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An Enzyme-Linked Immunosorbent Assay for Detection of *Vibrio vulnificus* Biotype 2: Development and Field Studies

ELENA G. BIOSCA,* ESTER MARCO-NOALES, CARMEN AMARO, AND ELENA ALCAIDE

Departamento Microbiología, Universidad de Valencia, Burjassot E-46100, Valencia, Spain

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***Vibrio vulnificus* biotype 2 is a primary eel pathogen which constitutes a lipopolysaccharide (LPS)-based homogeneous O serogroup within the species. In the present work, we have developed an enzyme-linked immunosorbent assay (ELISA) based on the specificity of LPS for the detection of this pathogen. The ELISA specificity was confirmed after testing 36 biotype 2 strains from laboratory cultures and environmental samples, 31 clinical and environmental biotype 1 isolates, and several strains of *Vibrio*, *Aeromonas*, and *Yersinia* species, including the fish pathogens *V. anguillarum*, *V. furnissii*, *A. hydrophila*, and *Y. ruckerii*. The detection limits for biotype 2 cells were around 10^4 to 10^5 cells/well, and the immunoassay was also able to detect cells in the nonculturable state. Artificially infected eels and environmental samples were analyzed, and the immunodetection was confirmed by cultural methods (isolation on selective and nonselective media before and after broth enrichment). With this methodology, *V. vulnificus* biotype 2 was successfully detected in infected eels and asymptomatic carriers, which suggests that eels can act as a reservoir for this pathogen.**

Vibrio vulnificus is a marine species that includes two biotypes, which have been defined on the basis of differences in host specificity (40). Biotype 1 is an opportunistic human pathogen inhabitant of estuarine environments, which can be transmitted by ingestion of shellfish or by contact with seawater, especially in temperate climates (25). During winter months, cells of this biotype become nonculturable in the aquatic environment but still retain virulence (26, 28). In contrast, biotype 2 has been classically considered an obligate eel pathogen, since to date isolation only from diseased eels has been reported (8, 35, 40). Thus, detection techniques have been focused on the recovery of biotype 1, usually by employing enrichment in alkaline peptone broth followed by isolation on selective media (21, 27, 33). Since these methods require checking of numerous colonies and are unable to recover nonculturable cells, recent molecular techniques, such as immunoassays and PCR, have been proposed to circumvent these problems (6, 16, 17, 29, 34, 38). Because clinical and environmental strains of biotype 1 are phenotypically indistinguishable and possess a high degree of DNA similarity (36, 39, 41), all of these methods have been based on species-specific traits of *V. vulnificus.*

Recent virulence studies have revealed that biotype 2 is not an obligate eel pathogen but a primary eel pathogen, able to infect both warm- and cold-water fish and mice (3, 5, 7). This biotype can behave as an opportunistic pathogen for humans as well (1, 42). In fact, the two biotypes share several virulence determinants, such as exotoxin production and expression of capsule and siderophore-dependent iron acquisition mechanisms (3, 5, 12, 14), and the exotoxins produced by the two biotypes are equally lethal for eels and mice (7). Encapsulated cells of biotype 2, like biotype 1 cells, can be transmitted to new hosts through brackish water (4), where they survive as free-living microorganisms for at least a month (9). The two biotypes also share many genotypic and phenotypic traits, including cytotoxin-hemolysin genes, immunologically related outer membrane proteins, soluble intracellular proteins, etc. (3, 13, 15, 22–24, 32, 34). As mentioned above, molecular techniques developed to detect *V. vulnificus* are based on antigenic or genetic traits specific to this species, which are shared by both biotypes. Thus, the biotypes cannot be distinguished at the biotype level. This would explain the lack of reports on the isolation of biotype 2 strains from sources others than diseased eels. In fact, we have recently identified a clinical isolate from the American Type Culture Collection as belonging to biotype 2 (1). Therefore, from an epidemiological point of view, the development of methods that allow the specific identification of biotype 2 isolates would be of interest.

From previous serological studies we know that strains of biotype 2 express a common lipopolysaccharide (LPS) profile, which is immunologically identical for each them and differs from that of the LPS for the biotype 1 strains we have tested (2, 15). We have recently proposed that biotype 2 constitutes an LPS-based homogeneous O serogroup, which comprises the strains that are pathogenic for eels (15). We have named this serogroup the E serogroup. In view of the serological homogeneity and specificity of this LPS, the objective of the present work has been to develop an indirect enzyme-linked immunosorbent assay (ELISA) based on this molecule to detect specifically biotype 2 cells. In addition, we have also evaluated our immunoassay for detection and quantification of this pathogen in environmental samples, using cultural methods for comparative purposes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 45 strains of both biotypes of *V. vulnificus* from different sources and origins as well as strains of other *Vibrio* species and other genera were used in this study (Table 1). Strains were routinely grown in Trypticase soy broth (TSB) or Trypticase soy agar (TSA) (Difco) supplemented with 0.5% (wt/vol) NaCl (TSB-1 and TSA-1, respectively). Unless otherwise indicated, isolates were incubated at 28°C.

LPS preparations, electrophoresis, and immunoblotting. LPS extracts from whole cells of selected strains were prepared as described before (2). Crude LPS was extracted from outer membrane fractions of strains treated for 2 h at 60°C with proteinase K (Sigma) (1 mg/ml) as previously described (15). LPS was purified by phenol-water extraction as described by Westphal and Jann (43). LPS samples were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (19), as previously described (2). LPS bands were visualized by immunoblotting with antisera raised against biotype 2 cells as described before (2).

* Corresponding author. Mailing address: Dpto. Microbiología, Universidad de Valencia, Av. Dr. Moliner, 50, 46100 Burjassot, Valencia, Spain. Phone: (6) 398 31 04. Fax: (6) 386 43 72.

TABLE 1. Bacterial species and isolates tested by the indirect ELISA

Species and strain(s)[a]	Source and origin	ELISA reaction[b] with antiserum against:	
		Whole cells	LPS
V. vulnificus biotype 1			
Clinical strains			
ATCC 27562^T	Blood, United States	−	−
ATCC 29306 (CDCA1402)	Corneal ulcer, United States	−	−
ATCC 33814	Blood, United States	−	−
ATCC 33815	Leg ulcer, United States	−	−
CDC 7184[c]	Human blood, United States	−	−
L-180[c]	Septicemia case, United States	−	−
VVL1[c]	Fatal wound infection, United States	−	−
374[c]	Septicemia case, United States	−	−
UMH1[c]	Fatal wound infection, United States	−	−
MO6-24 (FDA)[c]	Blood, United States	−	−
CDC H3308[c]	Clinical, United States	−	−
Environmental strains			
UNCC 890[c]	Environmental, United States	(+)	−
TW1[d]	Tank water from an eel farm, Spain	−	−
E109, E110, E112, E113, E114[d]	Surface of European eels, Spain	−	−
V. vulnificus biotype 2			
ATCC 33149	Diseased Japanese eel, Japan	+	+
ATCC 33817 (CDC B3547)	Leg wound, United States	+	+
NCIMB 2136	Diseased Japanese eel, Japan	+	+
NCIMB 2137	Diseased Japanese eel, Japan	+	+
NCIMB 2138	Diseased Japanese eel, Japan	+	+
UE516[e]	Diseased Japanese eel, Taiwan	+	+
E4, E12, E22, E24, E27, E32, E37, E39, E40, E52, E56, E58, E80, E86, E92, E103, E105, E106, E112, E113, E116[d]	Diseased European eel, Spain	+	+
V. aesturianus NCIMB 2236^T		−	−
V. alginolyticus NCIMB 1903^T		−	−
V. anguillarum NCIMB 6^T		−	−
V. mytilii CECT 632^T		−	−
V. campbellii NCIMB 1894^T		−	−
V. carchariae NCIMB 12705^T		−	−
V. cholerae NCTC 8021^T		(+)	−
V. cincinnatiensis NCTC 12012^T		−	−
V. damsela CECT 626^T		−	−
V. diazotrophicus NCIMB 2169^T		(+)	−
V. fischeri NCIMB 1281^T		−	−
V. fluvialis NCTC 11327^T		−	−
V. furnissii ATCC 35016^T		−	−
V. harveyi NCIMB 1280^T		−	−
V. hollisae ATCC 33564^T		(+)	−
V. logei NCIMB 2252^T		−	−
V. mediterranei CECT 621^T		−	−
V. metschnikovii ATCC 7708^T		−	−
V. mimicus NCTC 11435^T		−	−
V. natriegens CECT 526^T		−	−
V. nereis NCIMB 1897^T		−	−
V. nigripulchritudo NCIMB 1904^T		−	−
V. ordallii ATCC 33509^T		−	−
V. orientalis NCIMB 2195^T		−	−
V. parahaemolyticus CECT 511^T		−	−
V. pelagius ATCC 25916^T		−	−
V. proteolyticus NCIMB 1326^T		−	−
V. splendidus NCIMB 1^T		−	−
V. tubiashii NCIMB 1340^T		(+)	−
V. furnissii 145[f]	Diseased European eel, Spain	−	−
A. hydrophila EO19[f]	Diseased European eel, Spain	−	−
A. hydrophila EO63[f]	Diseased European eel, Spain	−	−
Y. ruckeri O1[g]	Diseased rainbow trout, Italy	−	−
Y. ruckeri O2[g]	Diseased rainbow trout, Italy	−	−
Y. ruckeri O3[g]	Diseased rainbow trout, Italy	−	−
E. coli CECT		−	−
Vibrio sp. isolates		−	−
Aeromonas sp. isolates		−	−
Unknown isolates		−	−

Continued on following page

Antisera and serological testing. Polyclonal antibodies against formalin-killed cells of *V. vulnificus* biotype 2 strains ATCC 33149, NCIMB 2137, E22, E39, E86, and E105 were generated as described before (2). Additional antisera were raised against crude and purified LPSs of selected biotype 2 strains as previously described (15). Crude and purified LPS samples were prepared from selected biotype 2 strains and examined by immunoblotting of SDS-PAGE gels before being used for rabbit immunization (10). Only LPS samples retaining the O side chain moiety responsible for the serogroup specificity and immunogenicity were used for immunization after confirmation of their lack of toxicity by using rats as an animal model. The immunization schedule was as follows. Rabbits were first immunized subcutaneously with 50 μg of crude LPS and 25 μg of purified LPS in Freund's complete adjuvant. Booster immunizations in incomplete Freund's adjuvant were administered 3 and 5 weeks later. One week after the last injection, rabbits were bled from the ear vein. All sera were stored in aliquots frozen at −20°C until used.

Development of the indirect ELISA. (i) Procedure. Due to the fact that biotype 2 shares thermolabile, immunologically related proteins with biotype 1 (13), two approaches were attempted in order to reduce unspecific reactions: (i) the adsorption temperature was increased to 69°C, and (ii) heated cells were used as antigens. After several coating parameters (coating buffer, plate type, antigen concentration, temperature, and time) were tested for optimization of antigen adsorption, the procedure selected was the following. Flat-bottomed plates (Nunc-ELISA Maxisorp) were coated for 2 h at 69°C with 100 μl of a whole-cell suspension of 10^8 cells/ml in phosphate-buffered saline (PBS) (pH 7.2) per well. The unbound material was washed off with PBS containing 0.05% (vol/vol) Tween 20 (PBST), and plates were incubated for 2 h at 37°C with 100 μl of anti-biotype 2 rabbit antiserum in PBST with 1% (wt/vol) bovine serum albumin (BSA) (Sigma) (PBST-BSA) per well. After being washed, wells were incubated for 1 h at 37°C with 100 μl of diluted alkaline phophatase-conjugated goat anti-rabbit immunoglobulins (Bio-Rad) in PBST-BSA. The plates were washed, and then 100 μl of substrate solution containing 1 mg of *p*-nitrophenyl phosphate (Sigma) per ml diluted in diethanolamine buffer (pH 8) was added to each well and incubated for 1 and 2 h at 37°C in the dark. Absorbance values were immediately determined with a microplate reader (Multiscan microtiter plate reader) set at 405 nm. Results were considered positive if absorbances were equal to or greater than 0.5 after subtraction of values for negative control samples. In all experiments *V. vulnificus* biotype 1, *Escherichia coli*, and *Aeromonas hydrophila* strains were used as negative controls. Bacterial cells were omitted from some control wells.

(ii) Specificity. The specificity of the ELISA was determined by using different antisera raised against several biotype 2 strains as well as different antigens, including biotype 2 strains from different geographical origins, biotype 1 strains, and some eel-pathogenic species such as *Vibrio anguillarum*, *Vibrio furnissii*, *A. hydrophila*, and *Yersinia ruckeri*. To avoid cross-reactivity with biotype 1 and to reduce the nonspecific background ELISA signal, two approaches based on the serological specificity of the LPS were used: (i) adsorption of nonspecific antibodies to biotype 1 cells (the two biotypes share antigenically related outer membrane proteins) and *Aeromonas* cells (aeromonads constitute part of the microbiota of eels and freshwater [18]) and (ii) raising of antisera against crude and purified LPSs of biotype 2 strains. The adsorption was performed by incubating antisera for 2 h at 37°C and overnight at 4°C with an acetone powder extract from whole-cell bacterial cultures of selected strains.

(iii) Sensitivity. To determine the detection limits of the ELISA, decreasing numbers of *V. vulnificus* biotype 2 cells (ranging from 10^7 to 10^1 cells/well) were applied to wells, and different dilutions of the appropriate antisera were used.

(iv) Assessment. Before field studies, an assessment was carried out by using kidneys and livers of healthy and artificially infected eels (average weight, 10 g) with intraperitoneal (14) or bath (4) challenge. Tissue samples were diluted 1:5 (wt/vol) in PBS and homogenized. Samples of water and mucus were occasionally taken as previously described (4). Aliquots of all types of samples were diluted 1:10 in sterile alkaline peptone water (APW) with 1, 2, and 3% (wt/vol) NaCl (APW-1, APW-2, and APW-3, respectively); TSB-1 was also employed for normally sterile sites (internal tissues). Both kind of media were incubated for 4 h at 28°C with gentle shaking and then processed for ELISA and plate counting on TSA-1, cellobiose-polymyxin B-colistin (CPC) agar, and thiosulfate-citrate-bile-sucrose (TCBS) agar, and plates were incubated at 28, 39, and 37°C, respectively.

The immunodetection was confirmed in all cases by identification of suspected colonies by slide agglutination with polyclonal antiserum against biotype 2 cells and inoculation with the API 20E system as previously described (4, 11).

(v) Detection of nonculturable cells. Since culture methods are unable to recover nonculturable cells, we also evaluated the ability of the immunoassay to detect dormant biotype 2 cells. The dormancy was induced in saline-water microcosms maintained at 4°C as previously described (9).

Immunodetection in environmental samples. One eel farm with a previous history of *V. vulnificus* infections was chosen for environmental sampling during the warmer months of 1994 and 1995. Samples, including livers and kidney of eels (average weight, 100 g/fish), glass eels (body weight, around 0.1 to 0.2 g/fish), tank water, and sediment, were collected in sterile containers and kept at 4°C until being processed, after about 1 h. Internal organs of eels and glass eels from other eel farms, without records of *V. vulnificus* infections, were employed as negative controls (18). All farm samples were tested for the presence of biotype 2 by both cell culture isolation and indirect ELISA, as mentioned above. Each sample was split into two subsamples, which were processed either immediately or after 4 h of enrichment in APW-1. Additionally, around 1 liter of tank water was filtered in aliquots of 200 ml throughout 0.45-μm-pore-size filters (Nucleopore). Filters were either placed directly on CPC agar or enriched in APW-1. In all cases, isolates that were cellobiose positive on CPC agar and sucrose negative on TCBS agar were evaluated by ELISA and by the API 20E system after subculturing on TSA-1. Positive results in the ELISA were confirmed by means of identification with the API 20E system after subculturing on TSA-1.

RESULTS AND DISCUSSION

ELISA specificity. Studies on the serology of *V. vulnificus* have clearly demonstrated that isolates of biotype 1 contain heterologous surface antigens (2, 13, 20, 31, 40). By contrast, biotype 2 isolates constitute a homogeneous O serogroup, which we have called serogroup E (from eels) (15). Thus, serologically different LPSs have been detected among biotype 1 strains (2, 15, 20), while eel isolates present the same LPS profile (2, 15). This LPS is immunologically distinguishable not only from the LPSs of biotype 1 strains tested but also from the LPSs of 29 reference *Vibrio* strains (15). In the present study we have confirmed our previous results, since the LPSs of representative serotypes of *A. hydrophila* and *Y. ruckerii* were not revealed by immunoblotting with antiserum against biotype 2 cells (Fig. 1). Thus, we chose this molecule to design an ELISA for the specific detection of this eel pathogen.

First, rabbit antiserum against whole cells of biotype 2 was tested. As expected, all biotype 2 isolates from different sources and geographic origins were positive in this immunoassay (Table 1). Nevertheless, a weak cross-reactivity (absorbance values at 405 nm of between 0.2 and 0.35) with some biotype 1 strains and other vibrios, particularly reference strains of *V. cholerae*, *V. diazotrophicus*, *V. hollisae*, *V. splendidus*, *V. fisherii*, and *V. orientalis*, as well as with several aeromonad strains was observed (Table 1). Since cells were coated at 69°C, biotype 2 must share thermostable antigens with these strains. In order to decrease cross-reactivity, the antiserum was adsorbed with dehydrated cells of some biotype 1 and aeromonad strains. Cross-reactions were diminished, although a large drop in titers was observed (data not shown). In view of these results, and due to some cross-reactivity with bacteria other than those adsorbed, this method was discarded. Sera against crude and purified LPSs were obtained. Cross-reactions were weaker or disappeared with sera against crude or purified LPS, respectively (Table 1). As expected, all biotype 2 strains gave a strong reaction with both kinds of antisera (Ta-

[a] ATCC, American Type Culture Collection, Rockville, Md.; CDC, Centers for Disease Control and Prevention, Atlanta, Ga.; FDA, Food and Drug Administration, Cincinnati, Ohio; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland; CECT, Coleccion Española de Cultivos Tipo, Valencia, Spain; NCTC, National Collection of Type Cultures, London, United Kingdom; T, type strain.

[b] +; positive reaction; (+), weakly positive reaction; −, negative reaction.

[c] Supplied by J. D. Oliver, University of North Carolina, Charlotte.

[d] See references.

[e] Supplied by Y.-L. Song, National Taiwan University, Taipei, Taiwan.

[f] Supplied by C. Esteve, University of Valencia, Valencia, Spain.

[g] Supplied by G. Bovo, Instituto Zooprofilactico Sperimentale of Venice, University of Padua, Padua, Italy.

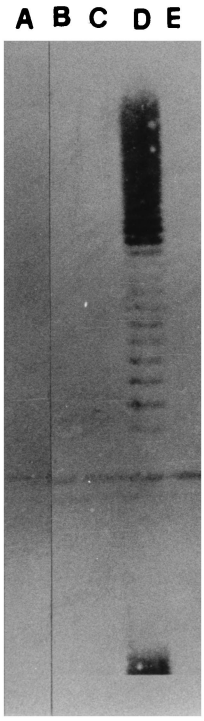

FIG. 1. SDS-PAGE of LPSs. Lane A, *Y. ruckerii* O1; lane B, *Y. ruckerii* O2; lane C, *Y. ruckerii* O3; lane D, *V. vulnificus* biotype 2 E86; lane E, *A. hydrophila* EO63. Gels were stained by immunoblotting with antiserum against crude LPS from *V. vulnificus* E86.

ble 1). Consistent with previous results with dot blot assays (15), several differences in absorbance values were observed among Japanese and European strains (data not shown). Both results suggest that two subserogroups could exist among biotype 2 isolates. Further characterization of lateral O side chains of purified LPSs of Japanese and European biotype 2 strains to confirm this hypothesis is in progress.

ELISA sensitivity. Absorbance values on ELISA plates containing different amounts of biotype 2 cells were determined by using three antisera raised against three kinds of biotype 2 antigens (formalized cells, crude LPS, and purified LPS). The detection limits were between 10^3 and 10^4 cells/well and between 10^4 and 10^5 cells/well for antisera raised against formalized cells and crude or purified LPS, respectively (Fig. 2). One of the reasons for the lower detection limit with anti-LPS sera could be the nature of the antigen we selected, that is, a T-cell-independent antigen. The levels of sensitivity coincide with those reported for other immunoassays (30, 37). The number of biotype 2 cells required to infect eels through water is around 10^5 to 10^7 CFU/ml when bacteria are experimentally challenged by immersion (4). This number is within the detection limits achieved by our ELISA. Nevertheless, the current levels reported for detection of *V. vulnificus* biotype 1 in environmental samples are between 10^1 and 10^4 CFU/ml (24, 38, 44). In an attempt to improve our ELISA, a sandwich ELISA was designed by using rat anti-LPS sera as capture antibodies, but the sensitivity was lower than that found with the indirect ELISA, so the method was discarded (unpublished data).

Assessment of ELISA. The assessment of our ELISA was carried out by using kidney and liver samples from healthy and artificially infected eels. In all cases, detection of biotype 2 was positive for liver and kidney samples from infected eels and negative for tissue samples from noninfected eels (Table 2). The immunoassay was evaluated against conventional culture procedures, and positive results were considered presumptive until confirmation by standard methodologies was achieved. Detection was further validated by a new PCR methodology that uses primers derived from the hemolysin gene of *V. vulnificus* (17). This method had been previously developed for detection of this species from oysters and eel tissues (17). Furthermore, samples from tank water and surfaces of moribund fish and survivors challenged by immersion were also analyzed.

We have confirmed the utility of the selective medium CPC agar for the recovery of biotype 2 cells from all kinds of samples, especially from water samples and the surfaces of diseased fish artificially infected by immersion (Table 2). *V. vulni-*

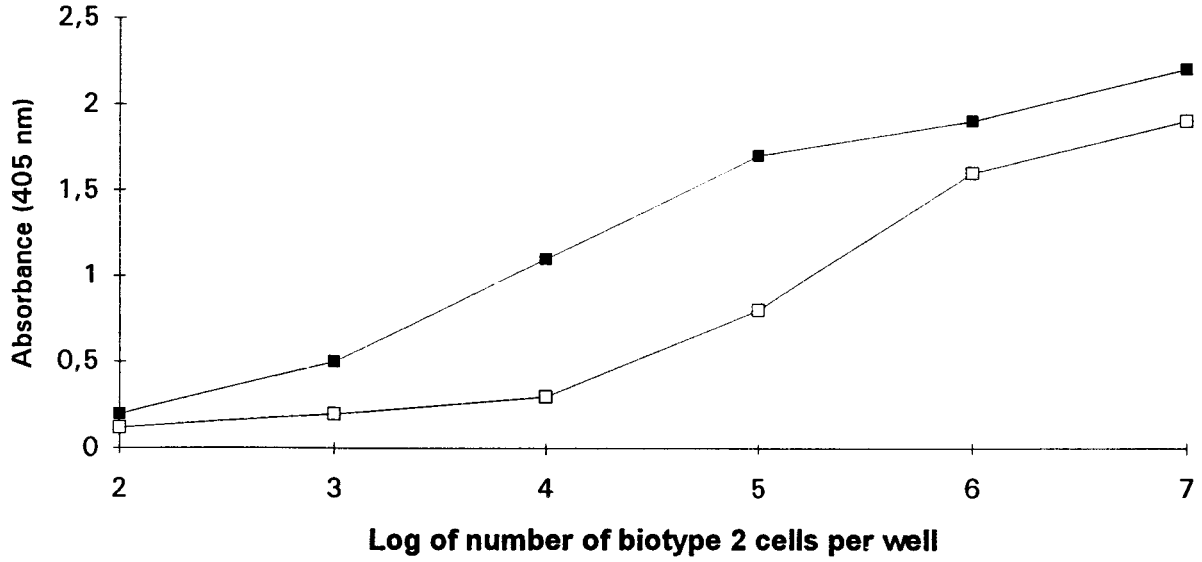

FIG. 2. Sensitivity of ELISA with antisera raised against whole cells (■) and crude LPS (□).

TABLE 2. Detection of *V. vulnificus* biotype 2 in artificially infected samples

Sample	Detection			
	Without enrichment[a]		With enrichment[b]	
	Cultural methods[c]	ELISA[d]	Cultural methods	ELISA
Liver, kidney, or mucus of negative control eels	−	−	−	−
Liver or kidney of carrier eels	−	−	+/−[e]	+/−
Liver or kidney of infected eels	+	+	+	+
Infected tank water	+	+	+	+
Mucus of bath-infected eels	+	+	+	+

[a] Samples were processed immediately.
[b] Samples were processed after 4 h of enrichment in APW-1 or TSB-1.
[c] Detection by cultural methods on TSA-1, TCBS, or CPC agar plates. +, detection after biochemical and serological identification; −, no detection.
[d] Detection by ELISA. +, detection (absorbance was greater than 0.5 after subtraction of value for negative controls); −, no detection.
[e] Detection was achieved in some samples.

ficus is not a predominant species in the aquatic environment (25). If this bacterium is present in the farm environment and/or in healthy eels, it must be in lower numbers, as happens with other fish pathogens (18). Thus, the use of selective media combined with enriched conditions might aid in the detection of the pathogen in environmental samples. Therefore, different enrichment conditions were tested for improvement of the signal for immunodetection and of recovery on selective media. The enrichment conditions that gave the best results were incubation of samples for 4 h in APW-1 at 28°C. These conditions of salinity and temperature coincide with the optimal ones needed for waterborne transmission of biotype 2 as well as with those recorded during outbreaks (3, 4, 8, 15). For internal organs, enrichment in TSB-1 was also employed. The use of enrichment in artificially infected eels did not improve the detection by either conventional procedures or ELISA. Nevertheless, sometimes a culture step was necessary before immunological detection with artificially infected fish, probably due to the physical limitation of the small weight (less than 0.5 g) of the internal organs employed.

Finally, while conventional methods allow detection only of culturable cells, the ELISA described in this work allowed us to detect nonculturable biotype 2 cells, suggesting that the antigenic specificity of biotype 2 must be conserved even in the nonculturable state. This may be explained by the fact that our ELISA is based on the immunological recognition of a constitutive component of the outer membrane, the LPS.

Environmental samples. Twelve sampling campaigns were carried out in the warmer months of 1994 and 1995. All samples were tested for the presence of *V. vulnificus* biotype 2 by both conventional methods and ELISA. *V. vulnificus* biotype 2 was detected in internal organs by either one or both methods. Occasionally, several discrepancies were noted, since false-negative results were detected by ELISA in enrichment broth from internal organs and vice versa. These cases could be explained (i) by competition with other bacteria that would not allow biotype 2 to multiply and reach a high enough number to produce a positive signal in ELISA and/or (ii) by the presence of biotype 2 cells in the nonculturable state. Except for in a punctual outbreak, which happened in September 1994, detection of biotype 2 from internal organs of asymptomatic fish was positive. These fish had been vaccinated against the vibriosis caused by *V. vulnificus* biotype 2 and were in a good immunological state. The positive detection of biotype 2 in asymptomatic carriers suggests that eels could be one of this pathogen's reservoirs. Further, this could explain the reappearance of the pathogen in the same farm at widely separate times from 1989 to 1994 (15). In fact, this is the first time that the isolation of biotype 2 from samples other than diseased eels has been reported. Recovery of biotype 2 cells from asyptomatic eels was achieved only after enrichment in TSB-1 medium and plating onto TCBS agar and TSA. This could be explained by the efficiency of recovery of biotype 2 observed with CPC agar, which was around 10^3 to 10^4 times lower than with TCBS agar and TSA.

Detection of biotype 2 in water and sediment was negative by both ELISA and culture methods. The farm environment is very different from the marine one in terms of salinity, suspended organic matter, temperature, microbiota, and presence of bacteria with multiple drug resistance (18). Moreover, from previous results we know that biotype 2 cells are not successfully recovered from freshwater or from eel surfaces challenged in freshwater (4). Thus, the present results are consistent with those previously reported, since farm tanks contain water that is almost the same as freshwater. However, some positive background was detected by ELISA with glass eel homogenates. In this case, none of the cellobiose-positive colonies on CPC agar, along with some cellobiose-negative colonies used as a control, had an API 20E profile which was consistent with *V. vulnificus* biotype 2. Since the background levels in glass eel homogenates were not correlated with isolation, we could not confirm the presence of the pathogen in elvers. However, it is possible that these negative results were due to overgrowth of other bacterial species, such as aeromonads, that are predominant in healthy elvers (18).

V. vulnificus biotype 1 was successfully recovered from CPC agar plates seeded with sediment, tank water, and surface eel (mucus) samples, and absorbance values detected in ELISA plates were similar to those observed for negative controls. Some of the cellobiose-positive strains had a typical *V. vulnificus* biotype 1 profile in the API 20E system, whereas none of the cellobiose-negative colonies gave *V. vulnificus* profiles. Finally, CPC medium gave the best results for the recovery of *V. vulnificus* biotype 1 from water, sediment, and eel surface samples, whereas TCBS agar was more suitable for the recovery of biotype 2 from internal organs.

Although the total heterotrophic bacterial population showed some variation during sampling events, our results are consistent with those reported in similar studies (18).

In conclusion, the ELISA developed in the present work constitutes the first molecular method which directly identifies *V. vulnificus* biotype 2, making it a useful tool for detection and identification, since previously described techniques do not discriminate between biotypes. This ELISA can be used instead of conventional biochemical and serological tests for detection of this biotype, at least in samples from eel farms. Due to the economic losses caused by this pathogen in eel farms in our area, and bearing in mind that cells of biotype 2 are able to invade and cause infection in eels, it seems evident that direct detection of biotype 2 is more valuable for sanitary controls than diagnosis at the species level. Further, accurate identification of biotype 2 would improve ecological and clinical investigations of this microorganism. Finally, as long as there is interest in characterizing new isolates of this pathogenic bacterium, isolation by culture must continue.

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