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Enzyme Immunoassay for Identification of *Vibrio vulnificus* in Seawater, Sediment, and Oysters

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Historically, methods used to identify *Vibrio vulnificus* in environmental samples have been inadequate because isolation and identification procedures are time-consuming and fail to separate *V. vulnificus* from other bacterial species. We describe an enzyme immunoassay (EIA) and culture techniques which identified *V. vulnificus* in seawater, sediment, and oysters. The EIA used monoclonal antibody FRBT37 to a species-specific epitope of *V. vulnificus*. No cross-reactions were observed among 72 non-*V. vulnificus* strains comprising 34 species and 15 genera. In field trials, the EIA identified correctly 99.7% of 348 biochemically confirmed *V. vulnificus* isolates. The epitope corresponding to FRBT37 was found in cells lysed by Triton X-100, deionized H_2O, and ultrasonication but was not found in culture supernatants, indicating that its location was intracellular. In addition, electron micrographs of *V. vulnificus* labeled with FRBT37-biotin-avidin-gold showed that epitope FRBT37 reacted with fragments of lysed cells but not whole cells. FRBT37 was expressed when *V. vulnificus* was cultured in different growth media. The minimum level of detection of the EIA was approximately 2,000 *V. vulnificus* cells per EIA well. Epitope FRBT37 was labile at 70°C for 30 min. Immunoblot and EIA plate formats reduced assay time and facilitated handling large numbers of test samples.

Vibrio vulnificus is an autochthonous bacterium of estuarine and marine waters of temperate and tropical climates (17, 21, 22), where seafoods and water can be vectors for transmission of *V. vulnificus* to humans (5). Early studies of the distribution of *V. vulnificus* in nature, as well as clinical investigations, used methods developed for other *Vibrio* species. Typically, those methods included incubation of test samples in alkaline peptone water (APW), followed by isolation on thiosulfate-citrate-bile salts-sucrose (TCBS) agar, and identification by a battery of biochemical and growth tests (11, 22).

The procedures for isolation and identification of *V. vulnificus* are complicated by the replication of other bacteria in APW and on various agars (14). Consequently, numerous colonies of *V. vulnificus* and non-*V. vulnificus* bacteria must be tested by using a number of biochemical assays (5, 26) which are frequently limited by time and cost.

Development of new isolation media and identification tests for *V. vulnificus* will probably improve environmental, clinical, and epidemiological studies. Colistin-polymyxin B-cellobiose (CPC) agar (15) and sodium dodecyl sulfate-polymyxin B-sucrose agar (3) have been reported as new selective media for *V. vulnificus*; however, few studies describe their use in the field. Microimmunodiffusion (16) and coagglutination (18) methods specific for *V. vulnificus* reduce biochemical testing but lack sensitivity and are tedious to perform on large numbers of isolates.

The present report describes an enzyme immunoassay (EIA) which uses *V. vulnificus*-specific monoclonal antibody (MAb) FRBT37 to identify relatively low numbers of *V. vulnificus* organisms in enrichment broths and agar cultures of oysters, sediment, and seawater.

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MATERIALS AND METHODS

Production of MAb FRBT37. *V. vulnificus* ATCC 27562 was cultured on tryptic soy agar (TSA; Difco Laboratories, Detroit, Mich.) for 24 h at 35°C. Colonies were suspended in phosphate-buffered saline (PBS; 5.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.13 M NaCl [pH 7.4]), treated with 1% formaldehyde for 30 min at room temperature, washed three times with PBS (5,000 × *g* for 15 min), and suspended in PBS to approximately 10^8 cells per ml (A_{420} = 0.64). BALB/c mice were immunized by intraperitoneal injection of 0.25 ml of the *V. vulnificus* suspension mixed 1:1 with Freund complete adjuvant (Sigma Chemical Co., St. Louis, Mo.). On day 21, the mice were injected with *V. vulnificus* and Freund incomplete adjuvant (Sigma), on day 35 they were injected with 0.5 ml of *V. vulnificus* in PBS, and 3 days before fusion they were injected with *V. vulnificus* in PBS. Splenocytes and myeloma SP2/0 were fused by using the protocol of Van Deusen and Whetstone (24). After selection with hypoxanthine-aminopterin-thymidine medium, hybridoma supernatants were screened by EIA against a panel of bacteria. Selected hybridomas were cloned by limiting dilution (1). The isotype of MAb FRBT37 was determined by using an EIA kit and following the manufacturer's (Boehringer Mannheim, Indianapolis, Ind.) instructions.

Specimens. Oysters (*Crassostrea virginica*) were collected from U.S. Gulf Coast estuaries. The shell stock used for isolation of *V. vulnificus* and testing of the specificity of the EIA was harvested primarily outside Mississippi and transported to Mississippi processing plants. The remaining studies were conducted with oysters, sediment, and seawater collected at Cedar Point, north of Dauphin Island, Ala. Specimens were transported to the laboratory and analyzed within 2 h. Oyster shells were scrubbed with a brush under running water, opened with a shucking knife, and transferred to sterile blender jars.

Isolation and enumeration of *V. vulnificus*. The procedures used and the formulations of the media and reagents used

have been described previously (12). Deviations from those procedures are discussed below. In each analysis, approximately 200 g of oyster meats (12 to 15 oysters), 10 g of sediment, and 10 ml of seawater were tested. Oyster meats and sediment were homogenized with an equal weight of sterile PBS (23). Dilutions of 10^{-1} to 10^{-6} were prepared by the procedure of Cook and Pabst (4), except that PBS was used as the diluent.

A three-tube most-probable-number technique using APW enrichment was used. APW was inoculated with 1.0-ml samples of the appropriate dilutions and incubated for 12 h at 35°C. APW exhibiting turbidity was streaked on CPC and/or TCBS agar and incubated for 10 to 12 h at 40 and 35°C, respectively. CPC agar was modified to contain 400,000 U of colistin methanesulfonate.

V. vulnificus-like colonies were transferred from CPC and TCBS agars and streaked for isolation on T_1N_1 agar (23). Isolated colonies from the highest dilution exhibiting *V. vulnificus*-like colonies on CPC from all three most-probable-number tubes and suspect colonies from higher dilutions were selected for further testing. TCBS colonies were used when *V. vulnificus*-like colonies were absent from CPC agar. Initial screening included a motility test (motility test medium; Difco), a β-D-galactosidase test using *o*-nitrophenyl-β-D-galactopyranoside, and a coagglutination test (18).

Slide agglutination tests were performed by the following procedures. Growth from an 18- to 24-h culture (TSA supplemented with 1% lactose) was suspended in 1 to 2 ml of Tris–EDTA–Triton X-100 buffer (18) and incubated at room temperature overnight. A drop of the cell suspension was mixed with a drop of coagglutination reagent on a glass slide. The mixture was observed for agglutination after the slide was rocked over indirect light.

Motile, β-D-galactosidase-positive colonies which agglutinated in the coagglutination test were subjected to biochemical tests, including salt requirement, oxidase, and indole tests; fermentation of sucrose, lactose, mannitol, mannose, arabinose, salicin, cellobiose, maltose, trehalose, galactose, and glucose; and decarboxylase reactions (arginine, ornithine, and lysine).

Salt requirements were determined in 1% tryptone broth containing 0, 3, 6, 8, and 10% NaCl. Carbohydrate fermentations were determined in purple broth base (Difco) containing 1% carbohydrate. Decarboxylase reactions were determined in decarboxylase base medium (Difco) containing 1% amino acid.

Representative isolates from each lot of oysters were stored at −60°C.

EIA procedure. Hybridoma supernatants were screened for MAbs by coating EIA plates (2595; Costar, Cambridge, Mass.) with 50-μl suspensions containing 10^8 CFU of bacteria (see Table 1) which were pretreated with 1% formaldehyde as described in the immunization protocol. Gram-negative suspensions were dried in wells of EIA plates in PBS at 35°C for 18 to 24 h. Gram-positive bacteria were adhered to EIA plates in 0.1 M Na_2CO_3 (pH 9.6) at 4°C for 18 to 24 h. After attachment of bacteria, EIA plates were treated sequentially for 1 h each with 200 μl of 1% bovine serum albumin in PBS, 50 μl of hybridoma supernatant, and 50 μl of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; heavy and light chain specific; Organon Teknika, Malvern, Pa.). After incubation with each reagent, EIA plates were washed three times with 0.15 M NaCl–0.05% Tween 20. Bound conjugate was observed by addition of 100 μl of substrate solution (0.003% H_2O_2, 0.05 M citric acid, [pH 4.0], 1 mg of 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid per ml, Sigma) to wells of EIA plates and measuring the A_{405} with a 96-well optical plate reader (BIO-TEK Instruments, Winooski, Vt.) after incubation at room temperature.

V. vulnificus-like colonies on CPC, TSA, or T_1N_1 agar were transferred with sterile wooden sticks to wells of a sterile 96-well culture plate (3595; CoStar) containing 100 μl of APW and incubated for approximately 4 to 6 h at 35°C. In some experiments, APW enrichment cultures of oyster meats, seawater, and sediment were tested directly by EIA. A 25-μl volume of an APW culture was mixed with 25 μl of 0.02% Triton X-100 (Eastman Kodak Chemical Co., Rochester, N.Y.) in wells of EIA plates and evaporated at 35°C for 18 h. The EIA procedures and reagents used were identical to those used to screen hybridomas.

The sensitivity of the EIA was determined by using twofold serial dilutions (25 μl) of *V. vulnificus* mixed 1:1 with 0.02% Triton X-100. The optimum concentration of Triton X-100 was determined by using twofold serial dilutions of 1% Triton X-100 (25 μl) mixed with APW (25 μl) containing 10^8 CFU of *V. vulnificus* per ml. The EIA was conducted as described above.

To determine whether the vehicle influenced the EIA reaction, 0.02% Triton X-100 was added to deionized H_2O, PBS, or APW. Each Triton X-100 solution was mixed 1:1 with an APW culture of *V. vulnificus* ATCC 27562 and tested by EIA as described above. Controls consisted of 25 μl of a *V. vulnificus* APW culture mixed 1:1 with deionized H_2O, PBS, or APW, without Triton X-100.

Preparation and use of peroxidase-conjugated MAb. IgG was purified from MAb FRBT37 ascites fluid by using protein A-Sepharose (10). The quantity of purified IgG was determined at A_{280} by using an extinction coefficient of 1.4 (1). We added 0.5 mg of type VI-A peroxidase (0.5 ml of a 2-mg/ml solution; Sigma) to 0.1 ml of purified MAb FRBT37 (1 mg/ml). Peroxidase was conjugated to MAb FRBT37 by a one-step glutaraldehyde method (25). The conjugate was diluted to 3.0 ml with Tris-HCl buffer (0.05 M, pH 8.0) containing 1.0% bovine serum albumin and 0.02% NaN_3 and stored at 4°C. The working dilution of conjugate was determined by EIA by using plates coated with *V. vulnificus* ATCC 27562 as described above.

Immunoblot. APW cultures were mixed with an equal volume of 0.02% Triton X-100 and incubated at room temperature for 15 min. Next, 100 μl of the APW-Triton X-100 mixture was transferred to wells of a microsample filtration manifold (Schleicher & Schuell, Inc., Keene, N.H.) containing a 0.45-μm-pore-size nitrocellulose membrane (BA85; Schleicher & Schuell) and filter paper (GB0003; Schleicher & Schuell). After 15 min at room temperature, the remaining contents of the wells were removed by filtration. The nitrocellulose membrane was then removed from the manifold, blocked for 30 min at 35°C in PBS containing 5% bovine serum albumin, washed for 5 min in PBS–0.5% Tween 20, and incubated with MAb FRBT37 diluted in PBS to a working dilution for 30 min at 35°C with gentle rocking. The membrane was washed for 15 min with three changes of PBS-Tween. Alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) in PBS was added to the membrane, incubated for 30 min at 35°C with gentle rocking, and washed for 15 min as described above, except that the last wash was made with alkaline phosphatase substrate buffer (10 μl of diethanolamine, 10 mg of $MgCl_2 \cdot 6H_2O$, 100 ml of deionized H_2O [pH 9.8]). Nitro Blue Tetrazolium chloride (44 μl; Bethesda Research Laboratories, Gaithersburg, Md.) and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (33 μl;

Bethesda Research Laboratories) were added to 10 ml of alkaline phosphatase substrate buffer and incubated with the membrane for 20 to 30 min at room temperature with gentle shaking. The enzyme substrate reaction was stopped by washing the membrane with distilled water. The results were observed visually.

Electron microscopy. A formaldehyde-fixed suspension of 10^8 CFU of *V. vulnificus* ATCC 27562 per ml was floated on a Formvar-coated, carbon-reinforced 300-mesh copper grid (EM Sciences, Fort Washington, Pa.) for 15 min, air dried for 3 min, and then treated with the following reagents for 30 min each: 1% bovine serum albumin, MAb FRBT37, biotin-conjugated goat anti-mouse IgG (Sigma), and streptavidin-gold (particle diameter, 20 nm; Polysciences, Warrington, Pa.). After each treatment, grids were washed with three changes of PBS for three min each and a then in deionized H_2O. Specimens were viewed on a transmission electron microscope (301; North American Philips, Mahwah, N.J.) at 60 kV and at magnifications of 34,000 to 110,000.

Characterization and expression of epitope FRBT37. The presence of epitope FRBT37 was evaluated after culture in broth and agar media. *V. vulnificus* ATCC 27562 and C7184 were grown on TSA at 35°C for 18 h and then transferred to tryptic soy broth (Difco), TSA, brain heart infusion broth and agar (Difco), heart infusion agar (Difco), or APW. Agar cultures were incubated for 18 h at 35°C; broth cultures were incubated for 4 h at 35°C on a rocking platform. Bacteria were washed with PBS, fixed with 1% formaldehyde, suspended at 10^8 CFU/ml in PBS, and tested by EIA.

Lysed suspensions of *V. vulnificus* ATCC 27562 were prepared by sonication or suspension in deionized H_2O. Cells were sonicated by using a 250 sonicator (Branson Ultrasonics, Danbury, Conn.) at 100 V for 15 min at 4°C. Alternatively, cells were lysed in deionized H_2O for 1 h at 25°C. Lysis was verified by staining preparations with acridine orange and examining them by epifluorescence microscopy (8). After both treatments, lysates were centrifuged at 5,000 × *g*. Pelleted material and supernatants were tested by EIA.

In separate experiments, the thermal stability of epitope FRBT37 was determined by treating 10^8 CFU of *V. vulnificus* per ml of PBS at 25, 40, 70, 80, and 100°C for 30 min, followed by EIA.

Expression of epitope FRBT37 by opaque and transparent morphological forms of *V. vulnificus* was determined. One opaque and three transparent strains of *V. vulnificus* 4965, an environmental isolate, were isolated on TSA containing 1% NaCl and incubated at 25°C for 18 h. A colony of bacteria was transferred to a new TSA plate, and after 18 h at 25°C, cells were suspended in PBS at a concentration of 10^8 CFU/ml. The EIA was performed as described above.

V. vulnificus 1005-0, purified 1005-0 lipopolysaccharide, purified flagellar core protein, and flagellar core protein-specifc MAb were provided kindly by R. J. Siebeling and J. Simonson, Department of Microbiology, Louisiana State University, Baton Rouge. Purified 1005-0 lipopolysaccharide was suspended in 0.05 M carbonate buffer (pH 9.6) at 100 μg/ml and adsorbed to EIA plates at 4°C. The EIA was performed as described above. EIA using flagellar core protein and flagellar core protein-specific MAb was performed by the method of Simonson and Siebeling (18).

Statistical tests. The statistical difference between the EIA and biochemical methods for identification of *V. vulnificus* was determined by the McNemar test at a probability level of 0.05 by using a one-tailed test (6).

TABLE 1. Bacterial species tested by EIA

Bacterial species and strain	EIA reaction
Vibrio vulnificus	
Clinical isolates ATCC 27562, LAM 624, A1402, E4125, C7184	+
Environmental isolates 4965, 5112, 4725, 4723, 2040-81, 4600, 4726, 5114, 4793, 4832, 5189	+
Vibrio cholerae serogroup O1 CA401, INEL, V69, 102, 569B	−
Vibrio cholerae serogroup non-O1 S7, E8498	−
Vibrio parahaemolyticus ATCC 17802, ATCC 17803	−
Vibrio damsela	−
Vibrio fluvialis ATCC 33812	−
Vibrio alginolyticus ATCC 17749	−
Vibrio carchariae ATCC 35084	−
Vibrio mimicus 48PT, 2002H, 2011H	−
Vibrio hollisae ATCC 33564	−
Escherichia coli HB101, 2348, 350	−
Pseudomonas alcaligenes 5A	−
Pseudomonas aeruginosa ATCC 27853	−
Pseudomonas fluorescens 6B	−
Alcaligenes faecalis ATCC 8750	−
Enterobacter faecalis ATCC 19433	−
Enterobacter cloacae 4E	−
Plesiomonas shigelloides ATCC 14029	−
Aeromonas sobria 124, ATCC 9071	−
Aeromonas caviae 14, 34	−
Aeromonas hydrophila 1A, ATCC 19570, SSU	−
Serratia marcescens 5F	−
Citrobacter freundii 3D	−
Hafnia alveoli 2B, 2F	−
Klebsiella pneumoniae 3C	−
Bacillus cereus ATCC 11778	−
Staphylococcus aureus ATCC 12598	−
Flavobacterium meningosepticum ATCC 13263	−
Listeria monocytogenes ATCC 19111, ATCC 19112	−
Listeria grayi ATCC 25400	−
Listeria innocua ATCC 33090	−
Listeria ivanovii ATCC 19119	−
Listeria murrayi ATCC 25402	−
Listeria seeligeri ATCC 35967	−
Listeria welshimeri SPRC 3441	−

RESULTS AND DISCUSSION

Production of *V. vulnificus*-specific MAb. Splenocyte-myeloma fusions produced more than 1,000 hybridomas. Seventy hybridomas produced antibody reacting with *V. vulnificus* ATCC 27562, the immunogen. Results of tests of hybridoma supernatants with *V. vulnificus* and non-*V. vulnificus* strains showed that three MAbs were specific for *V. vulnificus*. Hybridoma FRBT37, which produced the highest absorptivity in EIAs, was cloned by limiting dilution and used for further studies. MAb FRBT37 was identified as an IgG2a antibody with kappa light chains.

Specificity of MAb FRBT37. The specificity of MAb FRBT37 was determined by EIA by using 72 laboratory stock strains of bacteria, including 34 species and 15 genera (Table 1). Results showed that reactions were limited to *V. vulnificus*.

The specificity of FRBT37 was also determined in field tests by comparing the biochemical and EIA reactions of 452 *V. vulnificus*-like bacterial colonies isolated from oysters on CPC and TCBS agars. Of 452 isolates examined, 347 were positive by EIA. Ninety-nine isolates were negative by EIA and biochemical tests, 5 were positive by EIA and negative by biochemical tests, and 1 was negative by EIA and

TABLE 2. Biochemical and growth characteristics of environmental *V. vulnificus* isolates testing positive by EIA and biochemical tests

Test	% Positive among:	
	Test isolates (n = 347)	Reported reactions[a] (n = 124)
Motility	100.0	99
ONPG[b]	93.8	75
Agglutination	98.8	NR[c]
Growth in:		
0% NaCl	0.0	0
3% NaCl	100.0	NR
6% NaCl	81.3	65
8% NaCl	0.0	0
10% NaCl	0.0	0
Oxidase production	99.4	100
Growth in Hugh-Liefson glucose broth	99.7	NR
Arginine dehydrolase production	0.0	0
Ornithine decarboxylase production	38.2	55
Lysine decarboxylase production	94.1	99
Indole	99.4	97
Utilization of:		
Sucrose	10.1	15
Mannitol	14.1	45
Mannose	100.0	98
Arabinose	0.0	0
Cellobiose	100.0	99
Maltose	100.0	100
Trehalose	100.0	100
Galactose	100.0	96
Lactose	90.0	85
Salicin	98.3	95

[a] In reference 5.
[b] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside.
[c] NR, Not reported.

positive by biochemical tests. The EIA identified 99.7% (347 of 348) of the biochemically confirmed *V. vulnificus* isolates. The biochemical and growth characteristics of the 347 EIA-positive cultures were similar to those reported by others (5) (Table 2). Analysis of these data by the McNemar test at a 0.05 probability level showed no statistical difference between EIA and biochemical methods for identification of *V. vulnificus*.

EIA results. The EIA facilitated testing of hundreds of isolates in both the immunoblot and 96-well microdilution plate formats (Fig. 1). The minimum level of detection was approximately 2,000 cells with the microdilution plate format, and the reaction intensity reached a maximum at approximately 10^6 cells (Fig. 2).

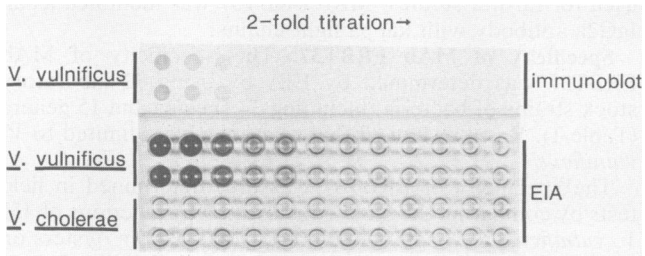

FIG. 1. Microdilution plate EIA and immunoblot formats for identification of *V. vulnificus*. APW enrichment broths of *V. vulnificus* ATCC 27562 and *V. cholerae* CA401 were diluted in twofold serial increments from left to right.

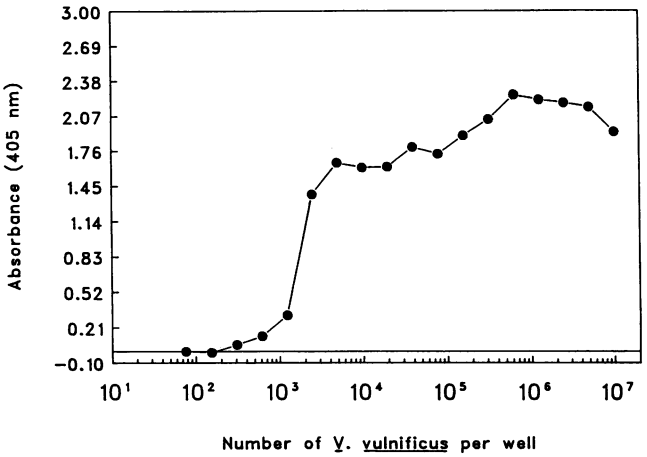

FIG. 2. Absorptivity of EIA reactions compared with concentration of *V. vulnificus*.

Samples collected from March to May showed that *V. vulnificus* could be identified by coating EIA plates with APW enrichments of seawater, sediment, and oysters, thus providing results in less than 24 h and eliminating the need for CPC agar. EIA results were identical to those of isolation methods involving plating of APW broth on CPC agar. However, beginning in June, some APW broths which yielded *V. vulnificus* on CPC agar were negative by EIA. We hypothesize that the changing bacterial flora of oysters during warmer months competed with *V. vulnificus* for attachment sites on EIA plates. Therefore, direct EIA measurements from APW are not recommended. Future studies intended to increase the selectivity of APW or develop a sandwich EIA to capture *V. vulnificus* from APW may overcome these limitations. Our attempts to develop a sandwich EIA by using FRBT37 as both the capture and signal antibodies or by using polyclonal anti-*V. vulnificus* serum as the capture antibody and FRBT37 as the signal antibody were unsuccessful, indicating that the number of epitopes per bacterial cell were low.

The protocol recommended for identification of *V. vulnificus* by EIA is as follows (Fig. 3): (i) incubate test portions in APW for 12 to 16 h at 35°C, (ii) plate turbid APW on modified CPC agar and incubate at 39 to 40°C for 10 to 24 h, (iii) transfer *V. vulnificus*-like colonies to APW and culture until turbid (4 to 6 h) at 35°C, and (iv) perform EIA. Assay time can be reduced by 1 day if a vacuum chamber is used to evaporate specimens in EIA plates or if the immunoblotting procedure is used. When MAb FRBT37 is conjugated to peroxidase, the assay time can be reduced by an additional hour.

Characterization of the epitope recognized by FRBT37. In early experiments, EIA reactions of whole cells were often weak for undefined reasons. These observations indicated that the epitope either was masked by a surface component or was intracellular. To enhance binding of FRBT37, non-Formalin-treated cells were suspended in Triton X-100, a nonionic detergent which solubilizes membranes but does not affect antibody-antigen interactions (7). Concentrations from 0.008 to 0.01% markedly increased the intensity of the EIA, with a maximum response at approximately 0.01% Triton X-100 (Fig. 4). We speculate that concentrations of

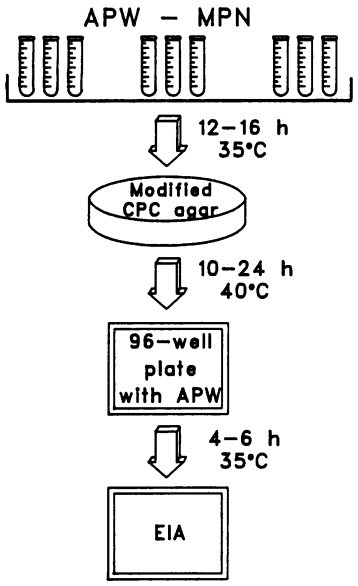

FIG. 3. Schematic diagram of isolation procedures used for EIA.

Triton X-100 above 0.01% inhibited binding of *V. vulnificus* to EIA wells and/or altered the structure of the epitope recognized by FRBT37.

Deionized H_2O, PBS, and APW were evaluated as solvents for Triton X-100 (Table 3). Of these, PBS–Triton X-100 produced the highest EIA reaction. The lytic effect of deionized H_2O without Triton X-100 also produced a strong EIA reaction but was not practical for testing of large numbers of colonies because isolates must be cultured in APW broth, isolated on CPC agar, washed, pelleted, and then suspended in deionized H_2O.

The epitope recognized by FRBT37 was produced in a variety of different culture media. EIA reactions were positive with *V. vulnificus* cultured in tryptic soy broth, TSA, brain heart infusion broth and agar, heart infusion agar, CPC, TCBS, and APW. In general, increased EIA reactions were observed for *V. vulnificus* grown in broth versus that grown in agar media.

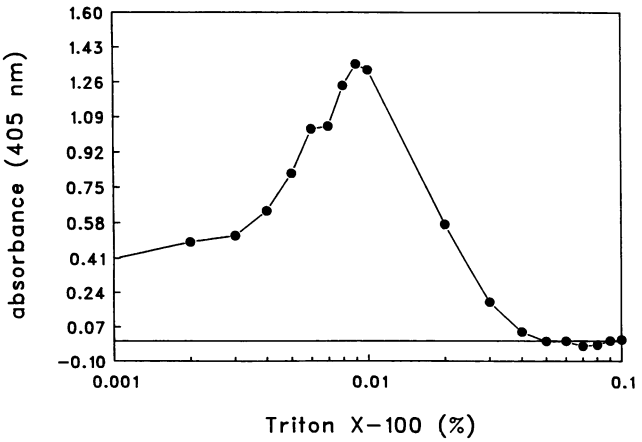

FIG. 4. Absorptivities of EIA reactions compared with Triton X-100 concentrations.

TABLE 3. Effect of Triton X-100 treatment on EIA reaction

Solvent	A_{405}	
	Control[a]	Triton X-100 treatment
PBS	0.662	1.248
Deionized H_2O	1.351	0.978
APW	0.552	0.866

[a] Treatment with solvent without Triton X-100.

Transmission electron micrographs of *V. vulnificus* labeled with MAb FRBT37, biotin-conjugated goat anti-mouse IgG, and avidin-gold showed that the epitope was intracellular and/or masked on the cell envelope. Avidin-gold label was observed on lysed cells and at points of apparent cell wall division. These observations were in agreement with previous experiments which showed that lysing of cells by Triton X-100 treatment (Table 3; Fig. 4), treatment with deionized H_2O (Table 3), or sonication (Table 4) markedly enhanced EIA reactions. We speculate that weak EIA reactions observed in experiments lacking lytic treatments resulted from exposure of the epitope recognized by FRBT37 in autolyzed, broken, or partially degraded *V. vulnificus* cells in broth and agar cultures.

The epitope recognized by MAb FRBT37 was sensitive to 70°C but not to 40°C. Nishibuchi and Seidler (16) described a *V. vulnificus*-specific radiomicrodiffusion identification method. Evidence indicated that it was an intracellular protein which precipitated in 70% but not 50% ammonium sulfate and was labile at 100°C but not at 70°C. Although the epitope recognized by FRBT37 is also apparently intracellular, experiments showed that it was a structure which precipitated in 50% ammonium sulfate and was labile at 70°C.

Epitope FRBT37 is also different from the *V. vulnificus*-specific epitope on flagellar core protein reported by Simonson and Siebeling (18). MAb FRBT37 did not react with purified *V. vulnificus* flagellar core protein (18). In addition, electron microscopy studies did not show binding of MAb FRBT37 to flagellar structures.

The epitope was not degraded when supernatants of sonicated *V. vulnificus* were treated with pronase or neuraminidase. Experiments intended to purify and characterize the epitope recognized by FRBT37 are in progress.

Presence of epitope on transparent- and opaque-phenotype colonies. *V. vulnificus* can display transparent and opaque phenotypes on laboratory agar. Opacity has been correlated directly with increased production of capsular polysaccharide and virulence (27). EIA reactions were similar for opaque and transparent colonies, indicating that capsular polysaccharide was not a structural component of the epitope.

Rapid identification tests will probably improve ecological and clinical investigations of *V. vulnificus*. These advancements will reduce the need for many biochemical tests. With

TABLE 4. Effect of sonication on EIA reaction

Bacterial fraction	A_{405}	
	Control[a]	Sonication
Supernatant	0.066	1.320
Pellet	0.825	0.762

[a] No sonication.

improvements in direct identification techniques using MAbs (2), nucleic acid probes (9, 13, 19), and the polymerase chain reaction (20), the need for culture techniques may be substantially reduced.

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