguillarum* after an experimental infection was evaluated under controlled conditions. Water and fish from a research aquarium facility, where a group of fish were experimentally infected as indicated below, were studied. Fish were kept in aquaria for 4 weeks before the start of the experiment in order to reduce transport stress. Then, 100 sea bream (6 cm in size) were intracoelomically injected with 0.1 ml of phosphate-buffered saline supplemented with 1.5% NaCl (PBSS) containing 4.6×10^3 CFU of *V. anguillarum* A018 (7) per fish. The second group was injected with sterile PBSS. The third fish group was not manipulated at all. Twenty fish were maintained in each aquarium at 20 to 22°C and 40‰ salinity. They were fed dry food only. Water was previously sterilized by filtration (5-μm pore size) and UV light irradiation to minimize bacterial entrance. The flow rate allowed complete renovation of aquarium water in 1 h. Aquarium water and samples from fish (kidney and intestine plus intestinal contents) were collected at predetermined time intervals for 30 days and additionally at any time that mortalities occurred. Mortalities associated with *V. anguillarum* infection occurred only during days 2, 3, and 4 postinfection (with four, two, and one dead fish, respectively) among fish injected with strain A018.

Second, two hatcheries, one situated in the north coast of Spain (Cantabric Sea) and the other situated in the northeast coast (Mediterranean Sea), were studied. Samples consisted of inflowing (before UV treatment) and effluent water in the installations, whole sea bream and turbot larvae, and live food (brine shrimps and rotifers) used for feeding fish larvae.

TABLE 1. Culture collection strains used in this study[a]

Species	Strain(s)
V. adaptus	ATCC 19263
V. aestuarianus	ATCC 35048
V. alginolyticus	ATCC 14582, 17749, 19108, 33838
V. anguillarum	ATCC 19264, 43305, 43306, 43307, 43308, 43309, 43310, 43311, 43312, 43313, 43314
V. campbellii	ATCC 25920, 25921, 33864
V. carchariae	ATCC 43515, 43516; LMG 7890
V. cincinnatiensis	ATCC 35912
V. costicola	ATCC 33508, 33508
V. damsella	ATCC 33536, 33539
V. diazotrophicus	ATCC 33466; NCIMB 2170, 2171
V. fisheri	ATCC 7744, 33983; NCIMB 1200, 1274, 1544
V. fluvialis	ATCC 33809, 33810, 33812; LMG 11656, 11657
V. furnissii	ATCC 33813, 33 841, 35016
V. harveyi	ATCC 14126, 25919, 33842, 33843, 33866; NCIMB 394, 993, 1847; LMG 11659
V. logei	ATCC 15382
V. marinus	ATCC 15381
V. mediterranei	ATCC 43341, 43342; LMG 11663
V. metschnikovii	LMG 11664, 11665
V. mimicus	ATCC 33653
V. mytili	CECT 632
V. natriegens	ATCC 14048, 33898; NCIMB 2273
V. navarrensis	CIP 103193
V. nereis	ATCC 25917, 33893, 33896; LMG 11669
V. nigripulchritudo	ATCC 27043
V. orientalis	ATCC 33934
V. ordalii	ATCC 33509; CIP 103205
V. parahaemolyticus	ATCC 17802, 17803, 27969, 33844
V. pelagius	ATCC 25916, 33504, 33782, 33784
V. proteolyticus	ATCC 15338
V. salmonicida	ATCC 43839
V. splendidus	ATCC 33125
V. tubiashii	ATCC 19105, 19106, 19109; NCIMB 2165
V. vulnificus	ATCC 27562, 33147
Aeromonas hydrophila	CECT 398
Pseudomonas aeruginosa	ATCC 15442

[a] ATCC, American Type Culture Collection, Rockville, Md.; CECT, Colección Española de Cultivos Tipo, Valencia, Spain; CIP, Collection Institute Pasteur, Paris, France; LMG, Laboratorium voor Microbiologie Gent, Ghent, Belgium; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland. Strains were kindly supplied by P. A. D. Grimont (Institut Pasteur), J. Swings (LMG), J. L. Larsen (Royal Veterinary & Agricultural University, Copenhagen, Denmark), and B. Austin (Heriot-Watt University, Edinburgh, Scotland).

Third, a completely open natural system was also studied. Samples of water, sediments, and dying mullets (*Mugil* sp.) were taken from the brackish lagoons in the Ebro Delta (northeast coast of Spain, Mediterranean Sea).

Detection by culture and colony hybridization. In order to detect culturable *V. anguillarum* cells, seawater samples were 10-fold serially diluted in sterile PBSS. Diluted and undiluted samples (100 μl) were spread on TSA1, TCBS, and VAM. Sediment samples were resuspended in sterile seawater thoroughly agitated with a magnetic stirrer for 15 to 30 min and then allowed to sediment. Bacterial analysis was performed on the supernatants. Fish tissues, live food, and larvae were homogenized in PBSS with a Potter homogenizer. Both supernatants of sediments and full homogenates were processed as indicated above for seawater samples.

After plates were spread, they were incubated at 25°C for 48 h and colonies were counted. Then, TSA1 and VAM plates were replicated on nylon membranes for RNA-DNA colony hybridization with a digoxigenin-labeled VaV3 probe. Blots of TSA1 plates were also replicated on VAM. The locations of probe-positive signals from autoradiograms were compared with the positions of yellow colonies on VAM. When hybridization with VaV3, differential growth in VAM, or both were obtained, the colonies were isolated and a phenotypic analysis was also performed, including use of the API-20E system (BioMérieux, Marcy l'Etoile, France) (14), plate growth in TCBS, TSA supplemented with 7.5% NaCl (TSA8), MacConkey agar, and starch-glutamate-ampicillin-penicillin agar (18) in order to assess the phenotypic identity. The following standard biochemical tests were also performed to confirm the API-20E results (1): Voges-Proskauer (11), arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase (23).

Detection by direct and post-PCR hybridization. For the detection of *V. anguillarum* without culture, seawater volumes ranging from 500 to 1,000 ml were aseptically pumped through 0.22-μl cylindrical filter membranes (Sterivex-GS; Millipore) at a pressure of 1 to 1.4 kg/cm^2, with DNA extraction occurring within the filter housing according to Somerville et al. (30). DNA was then purified with phenol-chloroform and by isopropanol precipitation (34). DNA from homogenated kidneys, intestines, rotifers, brine shrimps, and whole fish larvae (1 ml) was prepared as described by Valentine (33) but using a different 10× lysis buffer (0.1 M Tris-HCl [pH 8.0], 0.01 EDTA, 10% [wt/vol] sodium dodecyl sulfate [SDS], 1 mg of proteinase K per ml) and precipitation solution (0.2 M NaCl with isopropanol and 20 μg of glycogen). Fifty percent of the extracted DNA was directly hybridized, and the rest was amplified by PCR and then hybridized as described below.

Hybridizations and PCR. The VaV3 probe (22) was chemically synthesized by Genosys Biotechnologies, Inc. (The Woodlands, Texas) and labeled with digoxigenin-11–ddUTP by 3′-end labeling with terminal transferase according to the manufacturer's (Boehringer, Mannheim, Germany) specifications. Hybridizations were performed on nylon membranes as previously described (22) with a few modifications: hybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% [wt/vol] *N*-lauroylsarcosine, 0.02% [wt/vol] SDS, 2% blocking solution [proteolytic fragments of caseine in powder form]) contained 2.5 pmol of labeled probe per ml. After nylon membranes (Hybond N) were washed, they were treated for chemiluminescent detection with Lumigen-PPD according to the manufacturer's (Boehringer) instructions. When rRNA-DNA colony hybridization was performed, filters were preincubated in a solution containing 3× SSC plus 0.1% (wt/vol) SDS with shaking for 1 h at 68°C, and then surfaces were wiped with a moistened towel to remove cellular debris. When DNA-DNA hybridization was performed, 50% of solutions containing the extracted DNA, as described above, were directly blotted onto nylon membranes, using Minifold-I equipment (Schleicher & Schuell, Dassel, Germany), cross-linked, and hybridized with the VaV3 probe. When PCR was performed, 10, 1, and 0.1 μl of the extracted DNA was amplified with the universal primers 27f and 1494r (20). DNA amplification components were as described by Saiki (28) but with 0.5 μM (each) primer. The thermal cycling was as indicated by Relman (26), with a 4-min final elongation step. Between 10 and 20% of the amplified product was applied to a 0.8% (wt/vol) agarose gel. The rest of the product was processed by blotting on nylon membranes as described above for hybridization of raw DNA with VaV3.

Sensitivity controls. Tenfold serial dilutions of *V. anguillarum* in PBSS were tested in order to assess the sensitivity of the direct and post-PCR hybridization assays under more closely defined conditions. Cells were previously enumerated by direct fluorescence with orange acridine and plate culture in TSA1.

RESULTS

Specificity of the combined method. A total of 414 bacterial strains were studied. The results showed that those strains positive for both VAM differential growth (producing brilliant yellow colonies) and colony hybridization with VaV3 were biochemically identified as *V. anguillarum*. In contrast, none of the strains that were negative for both tests were *V. anguillarum* (Table 2). However, when only one of the tests was used, the number of misidentifications rose to 14.5% (60 of 414 strains). Of 314 strains of *Vibrio* sp. other than *V. anguillarum*, 49 were identified only by one test: either medium or probe. The phenotypic characterization indicated that those strains which were able to ferment sorbitol while growing on VAM medium were *V. fluvialis*, *V. harveyi*, *V. mediterranei*, *V. metschnikovii*, or *V. anguillarum*-like. Likewise, those strains that were recognized only by VaV3 probe were *V. diazotrophicus*, *V. ordalii*, or *V. navarrensis*. Eleven of 100 strains of *V. anguillarum* failed to be recognized by one of the two methods. These strains were either unable to ferment sorbitol or belonged to serovars O9 and O10 (22).

Sensitivity of the methods. When 10-fold serial dilutions of *V. anguillarum* in PBSS were tested in order to assess the sensitivity of the direct dot blot hybridization assay, we established the detection limit at around 2×10^6 cells, previously determined by plate culture in TSA1. Likewise, the detection

TABLE 2. Identification of *V. anguillarum* by molecular culture based on selective medium and biochemical analyses

Vibrios	Identification with:		Frequency (%)
	VAM medium	VaV3 probe	
V. anguillarum (n = 100)	+	+	89.0
	−	+	9.0[a]
	+	−	2.0[b]
	−	−	0
Other vibrios (n = 314)	+	+	0
	−	+	5.7[c]
	+	−	9.9[d]
	−	−	84.4

[a] *V. anguillarum* strains unable to ferment sorbitol.
[b] *V. anguillarum* strains belonging to serovars O9 and O10.
[c] Non-*V. anguillarum* strains recognized by VaV3 probe: *V. diazotrophicus*, *V. ordalii*, and *V. navarrensis*.
[d] Non-*V. anguillarum* strains able to grow on VAM and ferment sorbitol: *V. fluvialis*, *V. harveyi*, *V. mediterranei*, *V. metschnikovii*, and *V. anguillarum*-like.

TABLE 3. Water results from the induced vibriosis experiment

Sample group and day postinfection	Plate culture (log CFU/ml)			Colony hybridization (log CFU/ml)	Hybridization of PCR-amplified DNA[a]
	TSA1	TCBS	VAM[b]		
Infected fish tanks					
1	4.8	4.7	0	0	−
2	5.0	4.6	0	0	−
3	4.2	3.9	3.3	3.0[c]	+
4	4.2	3.7	0	0	−
5	4.1	3.8	0	0	−
6	4.9	4.4	0	0	NT[d]
10	3.2	2.6	0	0	+
15	2.7	2.3	0	0	−
20	2.4	2.2	0	0	−
30	3.9	3.9	0	0	−
Control fish tanks					
1	5.0	4.6	0	0	−
2	5.2	5.0	0	0	−
3	4.5	4.5	0	0	−
4	4.3	4.4	0	0	−
5	4.3	4.3	0	0	−
6	4.8	4.5	0	0	NT
10	2.0	1.6	0	0	−
15	3.7	3.0	0	0	−
20	2.8	2.2	0	0	−
30	4.9	4.9	0	0	−
Tanks without fish	1.3	1.0	0	NT	−
Unmanipulated fish	3.3	3.2	0	0	−

[a] Hybridization result with the specific probe (VaV3). Results with raw DNA were always negative.
[b] Only yellow colonies were counted.
[c] Colonies grown on TSA1 which gave positive signals with the VaV3 probe after autoradiography and conserved their differential growth in VAM.
[d] NT, sample not tested.

threshold of the PCR and hybridization assays was established at 8×10^2 cells, previously determined by direct fluorescence with orange acridine and plate culture in TSA1. The sensitivity of the combined method in environmental samples was 1 bacterium per 10^4 culturable bacteria.

Detection of *V. anguillarum* in samples from research aquaria. *V. anguillarum* was never detected by any method in water of the control aquaria (with PBSS-injected or unmanipulated fish or without fish). Neither the total heterotrophic bacterial population determined by plating on TSA1 nor presumptive vibrios which were able to grow on TCBS showed much variation among groups or throughout the period observed (Table 3). However, 10^3 CFU of *V. anguillarum* per ml was detected in water of aquaria with infected fish by both growth on VAM and VaV3 colony hybridization on TSA1 on day 3 postinfection. The hybridization of amplified DNA from water was also positive on day 3 postinfection and on day 10 postinfection among infected fish. No positive reaction was obtained by direct hybridization.

When fish kidneys and intestines were studied, *V. anguillarum* was clearly identified on days 2, 3, and 4 postinfection among infected fish by either the combined system or hybridization of amplified DNA (Table 4). Less sensitive results were obtained from hybridization with raw DNA, with only one positive sample being obtained. However, *V. anguillarum* was never detected on any other day among the infected fish or among the control fish studied irrespective of the method used.

Detection of *V. anguillarum* in samples from hatcheries. Neither inflowing nor effluent water showed the presence of *V. anguillarum* when VAM plus VaV3 was used, although 10^3 and 10^4 to 10^5 heterotrophic bacteria, respectively, mostly presumptive vibrios (according to differential growth in TCBS), were detected on culture plates (Table 5). Those isolates able to grow on VAM and ferment sorbitol were phenotypically characterized as *V. harveyi* or *V. anguillarum*-like. No positive result was obtained in water when hybridization techniques were used, even after PCR amplification.

V. anguillarum was detected in three of six brine shrimp and rotifer samples with less than 10 CFU per invertebrate when the combined method was used. Three of six samples presented some yellow colonies on VAM which did not hybridize with the probe. Those colonies were further identified by biochemical tests as *V. fluvialis* or *V. anguillarum*-like. No positive reaction was found by direct hybridization. However, four of five samples were positive after post-PCR hybridization. Meanwhile, detection in fish larvae, using VAM plus VaV3, was less than 50 CFU per larva for all samples. In one case, the combined method was sensitive enough to detect one *V. anguillarum* cell in 10^4 total cells able to grow on TSA1. Molecular results derived from direct hybridization of purified DNA showed no positive reaction, and post-PCR hybridization showed that only one of three samples was positive (Table 5).

Detection of *V. anguillarum* in samples from brackish lagoons. Although the number of culturable heterotrophic bacteria from water ranged from 10^3 to 10^5 CFU/ml, most of them being presumptive vibrios, *V. anguillarum* was detected only in two samples with less than 10 CFU/ml (Table 6). No hybridization of purified DNA, either raw or amplified, was detected. In sediments, the number of presumptive vibrios was higher than in water (10^4 to 10^6 CFU/ml), but only one sample was positive for *V. anguillarum* by the combined method, which coincided with the positive result for one of the water samples. *V. fluvialis* and *V. harveyi* cells grew on VAM and fermented sorbitol but they did not hybridize with the probe. No direct hybridization technique was applied to sediment samples.

Moribund adult fish had detectable levels of *V. anguillarum* in intestine (10^2 CFU/g), but not in kidney, using VAM plus VaV3. However, both results were positive after PCR-ampli-

TABLE 4. Tissue results from the induced vibriosis experiment

Study group and day postinfection	Sample from:	Plate culture (log CFU/ml)		Colony hybridization (log CFU/ml) with VaV3 from TSA1	Direct hybridization[a] of:	
		TSA1	VAM[b]		Raw DNA	PCR-amplified DNA[c]
Infected fish						
2	Kidney	6.9	7.5	7.2[d]	−	+
	Intestine	7.9	7.4	MD[e]	−	−
3	Kidney	6.5	7.2	6.4	−	+
	Intestine	6.7	6.6	6.6	+	+
4	Kidney	6.0	7.0	5.8	−	+
	Intestine	7.2	6.7	6.5	−	+
10	Kidney	0	0	0	−	−
	Intestine	7.1	0	0	−	−
15	Kidney	0	0	0	−	−
	Intestine	5.3	0	0	−	−
20	Kidney	0	0	0	−	−
	Intestine	5.8	0	0	−	−
30	Kidney	4.1	0	0	−	−
	Intestine	7.0	0	0	−	−
Control fish						
6	Kidney	4.6	0	0	NT[f]	NT
	Intestine	7.8	0	0	NT	NT
10	Kidney	0	0	0	−	−
	Intestine	3.4	0	0	−	−
15	Kidney	0	0	0	−	−
	Intestine	4.3	0	0	−	−
20	Kidney	0	0	0	−	−
	Intestine	7.0	0	0	−	−
30	Kidney	0	0	0	−	−
	Intestine	6.3	0	0	−	−
Unmanipulated fish						
	Kidney	0	0	0	−	−
	Intestine	NT	NT	NT	−	−

[a] Hybridization result with the specific probe (VaV3).
[b] Only yellow colonies were counted.
[c] Undiluted samples and 10-fold dilutions (1/10 and 1/100) were tested for PCR amplification of live food and larvae. Water samples were always undiluted. Then amplified DNA was hybridized with the specific probe (VaV3). A result was considered positive if any of the dilutions were positive.
[d] Colonies grown on TSA1 which gave positive signals with the VaV3 probe after autoradiography and conserved their differential growth in VAM.
[e] MD, missing data.
[f] NT, sample not tested.

fied DNA hybridization. Biochemical analysis revealed the presence of *V. diazotrophicus* in kidney. *V. anguillarum* and other VAM-positive strains (*V. fluvialis* and *V. anguillarum*-like) were detected in fish intestine.

DISCUSSION

A rapid, accurate combination of methods for the identification and enumeration of *V. anguillarum* within large populations of other vibrios is necessary to study its ecology in aquatic environments, especially for hygienic purposes in aquaculture. However, such a method has not been available to date.

Although other genetic probes to detect *V. anguillarum* have been proposed (4, 25), the present data show an example of the use of a DNA probe to identify *V. anguillarum* directly in environmental samples. The probe was also combined with the recently described *V. anguillarum* selective-differential medium, which allows better cell recovery than TCBS although lower recovery than marine agar (3).

VAM medium and the VaV3 probe were assayed together for the identification of *V. anguillarum* cultures among a large number of environmental *Vibrio* strains. When the results of both tests were positive, the strain studied was always *V. anguillarum*. A small number of strains were misidentified when only one of the tests was considered. On the one hand, the VaV3 probe failed to detect serovars O9 and O10 of *V. anguillarum*, as previously described (22). These serovars have been found rarely in aquatic environments and never in fish (31). In addition, the probe misidentified some strains of *V. diazotrophicus*, *V. navarrensis*, and *V. ordalii*. *V. ordalii* was described previously not to be recognized by the VaV3 probe with the strain tested (22). In this study, we tested additional strains and some of them reacted with the VaV3 probe. The position of *V. ordalii* in relation to *V. anguillarum* is still controversial. This situation is even reflected in the diversity of the 16S rRNA gene sequences deposited in the databases (e.g., sequences X74718 and D11214 in the EMBL data bank). On the other hand, the VAM medium failed to detect those strains of *V. anguillarum* which were unable to ferment sorbitol and misidentified others, mostly strains of *V. fluvialis*, *V. harveyi*, *V. mediterranei*, *V. metschnikovii*, and *V. anguillarum*-like species, which were able to grow on VAM (3). Nevertheless, with this combined method, these doubtful strains were detected, and

TABLE 5. Results from hatchery screening[a]

Sample	Plate culture (log CFU/ml)[a]			Colony hybridization with VaV3- and VAM-positive *V. anguillarum*	Direct hybridization[b] of:	
	TSA1	TCBS	VAM[c]		Raw DNA	PCR-amplified DNA[d]
Water						
Inflowing 1-H1	3.3	1.9	0	−	−	−
Inflowing 2-H1	3.0	3.0	0.7	−	−	−
Effluent 1-H1	4.6	3.4	0.7	−	−	−
Effluent 2-H1	4.9	5.0	1.6	−	−	−
Effluent 1-H2	4.1	4.2	0	−	−	−
Live food						
Rotifers 1-H1	MD	MD	MD	+[e]	−	−
Rotifers 2-H1	2.7	2.7	0.7	+	NT	NT
Rotifers 2-H2	4.1	2.9	0	−	−	+
Brine shrimps 1-H1	MD	MD	MD	−	−	+
Brine shrimps 2-H1	4.0	3.7	1.1	+	−	+
Brine shrimps 1-H2	3.7	3.8	−1.0	−	−	+
Fish larvae						
Turbot 1-H1	5.0	4.1	1.0	+	−	+
Turbot 1-H1	4.1	4.1	1.5	+	−	−
Sea bream-H1	4.3	4.4	1.0	+	−	−

[a] Results are reported as number of bacteria per milliliter of undiluted water, number of bacteria per invertebrate, or number of bacteria per larva. Rotifer samples were 10^4 organisms per ml, brine shrimp samples were 3×10^3 organisms per ml, and fish larva samples were 25 larvae per ml. Ten percent of these quantities was used for molecular detection, both direct hybridization and PCR. MD, missing data; NT, sample not tested; H1 or H2, hatchery 1 or 2, respectively.

[b] Hybridization result with the specific probe (VaV3).

[c] Only yellow colonies were counted.

[d] Undiluted samples and 10-fold dilutions (1/10 and 1/100) were tested for PCR amplification. Then amplified DNA was hybridized with the specific probe (VaV3). A result was considered positive if any of the dilutions were positive.

[e] Colonies grown on TSA1 which gave positive signals with the VaV3 probe after autoradiography and conserved their differential growth in VAM. In these cases, biochemical identification was always confirmed.

additional tests were then performed to complete their identification.

Therefore, we can conclude that with the combination of the two methods we have a very high probability of correctly detecting *V. anguillarum* in aquatic environments. The next step was then to determine the sensitivity of the single or combined methods when applied to natural samples.

The combined use of the selective medium and RNA-DNA colony hybridization provided satisfactory results when the method was applied to aquatic environments. This approach permitted us to detect one colony of *V. anguillarum* in about 10^4 colonies, even among other vibrios. It would have been extremely laborious to detect this low percentage of *V. anguillarum* with traditional methods. Moreover, the method allowed us to isolate the *V. anguillarum* strains detected. We observed that the number of *V. anguillarum* strains detected by colony hybridization in nonselective medium increased significantly with respect to the number obtained by direct plating on the selective medium because of the inhibitory effect of the VAM with respect to nonselective media (3). For this reason, although samples were initially spread on both TSA1 and VAM plates, those colonies recovered on TSA1, which were positive after VaV3 colony hybridization, were checked again on VAM, and their presence was confirmed biochemically. This verification allowed us to detect some *V. anguillarum* cells in the samples that were unable to grow directly on VAM (Table 6). No previous incubation in enrichment broth was performed, because although it could increase sensitivity in terms of presence or absence, it did not allow the quantification of *V. anguillarum* in the samples.

Sometimes, the number of positive colonies was higher in direct VAM plates than it was after VaV3 colony hybridization of cells grown in TSA1 (Tables 3 and 4). On some occasions, the colony hybridization was negative even though yellow colonies grew on VAM differential medium (Table 5). These results dealt with those strains (other than *V. anguillarum*) which could grow on VAM, producing yellow colonies, but which were never recognized by the VaV3 probe.

The results show that this combination of methods is fast and able to detect outbreaks of culturable *V. anguillarum* in fish farms or low numbers of *V. anguillarum* cells in marine and estuarine environments without subculture of isolates for complete phenotypic identification. Recently, we observed that hybridization of colonies grown on a membrane placed in the selective medium, after a short incubation in a nonselective medium, is highly sensitive and specific and, in our hands, the most rapid and reliable approach for the detection of *V. anguillarum* in the environment (data not shown).

Regarding the possible use of the probe to evaluate the presence of *V. anguillarum* directly by the application of molecular techniques, both direct hybridization and hybridization after PCR amplification were tested and compared with the combined method. Direct hybridization of purified DNA had lower sensitivity in well-defined experimental conditions (2×10^6 cells) with respect to other methods tested. This result is in agreement with other published data reporting 10^6 CFU as a direct hybridization threshold (21, 37). This low sensitivity was mainly observed in seawater samples because of the relatively small number of bacteria in relation to the probe sensitivity and DNA losses during nucleic acid extraction and purification. When we detected *V. anguillarum* from colony hybridization of fish tissue samples infected at levels ranging from 10^6 to

TABLE 6. Results from the brackish lagoon screening[a]

Sample	Plate culture (log CFU/ml)			Colony hybridization with VaV3- and VAM-positive *V. anguillarum*	Direct hybridization[b] of:	
	TSA1	TCBS	VAM[c]		Raw DNA	PCR-amplified DNA[d]
Water						
A1	3.4	2.7	NT	−	−	−
A2	4.7	3.3	NT	−	−	−
A3	3.9	3.5	0	−	−	−
A4	3.3	3.0	0	−	−	−
A5	3.5	2.9	0	−	−	−
A6	3.9	3.4	0	−	−	−
A7	3.1	2.5	0	+[e]	−	−
A8	2.5	0	0	−	−	−
A9	3.6	2.6	0	+	−	−
Sediments						
S2	6.4	4.9	NT	−	NT	NT
S3	5.9	5.6	3.0	−	NT	NT
S4	4.6	3.8	0	−	NT	NT
S5	4.1	3.4	0	−	NT	NT
S6	4.7	4.6	3.3	−	NT	NT
S7	5.0	3.6	0	+	NT	NT
S9	5.6	4.5	0	−	NT	NT
Dying fish (mullets)						
Kidney	4.0	2.4	0	−	−	+
Intestine	6.0	MD	2.5	+	−	+

[a] NT, sample not tested; MD, missing data.
[b] Hybridization result with the specific probe (VaV3).
[c] Only yellow colonies were counted.
[d] Undiluted samples and 10-fold dilutions (1/10 and 1/100) were tested for PCR amplification of tissues samples. Then amplified DNA was hybridized with the specific probe (VaV3). A result was considered positive if any of the dilutions were positive.
[e] Colonies grown on TSA1 which gave positive signals with the VaV3 probe after autoradiography and conserved their differential growth in VAM. All positive signals were less than 10 CFU/ml.

10^7 CFU/ml, results from direct hybridization were mostly negative (Table 4). We attribute this variability to the difficulty of obtaining precise results from environmental samples close to the lower threshold of detection.

PCR based on amplification of the 16S rRNA gene plus hybridization with the VaV3 probe offered the possibility of increasing the percentage of positive samples from 4% obtained by direct hybridization to 20% obtained by post-PCR hybridization. This percentage of positive results was similar to that obtained with the combined use of the selective medium and RNA-DNA colony hybridization. Results mostly agreed with those obtained under well-defined conditions (8×10^2 cells). Only one intestine sample with supposedly higher bacterial numbers failed to be identified with PCR (Table 4). In contrast, PCR was able to detect *V. anguillarum* DNA in one fish larva and one brine shrimp sample, even below the expected threshold indicated above (Table 5).

However, variable results could be obtained when PCR was used in natural complex substrates such as sediments, tissue homogenates, and even seawater; it may lack sensitivity because of inhibition of the *Taq* polymerase and/or primers (36). Unfortunately, those systems reducing inhibitory activity usually also limit the detection threshold (16). Serial dilutions of nonwater samples (32) allowed us to improve detection in 30% of the samples. To ensure the presence or absence of viable *V. anguillarum* cells, PCR following a preenrichment step could be used, as reported for other *Vibrio* and *Aeromonas* species (15, 17, 19). Finally, the use of universal eubacterial primers for PCR produced non-species-specific amplification, and consequently it increased the number of targets for the VaV3 probe, thus increasing the number of positive detections. Preferential amplification processes (27) could be involved, leading to biased results, although this has not been demonstrated. If we compare the results based on the detection of culturable cells with the results based on the direct application of molecular methods, we find 84% agreement, 9% of samples with positive results only for the former, and 7% with positive results only for the latter. Unfortunately, the sensitivity of molecular methods applied to natural samples is impossible to determine with precision, since it is impossible to obtain an exact count of the total number of a single species. This is because of the frequent presence of dead cells and also viable but nonculturable bacteria, which are detected by PCR but not by standard microbiological techniques. The presence of *V. diazotrophicus* was demonstrated in only 1 of 74 environmental samples tested. Neither *V. ordalii* nor *V. navarrensis* was detected in our environmental samples. Finally, molecular techniques may give rise to the detection of false-positives, as may have happened on day 10 in water samples collected from tanks containing infected fish. Although the exclusive use of molecular methods could lead to misidentification, the combined method did not, since it reveals the ambiguous strains for which additional phenotypic tests are required.

In summary, the combined method proposed, which coupled a selective-differential medium with rRNA-DNA colony hybridization, opens new possibilities for the diagnosis and monitoring of vibriosis in marine aquaculture and the study of the ecology of *V. anguillarum* in natural environments. For instance, the method might help to identify the natural reservoirs of aquaculture associated with *V. anguillarum* infections or facilitate the screening of live food which might be a potential carrier of the bacteria, affecting the growth and survival rates

of cultured fish (24). However, the identification of uncultured bacteria is still a challenge. Whole-cell identification of prokaryotic cells (6, 13) should be improved for application in diverse natural environments. Moreover, improved methods for DNA recovery and purification to eliminate inhibitors of PCR would represent further improvements in PCR detection of *V. anguillarum* cells in environmental samples.

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