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Broth Medium for Enrichment of *Vibrio fluvialis* from the Environment

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A medium was designed for the enrichment and enumeration of *Vibrio fluvialis* from environmental samples. The medium contains 1% peptone plus 4% sodium chloride and 5 μg of novobiocin per ml, pH 8.5. This *V. fluvialis* enrichment medium (FEM) was tested, in comparison with alkaline peptone (AP), in field samplings. A total of 177 samples (estuarine waters and sediment, sewage, and crabs) collected over a 14-month period were examined with FEM and with AP broth. Results showed that FEM was more effective than AP in detecting *V. fluvialis*, particularly from water and sewage samples with low salinities (<6‰). The best recovery of *V. fluvialis* occurred when both enrichment media were used simultaneously.

In 1977 Furniss et al. (2) described organisms isolated from water and human diarrhea and designated them as group F. These bacteria exhibited characteristics in common with both *Vibrio* and *Aeromonas* species. The organisms were further characterized (4, 5) and given the name *Vibrio fluvialis* (6). This species was isolated from a number of patients with diarrhea in Bangladesh (3, 13), from both human diarrhea cases and aquatic environments in India (9), and from polluted coastal waters of the United States (10). It has been reported that strains isolated from clinical specimens are anaerogenic and that the strains isolated from the environment are either aerogenic or anaerogenic (2, 5, 10). Recent studies have shown that this organism produces various virulence factors and enterotoxins (1, 7, 9–11). Since *V. fluvialis* is capable of causing human disease, it is desirable to have a procedure to detect this organism in natural environments, especially when there is a risk of transmission to humans. To the best of our knowledge, alkaline peptone (AP) broth and modified Rimler-Shotts were used as enrichment media in previous studies (10, 11). When the authors tried to isolate *V. fluvialis* from various aquatic samples with the above enrichment media, the former appeared superior to the latter. However, AP broth was not always satisfactory since other contaminating vibrios and terrestrial organisms, typically *Aeromonas* sp., competed with *V. fluvialis*. Diverse types of colonies appearing on thiosulfate-citrate-bile salts agar presumptive medium also made it difficult to detect *V. fluvialis*.

Therefore, an attempt was made to make AP enrichment broth more selective for the isolation and enumeration of this vibrio from environmental samples. In designing the improved enrichment medium the usefulness of sodium chloride, 2,4-diamino-6,7-diisopropylpteridine (0/129), and novobiocin was assessed as additives to the basal AP medium. *V. fluvialis*, like other vibrios, can grow in the presence of high concentrations of sodium chloride and, like *Aeromonas* sp., is not as sensitive to 0/129 and novobiocin as other *Vibrio* species (2, 5). A modified AP broth was developed in the laboratory and field tested. The overall results indicated that the new *V. fluvialis* enrichment medium (FEM) was more effective in detecting *V. fluvialis* than AP broth, particularly with low-salinity water samples and raw sewage.

MATERIALS AND METHODS

Bacterial strains. Reference strains used in this study are listed in Table 1. Aerogenic strains of *V. fluvialis* produced gas in methyl red–Voges-Proskauer broth (Difco Laboratories) and anaerogenic strains did not. Cultures were maintained in tryptic soy broth without dextrose (Difco) supplemented with 0.5% sodium chloride (TSB′) plus 5% glycerol and stored at −80°C. Frozen cultures were reconstituted on tryptic soy agar (Difco) supplemented with 0.5% sodium chloride (TSA′) and incubated at 25°C for 24 h.

Effect of novobiocin, 0/129, and sodium chloride on growth. A transfer was made from TSA′ into 10 ml of

TABLE 1. Bacterial strains studied

Organism	Strain no.	Origin	Source or reference
V. fluvialis, aerogenic[a]	DJVP 7225	Gastroenteritis, Indonesia	10
	2386	River water, England	5
	LSU 9-26a	Crab feces, Louisiana	R. J. Siebeling[b]
	LSU 12-1G	Crab feces, Louisiana	R. J. Siebeling
	LSU 12-17V	Crab feces, Louisiana	R. J. Siebeling
V. fluvialis, anaerogenic[c]	5125	Human feces, Bangladesh	9
	DJVP 7440	Gastroenteritis, Indonesia	10
	S50-1CC	Sediment, New York	10
	H-5	Estuarine water, Maryland	R. J. Colwell[d]
	LSU 10-30a	Crab feces, Louisiana	R. J. Siebeling
V. cholerae, non-O-1	N-3	Crab, Louisiana	This study
V. parahaemolyticus	CDC A8658	Gastroenteritis, Maryland	Centers for Disease Control
V. alginolyticus	ATCC 17749	Seawater, Japan	American Type Culture Collection
V. anguillarum	LS-174	Salmon, Oregon	J. Rohovec[e]
A. sobria	NMRI 6	Diver wound, Maryland	O. P. Daily[f]
A. hydrophila	NMRI 7	Diver wound, Maryland	O. P. Daily

[a] Gas producer in methyl red–Voges-Proskauer broth (Difco).
[b] Department of Microbiology, Louisiana State University, Baton Rouge.
[c] No gas production in methyl red–Voges-Proskauer broth.
[d] Department of Microbiology, University of Maryland, College Park.
[e] Department of Microbiology, Oregon State University, Corvallis.
[f] Office of Naval Research, Code 440B, Arlington, Va.

TSB′ and incubated statically at 25°C for 12 h. Then 0.1 ml of the broth culture was inoculated into 40 ml of TSB′ and incubated at 200 rpm and 25°C until the absorbance at 600 nm reached 1.0 to 1.2. The broth culture was diluted in phosphate-buffered saline (0.003 M NaH_2PO_4, 0.0067 M Na_2HPO_4, 0.13 M NaCl, pH 7.2) to about 5×10^2 CFU/ml. A 0.1-ml portion of the bacterial suspension was spread in duplicate over the surface of TSA′ and the test agar media, i.e., AP agar plates (peptone, 1%; sodium chloride, 1%; unless otherwise specified [pH 8.5]) containing different concentrations of novobiocin (sodium salt; Sigma Chemical Co.), 0/129 (Calbiochem), or sodium chloride. The inoculated plates were incubated at 35°C (customarily used for isolating this species from the environment; 10), and the number of colonies was counted after 24 h of incubation. Percent relative growth was determined by comparing the CFU on the test plate with that on the control TSA′ plate. For *V. alginolyticus* ATCC 17749, the control medium was AP agar with 5% sodium chloride.

Growth in FEM. FEM basal medium contained, per liter, 10 g of Bacto-Peptone (Difco) and 40 g of NaCl adjusted to pH 8.5. The medium was autoclaved for 15 min at 121°C. Novobiocin solution was prepared by dissolving 0.005 g in 10 ml of sterile distilled water and was added to the cooled basal medium. Test organisms were grown as described above. The broth culture in early stationary phase was diluted in phosphate-buffered saline to 10^{-6}, and 20 μl was inoculated into 5 ml of FEM in a colorimeter tube fitted with a plastic cap. The inoculated tube was incubated statistically at 35°C. Growth was monitored by measuring turbidity (absorbance at 600 nm) with a Spectronic 20 spectrophotometer (Bausch & Lomb). Absorbances below 0.03 were considered insignificant, i.e., within the variance of negative controls. Growth was also measured by determining total viable cell numbers in FEM at incubation times of 0 (inoculum) and 14 h when most *V. fluvialis* strains reached log phase. The number of viable cells was determined by the plate count method on TSA′.

Field tests. The efficacy of FEM, compared with AP, in recovering *V. fluvialis* from environmental samples was examined by the most-probable-number (MPN) technique. Environmental samples were inoculated into nine tubes of enrichment broth (three tubes for each of three 10-fold dilutions) and incubated at 35°C for 18 h. Then each broth culture was streaked onto thiosulfate-citrate-bile salts agar (Oxoid) and incubated at 35°C. Typical yellow colonies on thiosulfate-citrate-bile salts agar were tested in a screening medium (N. C. Roberts and R. J. Seidler, *Vibrios in the Environment*, in press), and representative isolates were submitted to a battery of biochemical tests to identify *V. fluvialis*. The MPN was calculated based on the number of tubes containing *V. fluvialis*. Sampling was performed at 20 stations in the state of Louisiana at a frequency of 2 to 23 times at each station in the period of November 1980 to December 1981 (N. C. Roberts, R. J. Seibeling, J. Kaper, and H. B. Bradford, Microb. Ecol., in press). Samples collected included natural water (river, pond, swamp, and estuary), sewage, sediment, and crabs. Temperature and salinity of water samples ranged from 7 to 32°C and from <1 to 32‰.

RESULTS AND DISCUSSION

AP broth, originally designed for the isolation of *V. cholerae* (8), is used at pH 8 to 9 for the isolation of *Vibrio* species because vibrios, unlike other organisms, generally can grow at high pH values. The effect of pH on the growth of four representative strains of *V. fluvialis* was examined to verify that AP can also be an effective basal medium for the enrichment of *V.*

TABLE 2. Relative growth of various organisms on AP agar with different concentrations of novobiocin, 0/129, or sodium chloride at 35°C

Organism		% Relative growth on given test medium											
		TSA′ (NaCl, 1%)	Control (AP with 1% NaCl)	AP (NaCl, 1%) with:								AP with NaCl (%)	
				Novobiocin (μg/ml)				0/129 (μg/ml)					
Species	Strain			2	5	10	20	2	5	10	20	3	5	7
V. fluvialis	DJVP 7225	100[a]	87	95	83	105	89	29	38	0	0	119	48	0
	S50-1CC	100	139	114	76	79	79	90	60	63	0	105	10	0
	5125	100	118	91	91	94	59	84	105	120	0	83	0	0
	2386	100	105	179	93	120	98	95	98	108	0	109	108	57
V. cholerae non-O-1	N-3	100	105	0	0	0	0	98	0	0	0	83	0	0
V. parahaemolyticus	CDC A8658	100	105	115	89	60	0	105	124	91	0	85	67	0
V. alginolyticus	ATCC 17749	SW[b]	0	0	0	0	0	0	0	0	0	SW	100	69
V. anguillarum	LS-174	100	100	0	0	0	0	0	0	0	0	0	0	0
A. sobria	NMRI 6	100	113	84	82	67	79	72	94	99	80	97	0	0
A. hydrophila	NMRI 7	100	51	116	78	64	76	81	69	72	76	74	0	0

[a] Percent relative growth was determined as (number of colonies on test medium/number of colonies on TSA′) × 100. Number of colonies on TSA′ was taken as 100% growth except for *V. alginolyticus* ATCC 17749, for which the numbers of colonies on AP agar with 5% NaCl was taken as 100%.

[b] No separate colonies were observed due to swarming.

fluvialis. All strains grew to approximately the same maximal levels in the pH range of 6 to 9. Therefore, a pH of 8.5 was chosen for further development of the selective enrichment medium.

With AP broth as a basal medium, the effects of novobiocin, 0/129, and sodium chloride on the growth of various vibrios and aeromonads were examined (Table 2). Strains of *V. fluvialis* and *Aeromonas* spp. were resistant to novobiocin, whereas *V. parahaemolyticus* CDC A 8658 was moderately sensitive (no growth at 20 μg/ml) and the other vibrios were extremely sensitive. Sensitivity to 0/129 varied among strains of *Vibrio* spp., including *V. fluvialis*, although all of the test strains were sensitive at the 20-μg/ml level. On the other hand, strains of *Aeromonas* spp. were not affected at this concentration. The growth of *Aeromonas* spp. was completely inhibited by 5% sodium chloride, whereas the growth of *V. fluvialis* strains was variable at the same concentration. From the data obtained it was assumed that a combination of novobiocin and sodium chloride at appropriate concentrations could be used in AP medium to inhibit the growth of undesirable species while allowing *V. fluvialis* to grow. A novobiocin concentration of 5 μg/ml was considered suitable because it totally inhibited the growth of three strains of *Vibrio* spp. and was moderately inhibitory to *V. parahaemolyticus* CDC A8658. Since the growth of *Aeromonas* spp. was influenced little by novobiocin or 3% NaCl, it appeared that 4% NaCl would be the concentration of choice.

In the next step, therefore, the growth of the reference strains in FEM containing 4% NaCl and 5 μg of novobiocin per ml was compared. Small numbers of bacterial cells were inoculated into FEM (Table 3) and incubated statically at 35°C to simulate an actual enrichment proce-

TABLE 3. Growth of *V. fluvialis*, *Vibrio* spp., and *Aeromonas* spp. in FEM as measured by number of viable cells

Test organism[a]	Strain	Total viable cell no. (CFU) at an incubation time of:[b]	
		0 h	14 h
V. fluvialis, aerogenic	DJVP 7225	88	1.9×10^8
	2386	56	2.7×10^8
	LSU 9-26a	34	5.0×10^7
	LSU 12-1G	78	6.9×10^8
	LSU 12-17V	50	8.0×10^8
V. fluvialis, anaerogenic	5125	72	<50
	DJVP 7440	2.0	4.9×10^7
	S50-1CC	58	1.4×10^8
	H-5	40	5.1×10^8
	LSU 10-30a	59	5.9×10^8
V. cholerae	N-3	74	<50
V. parahaemolyticus	CDC A8658	42	1.1×10^5
V. alginolyticus	ATCC 17749	44	<50
V. anguillarum	LS-174	52	<50
A. sobria	NMRI 6	190	<50
A. hydrophila	NMRI 7	38	<50

[a] Test organisms were grown in TSB′ to early-stationary phase (absorbance at 600 nm, 1.0 to 1.2) with shaking (200 rpm) at 25°C. Cultures were diluted to 10^{-6} in phosphate-buffered saline (pH 7.2), and 20 μl was inoculated into 5 ml of FEM and incubated statically at 35°C. Turbidity data are shown in Fig. 1.

[b] Number of viable cells per 5 ml was determined by the plate count method on TSA′.

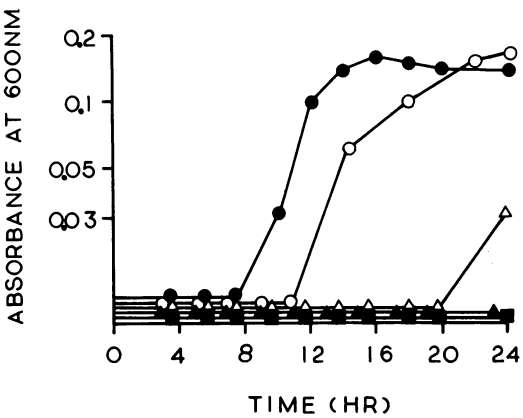

FIG. 1. Growth of *V. fluvialis*, *V. cholerae*, *V. parahaemolyticus*, and *A. hydrophila* in FEM measured turbidimetrically. Test organisms were grown in TSB' to early stationary phase and diluted to 10^{-6} in phosphate-buffered saline (pH 7.2), and 20 μl was inoculated into 5 ml of FEM. Growth at 35°C was monitored by measuring absorbance at 600 nm. Numbers of total viable cells at 0 and 14 h of incubation are shown in Table 3. Symbols: ●, *V. fluvialis* DJVP 7225 (aerogenic); ○, *V. fluvialis* S50-1CC (anaerogenic); ▲, *V. cholerae* N-3; △, *V. parahaemolyticus* CDC A8658; ■, *A. hydrophila* NMRI 7.

dure. When growth was monitored turbidimetrically, 9 of 10 *V. fluvialis* strains, regardless of origin or aerogenicity, exhibited signs of growth in 9 to 15 h. Representative growth curves are shown in Fig. 1. *V. parahaemolyticus* CDC A8658 increased to significant turbidity levels only after 24 h. The nine strains of *V. fluvialis*, which showed increased turbidity in 9 to 15 h, contained total viable cells in the order of 10^7 to 10^8 CFU at 14 h, whereas *V. parahaemolyticus* CDC A8658 was still in the range of 10^5 CFU at the same incubation time (Table 3). Viable cells were not detected for *V. fluvialis* 5125 and other strains of *Vibrio* and *Aeromonas* species.

TABLE 4. Isolation of *V. fluvialis* from environmental samples, using FEM and AP enrichment medium

Sample	No. studied	No. positive	Isolation with FEM/AP (%)[a]		
			+/+	+/−	−/+
Water					
Natural water	63	26	8 (30.8)	10 (38.5)	8 (30.8)
Sewage	45	14	0	13 (92.9)	1 (7.1)
Sediment	60	27	12 (44.4)	7 (25.6)	8 (29.6)
Crab	9	4	1 (25)	2 (50)	1 (25)
Total	177	71	21 (29.6)	32 (45.1)	18 (25.4)

[a] +, Presence, and −, absence, of *V. fluvialis*.

The above results strongly suggest that enrichment in FEM under these conditions (static incubation, 35°C, >14 h) should improve the detection of *V. fluvialis* by inhibiting the growth of other *Vibrio* and *Aeromonas* species.

FEM containing 4% sodium chloride and 5 μg of novobiocin per ml was examined in field tests in Louisiana where *V. fluvialis* is often isolated from aquatic environments. A total of 177 samples were processed in a 14-month period with both FEM and AP. *V. fluvialis* was isolated from 71 of these samples (Table 4). Among them, 29.6% were isolated by both enrichment media; 45.1%, through FEM only; and 25.4%, through AP only. The superior results obtained with FEM are mainly attributable to the almost exclusive detection by FEM of *V. fluvialis* from sewage samples. It is suspected that this higher isolation frequency by FEM broth is due to the inhibition of growth of terrestrial species such as *Aeromonas* by the high concentration of sodium chloride in FEM. Therefore, the isolation of *V. fluvialis* from water was analyzed with regard to the salinity of the sample (Fig. 2). At low salinities (<6‰) FEM was more effective than AP as illustrated by both a higher incidence of positive samples and a greater concentration recovered.

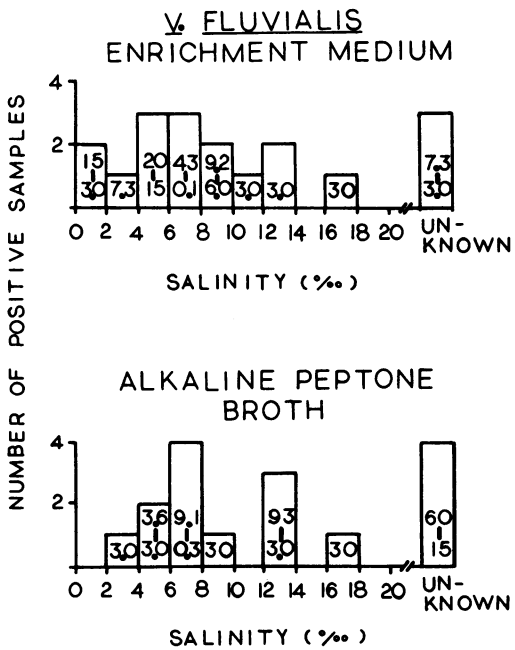

FIG. 2. Isolation of *V. fluvialis* from natural water samples with varying salinity, using FEM and AP enrichment medium. Height of the columns represents the number of positive samples. Numbers within each column indicate the MPN range of counts. A total of 63 samples were tested, of which 26 contained detectable counts of *V. fluvialis*.

At high salinities (>6‰), AP may be slightly more effective than FEM as the samples generally exhibited higher MPN values with AP than with FEM. This could be due to the overgrowth by novobiocin-resistant halophilic organisms. There was no apparent correlation between the MPN of *V. fluvialis* and other environmental factors measured such as water temperature, turbidity, dissolved oxygen, pH, and fecal coliforms (data not shown). To summarize, the results showed that FEM was an effective enrichment medium for detecting *V. fluvialis* from various environmental samples, particularly in samples with low salinities, including sewage.

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LITERATURE CITED

1. **Agarwal, R. K., and S. C. Sanyal.** 1981. Experimental studies on enteropathogenicity and pathogenesis of group 'F' vibrio infections. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **249**:392–399.
2. **Furniss, A. L., J. V. Lee, and T. J. Donovan.** 1977. Group F, a new vibrio? Lancet **ii**:565–566.
3. **Huq, M. I., A. K. M. J. Alam, D. J. Brenner, and G. K. Morris.** 1980. Isolation of vibrio-like group, EF-6, from patients with diarrhea. J. Clin. Microbiol. **11**:621–624.
4. **Jensen, M. J., P. Baumann, M. Mandel, and J. V. Lee.** 1980. Characterization of facultatively anaerobic marine bacteria belonging to group F of Lee, Donovan, and Furniss. Curr. Microbiol. **3**:373–376.
5. **Lee, J. V., T. J. Donovan, and A. L. Furniss.** 1978. Characterization, taxonomy, and emended description of *Vibrio metschnikovii*. Int. J. Syst. Bacteriol. **28**:99–111.
6. **Lee, J. V., P. Shread, A. L. Furniss, and T. N. Bryant.** 1981. Taxonomy and description of *Vibrio fluvialis* sp. nov. (synonym group F vibrios, group EF6). J. Appl. Bacteriol. **44**:73–94.
7. **Lockwood, D. E., A. S. Kreger, and S. H. Richardson.** 1982. Detection of toxins produced by *Vibrio fluvialis*. Infect. Immun. **35**:702–708.
8. **Pollitzer, R.** 1959. Cholera. W.H.O. Monogr. Ser. **43**:533–536.
9. **Sanyal, S. C., R. K. Agarwal, E. Annapurna, and J. V. Lee.** 1980. Enterotoxicity of group F vibrios. Jpn. J. Med. Sci. Biol. **33**:217–222.
10. **Seidler, R. J., D. A. Allen, R. R. Colwell, S. W. Joseph, and O. P. Daily.** 1980. Biochemical characteristics and virulence of environmental group F bacteria isolated in the United States. Appl. Environ. Microbiol. **40**:715–720.
11. **Spira, W. M., R. R. Daniel, Q. S. Ahmed, A. Huq, A. Yusuf, and D. A. Sack.** 1979. Clinical features and pathogenicity of O group 1 nonagglutinating *Vibrio cholerae* and other vibrios isolated from cases of diarrhea in Dacca, Bangladesh, p. 137–153. *In* D. Middleton and I. Librn, (ed.), Proceedings of the 14th Joint Conference, U.S.-Japan Cooperative Medical Science Program, Karatsu, Japan, 1978. Toho University, Karatsu.