

Comparison of two selective and differential media for the isolation of *Vibrio vulnificus* from the environment

T.C. Williams*, B.A. Froelich, and J.D. Oliver

University of North Carolina at Charlotte, 9201 University City Blvd, Charlotte, NC 28223 (tcwilli1@uncc.edu)

Introduction

Vibrio vulnificus is an opportunistic human pathogen that naturally inhabits estuarine and coastal waters worldwide. This gram-negative bacterium is ubiquitous in these environments and has been isolated from water, sediments, fish, and shellfish such as bivalve mollusks. *V. vulnificus* is a medically important pathogen due to its ability to cause fulminant and potentially fatal systemic infection when ingested via raw or undercooked shellfish. In addition, this water-borne pathogen can cause severe wound infections often resulting in necrotizing fasciitis. Considering the medical relevance of this pathogen, it is important to be able to isolate and identify this organism from the environment even when occurring at very low numbers. Our lab has previously developed and modified a selective and differential medium, CPC+, which is efficient at isolating *V. vulnificus* from the environment without the need for enrichment (Warner and Oliver, 2007). Recently, another medium, CHROMagar Vibrio (here denoted CaV) has come into use as a means for isolating vibrios from the environment. The goal of our study was to compare the efficacy of CPC+ and CaV in isolating *V. vulnificus*, and to highlight the benefits and downfalls of each one.

Methods

Comparison of growth on CaV and CPC+

Selected strains (Table 1) were plated from freezer stocks onto heart infusion (HI) agar, CaV, and CPC+ and allowed to incubate overnight (at 30°, 37°, and 40° C, respectively). The ability to grow and colony color was documented.

Cross checking efficacy of CHROMagar Vibrio and CPC+

Oyster homogenates were diluted and plated on CPC+ or CaV and allowed to incubate (at 40° and 37° C respectively). Presumptive *V. vulnificus* colonies from CPC+ (flat yellow colonies with brownish centers and yellow halos) were selected and plated onto CaV. In a similar manner, colonies of all representative colors from CaV were picked and plated onto CPC+. C/E Multiplex PCR was performed on all presumptive *V. vulnificus* colonies, as described by Warner and Oliver (2008), to definitively identify this species.

Identification of unknowns, V. vulnificus-like isolates

Biochemical testing was performed using the dichotomous key of Nogueroles and Blanch (2008).

Results/Discussion:

Efficacy of CPC+

CPC+ has been extensively utilized by our lab as an efficient means of isolating *V. vulnificus* from environmental samples (typically oysters and water), even when present in low numbers. Warner and Oliver (2007) found that, of 175 presumptive *V. vulnificus* colonies from oysters, 160 (91.4%) were confirmed by PCR to be this species. In addition, 75% of water isolates were confirmed as *V. vulnificus* from this medium. Similar studies from our lab between years 2008 and 2010 found that, Gulf coast oysters sampled with CPC+ returned positive *V. vulnificus* isolates >95% of the time. Using CPC+ not only increases the extent of recovery (thereby eliminating the need for enrichment), but also eliminates any selective growth of one of the two genotypes (C or E) known to occur in this pathogen. In addition, this medium inhibits the growth of a number of non-vulnificus *Vibrio* species (typically those that form blue/green colonies with a purple halo due to lack of cellobiose fermentation). Despite the benefits of

CPC+ there are some limitations. We have found that a small percentage of presumptive colonies that are not *V. vulnificus* can confound results significantly when *V. vulnificus* is present in low numbers. A recent study by our lab examined *V. vulnificus* populations in oysters collected from the North Carolina and Gulf coasts of the USA. We found that, of 21 presumptive *V. vulnificus* colonies from Gulf coast oysters obtained on CPC+, 21 (100%) were confirmed by PCR to be *V. vulnificus* (Figure 1; Lanes 17-26 and 28-36). However, the same procedure was performed on NC oysters and of 14 presumptive *V. vulnificus* colonies, none were confirmed by PCR to be this species (Figure 1; Lanes 2-15). This finding prompted us to perform a series of tests in order to identify these false positive, non-vulnificus organisms.

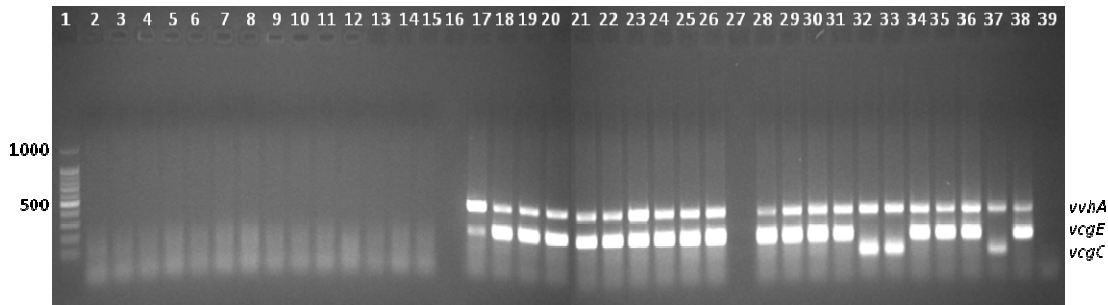


FIGURE 1. Lane 1: 100bp DNA ladder;
 Lanes 2-15: NC oyster samples from CPC+ agar, false positives;
 Lanes 17-26: Gulf coast oyster samples from CPC+, positive for *V. vulnificus*;
 Lanes 28-36: Gulf coast oyster samples from CaV, positive for *V. vulnificus*,
 Lane 37-39: *V. vulnificus* (C-genotype) positive control, E-genotype positive control, negative control, respectively

Efficacy of CHROMagar Vibrio

CHROMagar Vibrio (CHROMagar; Paris, France) uses chromogenic technology to allow for the isolation and detection of *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, and *V. alginolyticus*, resulting in colonies which can be distinguished (Table 1) based on color development (mauve, dark blue, light blue, and white/colorless, respectively). Indeed, the ability to isolate and identify these four medically relevant pathogenic vibrios with one medium is highly advantageous. Such a medium provides a considerably larger amount of information about the population structure of the environment being. Despite the advantages provided by CaV, we have encountered a similar problem with this medium as was seen with CPC+, that is, when *V. vulnificus* numbers are low it appears that a non-vulnificus false positive *Vibrio* species can be present in large numbers. These colonies grow dark blue on CaV, thus appearing to be *V. vulnificus*; however PCR reveals that they are not this species (data not shown).

TABLE 1. Comparison of growth on CPC+ and CHROMagar Vibrio

	CPC+	CaV
<i>Acinetobacter sp.</i>	NG	NG
<i>V. mimicus</i>	NG	NG
<i>V. vulnificus</i>	yellow	dark blue
<i>V. alginolyticus</i>	yellow ^a	white
<i>V. parahaemolyticus</i>	NG	purple ^b
<i>V. cholerae</i>	purple ^c	light blue

(NG = no growth)

a – 3/4 strains were yellow, 1 strain did not grow

b – 2/3 strains were purple, 1 strain did not grow

c – 3/4 strains were purple, 1 strain did not grow

Identification of unknowns

By cross plating colonies from CPC+ to CaV, and vice versa, we found that there are 3 bacterial species that yield false positives for *V. vulnificus*. Characteristics of these unknowns are seen in Table 2.

TABLE 2. Identification of unknowns (false positives for *V. vulnificus*)

	Characteristics of unknown on CPC+	Characteristics of unknown on CaV	Identified unknown
Type 1	yellow colonies with halo (+)	white colonies	<i>V. alginolyticus</i> or??
Type 2	no growth	dark blue colonies (+)	likely <i>V. metschnikovii</i> possibly <i>V. calviensis</i>
Type 3	yellow colonies with halo (+)	dark blue (+)	likely <i>V. coralliilyticus</i>

(+) – colonies that are considered presumptive *V. vulnificus* positives

As shown in Table 1, some but not all, strains of *V. alginolyticus* are able to ferment cellobiose on CPC+ and therefore appear as presumptive *V. vulnificus* colonies. We have shown here, however, that *V. alginolyticus* strains which appear to be *V. vulnificus* on CPC+ are clearly distinguishable as white colonies on CaV. Thus, we determined that *V. alginolyticus* at least partially contributes to the presence of false positives on CPC+.

Further testing of unknowns was performed using biochemical tests as outlined by Noguerola and Blanch (2008) and preliminary results are displayed in Table 2 (further confirmation of unknowns is currently in progress).

Conclusions

Elucidating the population structure of *Vibrio vulnificus* in its natural environment is essential for our understanding of the ecology of this organism. From this study, we now better understand the perks and pitfalls of CPC+ and CHROMagar *Vibrio* as selective and differential media for the isolation and identification of this important pathogen. As we have shown, neither medium is flawless, due to the potential for false positives that arise on both media, particularly in the event that *V. vulnificus* levels are low. Unfortunately, we cannot use the two media in conjunction to rule out false positives due to strains that yield false positives on both media. Nevertheless, both media have distinct benefits. As seen in Table 1, CaV has the ability to clearly distinguish *V. alginolyticus* (as white colonies) which can appear as a false positive on CPC+. In addition, CaV allows us to presumptively isolate and distinguish between four medically relevant *Vibrio* spp. CPC+ has been shown to isolate *V. vulnificus* with a high rate of recovery when this bacterium is present in sufficient numbers. In addition, it has been demonstrated that CPC+ does not discriminate between C- or E-genotypes, which is essential for understanding the dynamics of these two genotypes in the environment.

References

- Noguerola, I. and A.R. Blanch. (2008). Identification of *Vibrio* spp. with a set of dichotomous keys. *J. Appl. Microbiol.* 105(1): 175-185.
- Warner E. and J.D. Oliver. (2007). Refined medium for direct isolation of *Vibrio vulnificus* from oyster tissue and seawater. *Appl. Environ. Microbiol.* . 73(9): 3098-3100.
- Warner, E.B. and J.D. Oliver. (2008). Multiplex PCR assay for detection and simultaneous differentiation of genotypes of *Vibrio vulnificus* biotype 1. *Food. Path. Dis.* 5(5): 691-693.