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Refined Medium for Direct Isolation of *Vibrio vulnificus* from Oyster Tissue and Seawater[▽]

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We have developed a new medium for the direct isolation of *Vibrio vulnificus* from water and oyster samples. The medium was shown in laboratory and field studies to be highly selective without providing preferential isolation of either *V. vulnificus* genotype.

Vibrio vulnificus, biotype 1, causes invasive wound infections and life-threatening systemic disease, particularly in individuals with underlying health risks (6, 12, 17). Infection is associated with the consumption of raw shellfish or exposure of preexisting wounds to seawater or seafood products (6, 17). Biotype 1 has been further classified into two genotypes by sequence variations identified in both the 16S rRNA gene (7, 14) and a virulence-correlated gene (*vcg*) (15, 16). We have shown (16) a high correlation between clinically isolated strains and possession of the *vcgC* variation (C genotype, corresponding to the 16S B variant), while most cells isolated from the environment have the *vcgE* variation (E genotype, corresponding to the 16S A variant) (14, 16). *V. vulnificus* samples isolated from oysters exhibit a high level of genetic diversity. However, strains isolated from clinical cases appear to arise from single strains (11). This implies that not all strains are equally virulent and highlights an important reason for the accurate enumeration and differentiation of the strains isolated.

Currently, there is no medium available that has both the specificity and the sensitivity required to detect low numbers of *V. vulnificus* in the environment (8). Several plating media have been developed based on the fermentation of cellobiose by *Vibrio vulnificus*, including two derivatives of cellobiose-polymyxin B-colistin (CPC) (modified CPC [mCPC] and cellobiose-colistin [CC]) and *Vibrio vulnificus* medium (VVM) (1, 4, 9, 13). While such media are sometimes used for direct plating, enrichment in alkaline peptone water (APW) (4) is often utilized to elevate the number of bacteria in a sample. Preliminary studies (unpublished) indicated that enrichment followed by plating to our original CPC agar allowed for a significant ($P < 0.05$) selective advantage of C-genotype strains over those of the E genotype. Our primary goal was to eliminate the enrichment step and develop a medium for the efficient direct plating of samples. This would facilitate the accurate enumeration of total *V. vulnificus* numbers in oysters and water samples without providing any selective advantage to either genotype. In our new formulation, we have modified the total salt concentration of CPC agar, added Mg^{2+} and K^{+}, and adjusted the antibiotic concentrations. To evaluate these modifications, we compared our revised CPC (CPC+) to other CPC derivatives and VVM.

V. vulnificus strains employed were C7184K, CMCP6, YJ016, LSU1866, and SPRC10143 (C-genotype strains) and Env1 (SS109B-3B2), JY1305, 3001C1, SS108A3A, and JY1701 (E-genotype strains). Log-phase cells of the above-named strains were grown in heart infusion (HI) broth. They were then either washed with one-half-strength artificial seawater (ASW) (18 ppt) and starved in one-half-strength ASW prior to inoculation into APW or added unwashed into APW to a final concentration of 1:1,000. Cells were incubated for 18 to 24 h at 37°C (4). Serial dilutions were made and plated to both HI and selective media and incubated overnight at 37°C. Comparisons of log-phase cultures inoculated directly into one-half-strength ASW (no APW enrichment) were made with all selective media. Growth on each medium was compared to that on HI, with the resultant ratio calculated as percent recovery.

Log-phase cells of two C-genotype and two E-genotype strains were diluted and added to APW with or without KCl and $MgCl_2$ (each at 4 ppt) and containing colistin methanesulfonate at 0 to 5,000 units/ml to provide a final concentration of 10^5 CFU/ml. Cultures were incubated at 37°C for 24 h. Serial dilutions were plated onto HI agar, and the numbers of CFU were compared to the numbers of CFU obtained from broth cultures lacking colistin. The resultant ratio was taken as the percent recovery.

CPC+ medium is prepared from two solutions. Solution A contains Bacto peptone (10 g), proteose peptone (5 g), NaCl (10 g), $MgCl_2 \cdot 6H_2O$ (4 g), KCl (4 g), bromothymol blue (0.04 g), cresol red (0.04 g), and distilled water (900 ml). The pH is adjusted to 7.6, and 15 g of agar is added. The solution is autoclaved for 20 min and then cooled to between 55 and 60°C. Solution B contains D-cellobiose (15 g), colistin methanesulfonate (1×10^6 units), and polymyxin B (4×10^5 units) dissolved in 100 ml distilled water by heat treatment at a low temperature. This solution is cooled to 55 to 60°C before the antibiotics are added and then filter sterilized and added to solution A.

Ten oysters were collected from Alligator Bay, NC. They were washed, shucked, and processed within 1 hour of collection along with a water sample taken from the same site. Minimal amounts of sterile one-half-strength ASW were added as required to allow the homogenization of oyster tissue.

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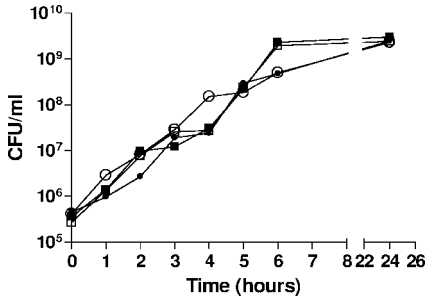

FIG. 1. Plating on HI agar following the growth of strains of the C (●, CMCP6; ■, C7184K) and E (○, Env1; □, JY1305) genotypes in APW.

One hundred microliters of homogenate was plated to CPC, mCPC, CC, VVM, and CPC+ agars. The water samples (1- and 10-ml aliquots) were filtered through 0.22-μm filters, which were directly plated to each medium. Following incubation overnight at 37°C, flat yellow colonies with a yellow halo on all *V. vulnificus*-specific media were designated presumptive for this species. A total of 175 of 303 presumptive colonies on CPC+ from oysters and all 12 colonies from the 10-ml seawater sample were picked for PCR analysis. Template DNA was prepared by boiling 1 ml of the overnight culture for 5 min, which was followed by centrifugation, with supernatants being transferred to fresh tubes for storage at 4°C. Separate PCRs were preformed using the high-fidelity TaKaRa *Ex Taq* system, with primers and cycling profiles for *vcgC*, *vcgE*, and *vvhA* as previously described (16).

We performed a one-way analysis of variance with Dunnett's post hoc test comparing results on all medium types and a two-way analysis of variance for medium and genotype results with the Bonferroni post hoc test, as applicable.

Colonies of *V. vulnificus* on CPC+ agar appeared flat and yellow with a yellow halo. Colony appearance was essentially the same for all media tested, as they all rely on the fermentation of D-cellobiose and the resultant pH change (4, 13). Previous tests of CPC and VVM indicated that *V. cholerae* and other *Vibrio* species either did not grow or produced blue-green colonies with a purple halo due to the lack of fermentation (1, 13). Because the carbohydrate source was unchanged, CPC+ remained differential for other *Vibrio* species.

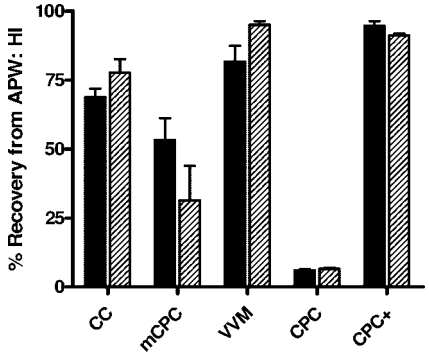

FIG. 2. APW enrichment employed prior to plating to the various *V. vulnificus*-selective media. Black bars, C genotype; gray bars, E genotype.

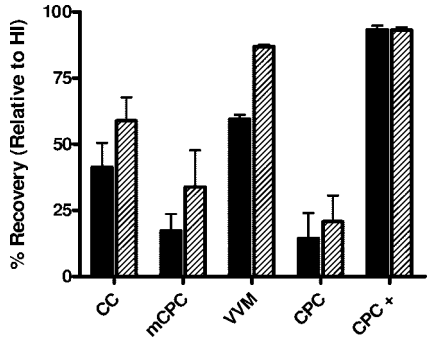

FIG. 3. Comparison of levels of direct recovery of each genotype on selective media following starvation. Black bars, C genotype; gray bars, E genotype.

When previously isolated C-genotype and E-genotype strains were grown in APW and then plated to HI agar, no significant difference in growth rates over a 24-h period was observed (Fig. 1). Thus, APW enrichment does not appear to select for either genotype. However, when APW-enriched cells were plated to the various *V. vulnificus*-selective media (Fig. 2), the reduced levels of recovery of both genotypes on CPC, mCPC, and CC were highly significant ($P < 0.01$) compared to the levels of recovery on CPC+. For mCPC, a significant ($P < 0.05$) selective advantage was seen for the recovery of C-genotype over E-genotype cells. The recovery of either genotype from enrichment on VVM was not significantly different from that on CPC+ agar.

Following incubation under starvation conditions, 93.3% of C-genotype strains and 93.1% of E-genotype strains were recovered on CPC+, compared to 59.4% and 86.6%, respectively, on VVM. The variations in growth of these strains on CPC, mCPC, and CC were highly significant ($P < 0.01$) (Fig. 3). In addition, the levels of recovery of the strains of the E genotype on CPC, mCPC, VVM, and CC were significantly higher ($P < 0.01$). This differential isolation suggested different sensitivities of the two genotypes to the peptide antibiotics employed. Colistin methanesulfonate and polymyxin B, antibiotics that affect primarily gram-negative bacteria by altering membrane permeability, leading to cell lysis (2, 5), are often used to enhance the selection of *V. vulnificus* (13). A study of the two genotypes revealed significant differences in their re-

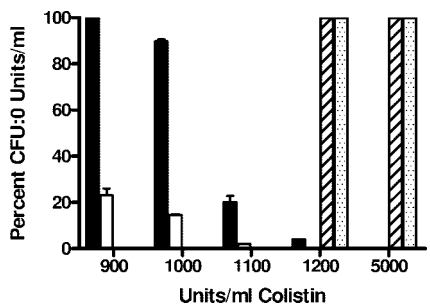

FIG. 4. Comparison of levels of growth of C ($n = 2$)- and E ($n = 2$)-genotype strains at various concentrations of colistin. Black bars, C-genotype strains; white bars, E-genotype strains; hatched bars, C-genotype strains with K and Mg added; gray bars, E-genotype strains with K and Mg added.

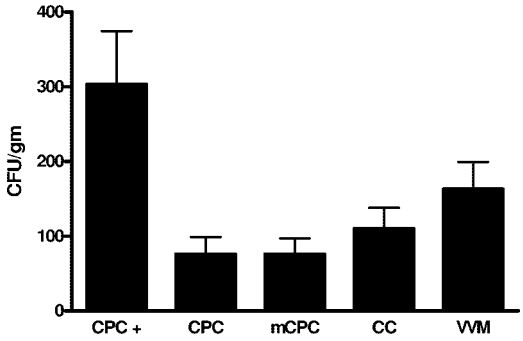

FIG. 5. Comparison of selective media for isolation of *V. vulnificus* from oysters.

sponses to colistin methanesulfonate (Fig. 4). This difference was eliminated upon the addition of $MgCl_2 \cdot 6H_2O$ and KCl salts to the medium, with both genotypes remaining fully culturable at a dosage of 5,000 units colistin/ml. Such an effect has previously been reported for these antibiotics (2). Furthermore, the addition of these salts altered the formulation of the medium to one more comparable with seawater.

Little variation was seen in the numbers of *V. vulnificus* cells isolated from oysters on CPC, mCPC, and CC, while their overall recovery rates were significantly lower ($P < 0.01$) than on CPC+ (Fig. 5). The difference in recovery of presumptive colonies on CPC+ and VVM approached significance ($P = 0.0563$). Low numbers of *V. vulnificus* are typically recovered from water, and only a single colony was isolated on CPC+ from the 1-ml sample, with no colonies being isolated at this volume on the other media tested. There were 12 colonies identified on CPC+ and 8 on VVM from the 10-ml sample. At this volume, only a single colony was identified on CPC, and three colonies each were identified on CC and mCPC.

In the current formulation, we modified CPC by reducing NaCl from 20 ppt to 10 ppt, adding 4 ppt each $MgCl_2 \cdot 6H_2O$ and KCl, and adjusting total peptide antibiotic levels to 1,400 units/ml. The added potassium and magnesium salts allowed the use of high levels of peptide antibiotics without interfering with *V. vulnificus* isolation. CPC+ demonstrated a better recovery of cells from oyster and water samples without enrichment and did not provide a selective advantage to either genotype. The use of peptones in CPC+ constitutes the only difference between VVM and CPC+. VVM utilizes yeast extract, which is contraindicated for use in fermentation and single-carbon-source media (3). This variation in nutrient source may explain the differences seen in the levels of recovery of *V. vulnificus* on VVM and CPC+ (10). Less growth of nontarget bacteria was observed on CPC+ than on the other media; in all oysters yielding presumptive *V. vulnificus* colonies, only 8 to 10% of the total bacterial counts were found to represent bacterial species other than *V. vulnificus*. In contrast, typically 20 to 50% of the total colonies on CC, mCPC, and VVM were found to be species other than V. *vulnificus*. Of the 175 presumptive colonies collected from oysters on CPC+ agar, 160 (91.4%) were confirmed by PCR as *V. vulnificus*. Similarly, 9 of 12 (75%) water isolates cultured on CPC+ were identified as *V. vulnificus*.

Our results suggest that the nutritional status of *V. vulnificus* cells can have an impact on the recovery of the two genotypes and that enrichment can influence the selection for one or the other genotype when organisms are plated to some *V. vulnificus*-selective media. There was a selective advantage for the C genotype when cells were taken from either a nutrient-rich environment, such as that found in an oyster, or a nutrient-poor environment, such as that found in seawater, and then enriched in APW and plated to selective media. This advantage was eliminated with CPC+.

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