Comparison of Five Selective Enrichment Broths and Two Selective Agars For Recovery of \textit{Vibrio vulnificus} From Oysters

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\textbf{ABSTRACT}

The Bacteriological Analytical Manual (6th edition) specifies use of glucose-salt-teepol (GST) broth for detection of \textit{Vibrio vulnificus} and other halophilic vibrios in seafood. Since teepol is no longer commercially available, this study compared five enrichment broths for their ability to recover \textit{V. vulnificus}. Ten samples of seeded oysters were analyzed using a three-tube MPN and enriched in each of five broths in duplicate. Broth cultures were then streaked onto cellobiose-polymyxin B-colistin (CPC) agar and sodium dodecyl sulfate-polymyxin B-sucrose (SDS) agar plates. Average (± standard error) recovery (log MPN/g) from each broth was as follows: Alkaline-peptone-water (APW), 4.16 ± 0.20; Marine (MRN) broth, 3.63 ± 0.16; Horie’s broth, 2.88 ± 0.17; Monsur’s broth, 2.43 ± 0.16; and GST broth, 1.28 ± 0.28. APW and MRN broths yielded significantly (P<0.05) higher recovery than other broths by the Kruskal-Wallis nonparametric rank test. \textit{Vibrio vulnificus} was isolated with higher frequency from CPC (81%) as compared with SDS (61%) agar plates. Background growth was minimal on CPC agar, facilitating selection of \textit{V. vulnificus} colonies. Based on these results, APW enrichment broth and CPC isolation agar were more efficient for recovery of \textit{V. vulnificus} from oysters than other broth and agar combinations.

\textit{Vibrio vulnificus} is a halophilic marine bacterium first described in 1976 (7). It is phenotypically similar to \textit{Vibrio parahaemolyticus} and has been recognized as a highly virulent pathogen (4,21,30,31). The organism occurs naturally in marine and estuarine environments surrounding the United States (26) and other countries, occasionally causing life threatening human infections in susceptible hosts. It has received increased attention in recent years and is considered as one of the most pathogenic vibrios (9,20,22).

\textit{V. vulnificus} has been associated with wound infections, foodborne septicemia, and gastroenteritis (23,24). A survey of patients with primary septicemia found that 89% had a chronic underlying illness associated with elevated serum iron levels and/or immunosuppression. Moreover, 87% of these patients had eaten raw oysters within 2 weeks prior to onset of symptoms. The infection was fatal for 61% of the patients (9,30,31). Since it is an estuarine bacterium, \textit{V. vulnificus} can easily contaminate filter-feeding seafood such as oysters and clams (25,26). The incidence of this pathogen in shellfish is higher during the summer, and it was found to survive for up to 2 weeks in commercial shellstock and at least 6 d in shucked oysters under refrigeration (15).

The sixth edition of the Bacteriological Analytical Manual of the Food and Drug Administration (FDA) specifies use of glucose-salt-teepol (GST) broth for enrichment in detection of halophilic vibrios, including \textit{V. vulnificus} (33). The FDA has modified this procedure by substituting an alkaline-peptone-salt broth (APS) containing 3% NaCl for GST broth because teepol is no longer commercially available (34). Following a 16-h enrichment at 35-37°C, a 3- mm loopful from the top cm of APS broth tubes is streaked onto cellobiose-polymyxin B-colistin (CPC) and thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates to obtain isolated colonies. Typically, \textit{V. parahaemolyticus} does not grow on CPC, but grows on TCBS, while \textit{V. vulnificus} grows on CPC and on TCBS.

Several publications (1,3,11,27,28) have presented data from studies evaluating several enrichment broths and agar media for their ability to detect \textit{Vibrio} spp. in seafood products. They include arabinose-ethyl violet-based broth (8,13), Monsur’s broth (14,19), and alkaline-peptone-water (APW) (12,14,33). The most commonly used selective isolation agar of pathogenic \textit{Vibrio} spp. is TCBS agar but its selectivity has been questioned, and there is a need for a better isolation medium. Massad and Oliver (18) described CPC agar, which takes advantage of the resistance of \textit{V. vulnificus} and \textit{V. cholerae} to colistin and polymyxin B, and of cellobiose fermentation as a differential element. Recommended incubation at 40°C also eliminates other marine bacteria, although Kaysner et al. (15) indicated that even at 35°C, CPC was inhibitory and allowed growth of pure \textit{V. vulnificus} cultures. Massad and Oliver (18) compared growth of 136 strains from 19 \textit{Vibrio} spp. on CPC,
ENRICHMENT BROTHS FOR V. VULNIFICUS

TCBS, and sodium dodecyl sulfate-polymyxin B-sucrose (SDS) agar. SDS agar had been suggested by Kitaura et al. (16) for detection of V. vulnificus and V. cholerae. Colonies of V. vulnificus on CPC were yellow, surrounded by a yellow zone due to cellobiose fermentation. V. cholerae colonies on CPC were purple surrounded by a blue zone because cellobiose is not fermented. The plating efficiency of CPC agar was found to be greater than that of TCBS and comparable to SDS agar (18).

Use of SDS agar for direct plating isolation resulted in detection of V. vulnificus in six of 9 samples of shellfish (5). In another study, direct plating on TCBS, CPC, and SDS agar media for detection of V. vulnificus in oysters resulted in counts one-tenth of those obtained by the MPN enrichment procedure in APW (15). It was then recommended that an enrichment be developed for the recovery of V. vulnificus from oysters to facilitate selection of typical colonies for identification. The selective properties of CPC agar gave less background microflora than SDS agar, resulting in better recovery of V. vulnificus from enrichment broths (15).

The objective of this study was to compare five enrichment broths and two isolation agar media for their ability to detect and enumerate V. vulnificus in oysters.

MATERIALS AND METHODS

Reference culture

A culture of V. vulnificus (LA M624), recovered from a clinical source, served as the reference or control strain. It was provided by the FDA, Seafood Products Research Center (Bothell, WA). The culture was maintained in motility medium (Difco Laboratories, Detroit, MI) with 2% NaCl, and propagated in tryptic soy agar (TSA, Difco) slants with 2% NaCl at 35°C for 24 h or 24°C for 72 h. The slants were washed with a 2% NaCl solution and the concentration of cells in the suspension was estimated against a standard curve by absorbance readings at 530 nm using a Pye Unicam spectrophotometer (Sargent-Welch, Cambridge, England). The concentration was confirmed by duplicate plate counts on TSA with 2% NaCl.

Culture media

The enrichment broths tested were alkaline-peptone-water (APW) (33); glucose-salt-teepol (GST) broth (33); Marine (MRN) broth (Difco); Horie's arabinose-ethyl violet (HAE) broth (8); and Monsur's (MNS) broth (14,19). The two isolation agar media were cellulbiose-polyoxymyxin B-colistin (CPC) agar (18), and sodium dodecyl sulfate-polyoxymyxin B-sucrose (SDS) agar (16).

Oysters

Ten samples of commercial oysters (Crassostrea virginica) from Maryland were obtained within 4-5 d of harvest from a local seafood distributor in Denver, CO, during the period of June through September. Immediately upon receipt in the laboratory, the oysters were scrubbed under cold tap water, shucked, prepared for inoculation with the reference culture, and analyzed.

Procedure

Duplicate 50-g subsamples from each of the 10 samples of oysters were inoculated with approximately 100 V. vulnificus (LA M624) cells per g. An additional 50-g uninoculated subsample (except for sample 2) was also analyzed as the control. The subsamples were blended for 60 s and serially diluted in 2% sterile saline. Portions (1-ml) were transferred into triplicate tubes (3-tube MPN) of each of the five enrichment broths tested, which were then incubated at 35°C for 16 h. One loopful from each tube was then streaked onto prepared plates of each of the two selective isolation agar media. Inoculated plates of CPC and SDS agar were incubated at 40 and 35°C, respectively, for 20-24 h.

Typical colonies of V. vulnificus are yellow, flat, translucent, often with orange center, and approximately 1-2 mm in diameter on CPC agar. On SDS agar, the colonies are purple, approximately 2-4 mm in diameter, and have distinct halos. Sucrose-fermenting V. vulnificus colonies are yellow with halos on SDS agar.

One to four typical colonies from each CPC or SDS agar plate were picked and inoculated onto triple sugar-iron (TSI) agar (Difco) with 2% NaCl, TSA with 2% NaCl, and modified presumptive Vibrio parahaemolyticus (mpPVP) agar (10) slants, and incubated at 35°C for 20-24 h. Modification of PVP involved elimination of sucrose and lactose, and decreasing the NaCl concentration from 3% to 2% (Kayser, 1988; Laboratory Information Bulletin No. 3205, FDA).

Typical isolates from TSI, TSA, and mpPVP slants were tested for β-galactosidase and oxidase production with Bacto-differentiation discs (Difco), and for agglutination with antisera provided by R. J. Siebeling, Louisiana State University, Baton Rouge, LA (29,32). A representative number of isolates (i.e., at least one from each broth and subsample) was confirmed as V. vulnificus using the API 20E rapid identification system (Analytab Products, Plainview, NY). Suspensions for API 20E inoculation were prepared using sterile 2% saline (17). Suspect isolates were considered positive on TSI agar slants if they gave alkaline-slant/acid-deep reactions, produced no hydrogen sulfide and very little or no gas. Atypical (sucrose-fermenting) V. vulnificus produced an acid/acid reaction on TSI. Additional requirements for confirmation of V. vulnificus isolates included alkaline-slant/acid-deep reactions on mpPVP, motility (positive), oxidase production, β-galactosidase production, and agglutination with V. vulnificus antiserum. Motility was tested in motility (SIM) medium (Difco) with 2% NaCl. For each enrichment broth, most probable numbers (MPN) were determined from the number of tubes containing confirmed isolates.

Statistical analysis

A nonparametric (Kruskal-Wallis) test of significance (6) was performed to compare the ability of each of the five enrichment broths to recover V. vulnificus in oysters. The Kruskal-Wallis test can be used to determine whether random samples represent identical populations when it is assumed that the populations are not normal. The actual data of each sample were ranked (i.e., 1, 2,...n) according to increasing numbers (MPN per broth), and average ranks were computed for each broth, then analyzed for significance (α = 0.05) with the Kruskal-Wallis nonparametric test.

RESULTS AND DISCUSSION

The oyster samples were collected and analyzed in the months of June through September. As the data indicate (Fig. 1), the level of natural contamination was so high that the added inoculum (100 cells/g) contributed very little to the total contamination with V. vulnificus cells. The objective of the study, however, was to evaluate five enrichment broths and two isolation agar media for their ability to detect V. vulnificus in oysters. The log MPN number of organisms detected per g of oysters decreased with enrichment broth in the sequence of APW > MRN > HAE > MNS > GST (Fig. 1). The average log MPN of V. vulnificus cells in inoculated oysters detected by GST broth was only
1.28/g, while the log MPN detected with APW was 4.16/g (Fig. 1). The average log MPN for the other broth cultures were between these two extremes.

Figure 1. Comparison of five selective enrichment broths (GST, MNS, HAE, MRN, and APW) for recovery of V. vulnificus from oysters by a 3-tube MPN procedure.

Figure 2 shows the statistical analyses of the results for uninoculated (control) and inoculated oysters, respectively. It is obvious that there were significant differences in V. vulnificus recovery among various broths tested. These differences are represented by the average rank of each broth calculated according to the Kruskal-Wallis nonparametric test. For the ten inoculated and nine naturally contaminated control samples, the average rank (Kruskal-Wallis test) for APW was significantly (α = 0.05 level) higher than the average ranks of GST, MNS, and HAE. MRN broth gave statistically equivalent, but lower, counts compared to APW. Thus, APW could be recommended as an appropriate broth for recovery of V. vulnificus cells from oysters.

Background, atypical growth was less on CPC agar plates than on SDS plates, which facilitated selection of V. vulnificus colonies. Cells of V. vulnificus were isolated from CPC agar plates more frequently than from SDS agar plates (Table 1). Of 580 total "typical" (cellobiose fermenters) colonies selected from CPC plates, 468 isolates (81%) were identified as V. vulnificus. Most of the nonconfirmed isolates, although cellobiose fermenters (yellow), were not typical (1-2 mm in dia., flat, and translucent) in morphology. Only 207 of 342 "typical" colonies (61%) from SDS agar plates were confirmed as V. vulnificus.

Based on these results, APW enrichment broth with CPC isolation agar was more efficient than other media for recovery of V. vulnificus in oysters. These results are consistent with FDA's current procedure for Vibrio spp. analysis of seafoods. Although these results are based on recovery of V. vulnificus from C. virginica, the combination of APW broth and CPC agar may be suitable for the recovery and enumeration of V. vulnificus in other species of oysters, other seafoods, or environmental samples.

ACKNOWLEDGMENTS

We thank C. A. Kaysner (Seafood Products Research Center, U.S. Food and Drug Administration, Seattle, WA) and M. L. Tamplin (formerly with Fisheries Research Branch, U.S. Food and Drug Administration, Dauphin Island, AL) for initial methodology, "Isolation and Identification of Vibrio vulnificus" (1988, unpublished), and R. J. Siebeling (Department of Microbiology, Louisiana State University, Baton Rouge, LA) for supplying the V. vulnificus antiserum.

TABLE 1. Efficiency of cellobiose-polymyxin B-colistin (CPC) and sodium dodecyl sulfate-polymyxin B-sucrose (SDS) isolation agar media for detection of V. vulnificus with five enrichment broths from oysters.

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<th>Sample No.</th>
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<th>SDS agar</th>
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<tr>
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<td>No. of colonies confirmed/No. of colonies picked</td>
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<td>Overall</td>
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REFERENCES


