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Enhanced Broth Media for Selective Growth of *Vibrio vulnificus*†

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Rapid detection of *Vibrio vulnificus* can be enhanced by optimizing the components of enrichment broth. PNC (5% peptone, 1% NaCl, and 0.08% cellobiose [pH 8.0]) enhanced the growth of *V. vulnificus* compared to alkaline peptone broth. PNCC (PNC with 1.0 to 4.1 U of colistin methanesulfonate per ml) increased the growth of low levels of *V. vulnificus* while suppressing non-target bacteria.

Vibrio vulnificus causes invasive and life-threatening disease in persons with weakened host defenses (7, 10, 13). Preventing infection depends in part on early detection. Currently, environmental and clinical data indicate that a level of ≥1,000 *V. vulnificus* organisms per g of oyster meat is associated with human infections (6). This concentration is below or near the minimum detection limits of most diagnostic assays, necessitating the use of enrichment broths.

Virtually all bacterial cultivations can be complicated by replication of non-target bacteria that compete for limited nutrients, produce bacteriocins, and/or effect pH (8, 11, 17). Another factor affecting method sensitivity is differentiation of target organisms among various colonies on agar. For example, TCBS (thiosulfate-citrate-bile salt-sucrose) agar is an excellent medium for selective isolation of vibrios; however, because marine samples normally contain multiple *Vibrio* spp., especially dominant vibrios such as *Vibrio parahaemolyticus* and *Vibrio alginolyticus*, the target vibrio is often difficult to locate among colonies (3, 15–17).

Currently, diagnostic assays require sample enrichment to elevate low numbers of bacteria to minimum detection limits (1, 3–5, 12). For example, the lower limit of detection for PCR in oysters is 10,000 CFU/ml-g (1). Immunoassays usually require 10^5 cells/ml, with false-negative reactions observed in low dilutions of samples, possibly resulting from overgrowth by non-target organisms and/or inhibitory substances in sample homogenates (15).

The purpose of this study was to define components of enrichment broth that enhance and select for growth of *V. vulnificus*. First, the effects of alkaline peptone broth (APB) components on *V. vulnificus* growth were evaluated. From these results, a basal broth was prepared and used to evaluate additional medium supplements (i.e., selected antibiotics and carbohydrates).

Bacterial strains. *V. vulnificus* environmental strains 4600, 4832, and 4965 (provided by D. A. Cook, U.S. Food and Drug Administration [FDA]), *V. parahaemolyticus* HC5C-1C (provided by C. Kaysner, U.S. FDA), and *V. alginolyticus* ATCC 17749 were studied. All strains were authenticated by established techniques (3, 15). One day prior to experimentation, frozen cultures were plated on tryptic soy agar (TSA) (Difco Laboratories, Detroit, Mich.) containing 1.0% NaCl. Results showed that the three *V. vulnificus* strains had indistinguishable growth patterns under all test conditions; unless otherwise specified, only *V. vulnificus* 4832 data are shown.

V. vulnificus strains were incubated on TSA–1% NaCl at 35°C for 16 to 24 h, and then one isolated colony was transferred to 10 ml of APB and incubated until logarithmic growth phase. Twenty microliters was transferred to 8 ml of experimental broth, and bacterial growth was monitored by the absorbance at 420 nm. All experiments consisted of static cultures and were conducted in triplicate.

Effects of peptone. Deionized water containing 1% NaCl was supplemented with 0.5 to 5.0% peptone at pH 8.4.

Effects of NaCl. Deionized water containing peptone was supplemented with 0 to 5.0% NaCl.

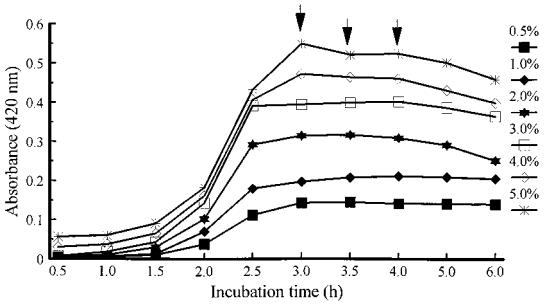

FIG. 1. Effects of peptone concentration on growth of *V. vulnificus* 4832 in broth containing 1% NaCl, pH 8.4. ▼, statistical analysis performed at time point.

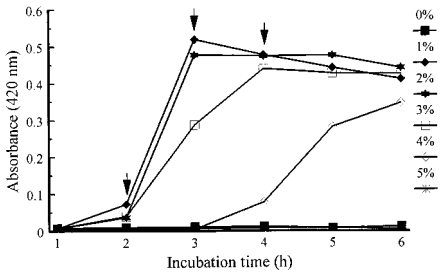

FIG. 2. Effects of NaCl concentration on growth of *V. vulnificus* 4832 in broth containing 5% peptone, pH 8.4. ▼, statistical analysis performed at time point.

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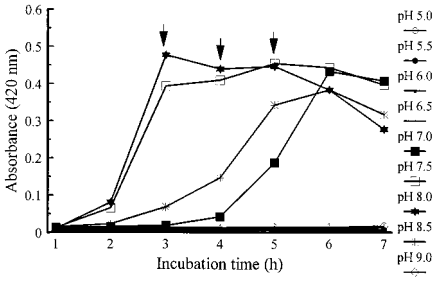

FIG. 3. Effects of medium pH on growth of *V. vulnificus* 4832 in broth containing 5% peptone and 1% NaCl. ▼, statistical analysis performed at time point.

TABLE 1. Inhibitory effects of CM on *V. vulnificus* 4832, *V. parahaemolyticus*, and *V. alginolyticus* at various inoculum sizes

No. of cells inoculated/ml	Effect of CM (U/ml)[a]											
	8.2			4.1			2.1			1.0		
	Vv	Va	Vp	Vv	Va	Vp	Vv	Va	Vp	Vv	Va	Vp
10^0	−	−	−	−	−	−	−	−	−	−	−	−
10^1	−	−	−	−	−	−	−	−	−	+	−	−
10^2	−	−	−	+	−	−	+	−	+	+	−	+
10^3	−	−	−	+	−	+	+	−	+	+	−	+
10^4	−	−	−	+	−	+	+	−	+	+	−	+

[a] Bacterial cultures were added to broth containing 5% peptone–1% NaCl (pH 8.0) and CM. Cultures were incubated at 35°C for 8 h. Vv, *V. vulnificus*; Va, *V. alginolyticus*; Vp, *V. parahaemolyticus*; −, no growth; +, growth (turbidity).

Effects of pH. Two experiments were conducted to determine the effects of pH. First, media containing 5.0% peptone and 1.0% NaCl were made in 0.1 M citrate-phosphate, potassium phosphate, or Tris-HCl to produce respective pH values of 5.0 and 5.5; 6.0, 6.5, 7.0, 7.5, and 8.0; and 8.5 and 9.0. Second, media containing 5% peptone and 1% NaCl were made in 0.01, 0.03, 0.05, and 0.1 M Tris buffer to produce pH values of 7.5, 8.0, and 8.5 for each buffer concentration.

Effects of temperature. Media containing 5.0% peptone–1.0% NaCl (pH 8.0) were incubated at 25, 30, 35, 40, and 45°C to determine the effects of temperature.

Effects of selective carbohydrates. Media containing 0.1, 1.0, or 5.0% peptone and 1.0% NaCl (pH 8.0) were supplemented with salicin (0 to 0.5%), lactose (0 to 2.5%), or cellobiose (0 to 5.0%). A simplex-centroid experimental design was used to determine if combinations of carbohydrates had synergistic effects (2). Concentrations of each carbohydrate that supported optimum growth were chosen from single carbohydrate studies. Seven combinations of three carbohydrates (0.1% lactose, 0.1% salicin, 0.08% cellobiose) were studied.

Effects of antibiotics. The susceptibilities of *V. vulnificus*, *V. alginolyticus*, and *V. parahaemolyticus* to polymyxin B (PB) and colistin methanesulfonate (CM) were determined. The three species were separately prepared at 10^1 to 10^5 CFU/ml, and 1 ml was added to 10 ml of broth containing 5% peptone–1% NaCl (pH 8.0) supplemented with PB at 0.25, 0.31, 0.50, or 0.62 U/ml or CM at 1.0, 2.1, 4.1, or 8.2 U/ml. Triplicate tubes were used for each bacterial strain at each inoculum dose and for each antibiotic concentration. Following incubation at 35°C for 4 and 8 h, broth absorbance was recorded.

Statistics. All statistical tests were conducted with the General Linear Model procedures of the Statistical Analysis System (SAS Institute, Inc., Cary, N.C.). In laboratory studies of the effects of peptone, NaCl, pH, and temperature, mean comparisons were determined by Duncan's multiple-range test. Data for carbohydrate studies were determined by Dunnett's test (2).

PNC medium (5.0% peptone, 1.0% NaCl, 0.08% cellobiose [35°C, pH 8.0]) increased *V. vulnificus* growth compared to APB. PNCC medium (PNC with 1.0 to 4.1 U of CM/ml) inhibited growth of *V. parahaemolyticus* and *V. alginolyticus* while promoting growth of *V. vulnificus* strains.

Normal growth patterns were observed at peptone concentrations of 2.0 to 5.0% for all three strains (Fig. 1). Five percent peptone produced the highest turbidity at 3, 3.5, and 4 h for all three strains tested ($P < 0.05$).

One and 2.0% NaCl supported high growth for all strains in 1.0, 3.0, 4.0 (not shown), and 5.0% peptone (Fig. 2), with 5.0% peptone and 1.0% NaCl being optimal.

Results showed that pHs of 7.5 and 8.0 produced fast and abundant growth (Fig. 3). In 0.01, 0.03, and 0.05 M Tris, the growth rate was higher at pHs of 8.0 and 8.5 but lower at pH 7.5. In 0.1 M Tris, a lower growth rate occurred at pH 8.5.

The effects of incubation temperature were measured in 5.0% peptone–1.0% NaCl (pH 8.0) at a temperature range of 25 to 45°C. Maximum growth occurred at 35°C for all strains (not shown).

Lactose, salicin, and cellobiose are commonly used to differentiate *V. vulnificus* from other *Vibrio* spp. (9). Five percent peptone had a protective effect against the inhibitory properties of lactose observed at lower peptone concentrations (Fig. 4). Growth was enhanced in 0.1% peptone containing 0.5 or 1.0% lactose (Fig. 4C). Salicin showed effects similar to those of lactose (not shown).

In contrast to lactose and salicin, cellobiose markedly inhibited growth in 0.1% peptone at concentrations of 1.0% or

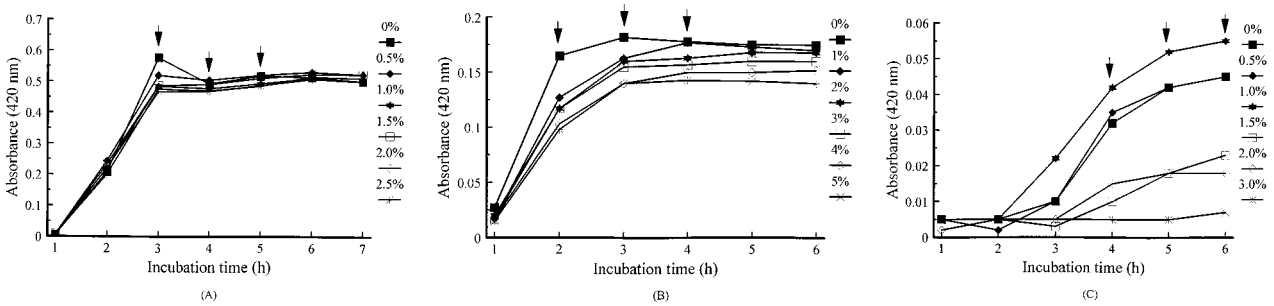

FIG. 4. Effects of lactose supplementation on growth of *V. vulnificus* 4832 in broth containing various peptone and lactose concentrations: 5% peptone with 0 to 2.5% lactose (A), 1% peptone with 0 to 5% lactose (B), and 0.1% peptone with 0 to 3% lactose (C). ▼, statistical analysis performed at time interval.

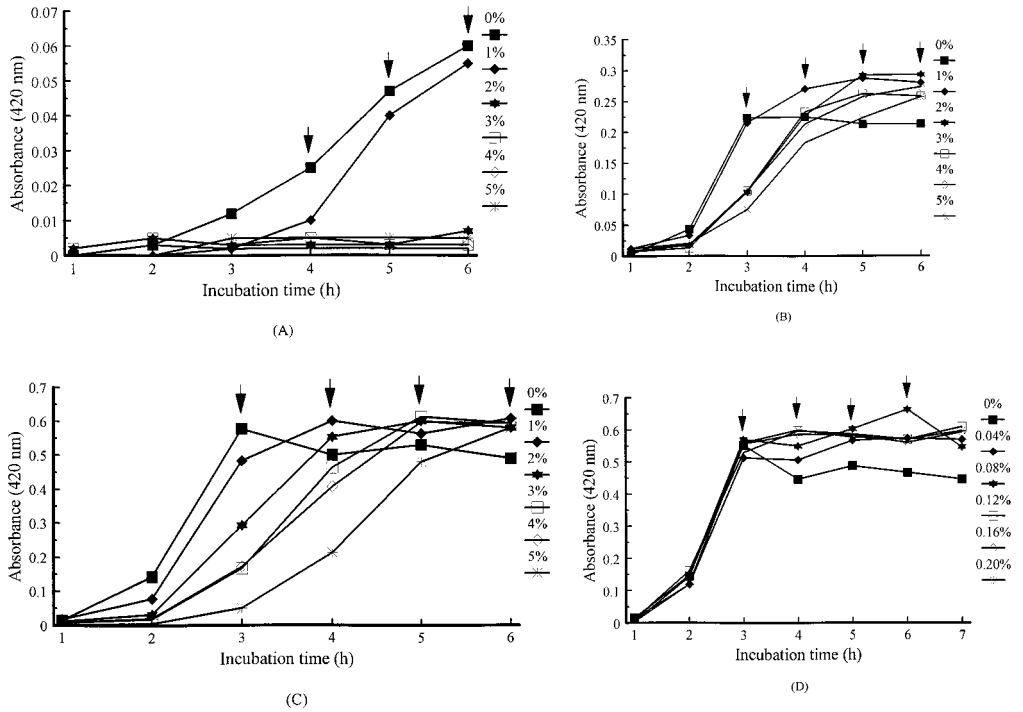

FIG. 5. Effects of cellobiose supplementation on growth of *V. vulnificus* 4832 in broth containing various peptone and cellobiose concentrations: 0.1% peptone with 0 to 5% cellobiose (A), 1% peptone with 0 to 5% cellobiose (B), 5% peptone with 0 to 5% cellobiose (C), and 5% peptone with 0 to 0.2% cellobiose (D). ▼, statistical analysis performed at time interval.

greater (Fig. 5A). However, in 1.0% peptone, 1.0% cellobiose enhanced growth after 3 h (Fig. 5B). Higher concentrations of cellobiose increased growth after 4 h in comparison to the control (Fig. 5B). In 5.0% peptone, 2.0% or higher cellobiose markedly delayed growth (Fig. 5C). In contrast, greater growth occurred at 0.04 to 0.20% cellobiose, particularly at 0.08% (Fig. 5D). No synergy among carbohydrates was observed (not shown).

Approximately 3% of *V. vulnificus* strains are susceptible to polymyxin antibiotics, in contrast to other *Vibrio* species (9). At inocula of 10^4 CFU/ml of broth (each), *V. vulnificus*, *V. alginolyticus*, and *V. parahaemolyticus* were inhibited at all test concentrations of CM (1.0 to 8.2 U/ml) and PB (0.2 to 0.6 U/ml) during 4 h of incubation (data not shown). At 8 h, 8.2 U of CM/ml inhibited all three *Vibrio* spp. at inoculum levels of 1 to 10,000 CFU/ml (Table 1); an initial inoculum of 100 *V. vulnificus* organisms per ml of broth was required for 8 h of growth in 1.0 to 4.1 U of CM/ml, while 10^2 *V. parahaemolyticus* organisms per ml and more than 10^4 *V. alginolyticus* organisms per ml were necessary under the same conditions.

TABLE 2. Inhibitory effects of PB on *V. vulnificus* 4832, *V. parahaemolyticus*, and *V. alginolyticus* at various inoculum sizes

No. of cells inoculated/ml	Effect of PB (U/ml)[a]											
	0.6			0.5			0.3			0.2		
	Vv	Va	Vp	Vv	Va	Vp	Vv	Va	Vp	Vv	Va	Vp
10^0	−	−	−	−	−	−	−	−	−	−	−	−
10^1	−	−	−	−	−	−	−	−	−	+	−	+
10^2	−	−	−	−	−	−	+	−	+	+	−	+
10^3	+	−	+	+	−	+	+	−	+	+	−	+
10^4	+	−	+	+	−	+	+	−	+	+	−	+

[a] Bacterial cultures were added to broth containing 5% peptone–1% NaCl (pH 8.0) and PB. Cultures were incubated at 35°C for 8 h. Vv, *V. vulnificus*; Va, *V. alginolyticus*; Vp, *V. parahaemolyticus*; −, no growth; +, growth (turbidity).

PB at 0.2 to 0.6 U/ml inhibited *V. alginolyticus* at all inoculum levels during 8 h of incubation (Table 2); *V. parahaemolyticus* was able to grow in PB up to 0.6 U/ml. An inoculum of 10^3 *V. vulnificus* CFU/ml was needed for growth in 0.6 U CM/ml. Overall, CM was more inhibitory than PB for *V. alginolyticus* and *V. parahaemolyticus* and less inhibitory than PB for *V. vulnificus*. Therefore, PNC with 1.0 to 4.1 U of CM/ml (PNCC medium) is proposed for field evaluations.

Although originally developed for *Vibrio cholerae*, APB has been routinely used as an enrichment broth for detection and enumeration of *V. vulnificus* and other *Vibrio* spp. in shellfish meats and environmental samples (3, 15). Sloan et al. (14) compared five selective enrichment broths for recovery of *V. vulnificus* in oysters and reported that APB produced the highest most probable number. The present study is the first to optimize the components of APB and supplements for enhanced recovery of *V. vulnificus*.

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REFERENCES

1. **Brauns, L. A., M. C. Hudson, and J. D. Oliver.** 1991. Use of the polymerase chain reaction in detection of culturable and nonculturable *Vibrio vulnificus* cells. Appl. Environ. Microbiol. **57:**2651–2655.
2. **Cornell, J. A.** 1990. Experiments with mixtures: designs, models, and the analysis of mixture data, 2nd ed., p. 22–65. John Wiley & Sons, Inc., New York, N.Y.
3. **Elliot, E. L., C. A. Kaysner, and M. L. Tamplin.** 1992. *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and other *Vibrio* spp., p. 111–140. *In* U.S. Food and Drug Administration, Bacteriological analytical manual, 7th ed. AOAC International, Arlington, Va.
4. **Hill, W. E., A. R. Datta, P. Feng, K. A. Lampel, and W. L. Payne.** 1992.

Identification of foodborne bacterial pathogens by gene probes, p. 383–417. *In* U.S. Food and Drug Administration, Bacteriological analytical manual, 7th ed. AOAC International, Arlington, Va.
5. **Hill, W. E., S. P. Keasler, M. W. Trucksess, P. Feng, C. A. Kaysner, and K. A. Lampel.** 1991. Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. Appl. Environ. Microbiol. **57:**707–711.
6. **Jackson, J. K., R. L. Murphree, and M. L. Tamplin.** 1997. Evidence that mortality from *Vibrio vulnificus* infection results from single strains among heterogeneous populations in shellfish. J. Clin. Microbiol. **35:**2098–2101.
7. **Janda, J. M., C. Powers, R. Bryant, and S. Abbott.** 1988. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. Clin. Microbiol. Rev. **1:**245–267.
8. **Kaysner, C. A., M. L. Tamplin, M. M. Wekell, R. F. Stott, and K. G. Colburn.** 1989. Survival of *Vibrio vulnificus* in shellfish and shucked oysters (*Crassostrea gigas* and *Crassostrea virginica*) and effects of isolation medium on recovery. Appl. Environ. Microbiol. **55:**3072–3079.
9. **Kelly, M. T., F. W. Hickman-Brenner, and J. J. Farmer III.** 1985. *Vibrio*, p. 282–301. *In* E. H. Lennette, A. Balows, W. J. Hausler, and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
10. **Kelly, M. T., and W. F. McCormick.** 1981. Acute bacterial myositis caused by *Vibrio vulnificus*. JAMA **264:**72–73.
11. **Lotz, M. J., M. L. Tamplin, and G. E. Rodrick.** 1983. Thiosulfate-citrate-bile salts-sucrose agar and its selectivity for clinical and marine *Vibrio* organisms. Ann. Clin. Lab. Sci. **13:**45–48.
12. **Morris, J. G., A. C. Wright, D. M. Roberts, P. K. Wood, L. M. Simpson, and J. D. Oliver.** 1987. Identification of environmental *Vibrio vulnificus* isolates with a DNA probe for the cytotoxin-hemolysin gene. Appl. Environ. Microbiol. **53:**193–195.
13. **Rippey, S. R.** 1992. Shellfish borne disease outbreaks, July 1992 compilation. FDA Report. Northeast Seafood Laboratory, North Kingston, R.I.
14. **Sloan, E. M., C. J. Hagen, G. A. Lancette, J. T. Peeler, and J. N. Sofos.** 1992. Comparison of five selective enrichment broths and two selective agars for recovery of *Vibrio vulnificus* from oysters. J. Food Prot. **55:**356–359.
15. **Tamplin, M. L., A. L. Martin, A. D. Ruple, D. W. Cook, and C. W. Kaspar.** 1991. Enzyme immunoassay for identification of *Vibrio vulnificus* in seawater, sediment, and oysters. Appl. Environ. Microbiol. **57:**1235–1240.
16. **Tamplin, M. L., G. E. Rodrick, N. J. Blake, D. A. P. Bundy, and L. Alexander.** 1983. Public health aspects of halophilic *Vibrio* in Jamaica. West Indian Med. J. **32:**147–151.
17. **Tamplin, M. L., G. E. Rodrick, N. J. Blake, and T. Cuba.** 1982. Isolation and characterization of *Vibrio vulnificus* from two Florida estuaries. Appl. Environ. Microbiol. **44:**1466–1470.