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A new culture-based method for the improved identification of *Vibrio vulnificus* from environmental samples, reducing the need for molecular confirmation

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ABSTRACT

Vibrio vulnificus is an opportunistic human pathogen responsible for 95% of seafood related deaths in the US. Monitoring the presence of this bacterium in estuarine waters and shellfish is of medical and economic importance due to its ability to cause severe wound infections and fulminant septicemia. Current methods for isolating *V. vulnificus* from environmental samples typically employ an initial selective medium which requires subsequent molecular confirmation of presumptive *V. vulnificus* isolates. Although culture-based methods are accessible and inexpensive, they lack the specificity needed to definitively identify *V. vulnificus.* The goal of this study was to develop a more accurate, culture-based method for the initial detection of *V. vulnificus,* thereby decreasing or eliminating the requirement for confirmatory molecular tests. Colony color characteristics of a variety of *Vibrio* species were determined on three commonly employed media to identify those which present as false-positive isolates for *V. vulnificus*. We subsequently developed a triple-plating method which utilizes three media in combination to greatly decrease the number of false positive isolates. The number of isolates positively identified as *V. vulnificus* using the triple-plating method were compared to a typical single-plating method and revealed over a 2-fold increase in ability to accurately predict *V. vulnificus* isolates. We suggest that this new method will enhance the predictive power of culture-based methods, reduce the cost and time spent on additional detection methods, and may be a valuable alternative when molecular methods are not available or unaffordable.

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1. Introduction

Vibrio vulnificus is ubiquitous in estuarine environments and has been isolated from water, sediments, fish, and shellfish (Daniels, 2011; Oliver, 2006a). This bacterium is a medically relevant pathogen due to its ability to cause fulminant and potentially fatal systemic infection when ingested, usually via raw or undercooked shellfish (Oliver, 2006b). In an analysis of data provided by the U.S. FDA between 1992 and 2007 in which 459 cases were examined, this bacterium was documented to have a 51.6% fatality rate (Jones and Oliver, 2009; Oliver, 2006b), the highest of any food-borne disease (Oliver, 2012a). Furthermore, this water-borne pathogen can cause severe wound infections such as necrotizing fasciitis, although with a lower mortality rate (ca. 25%) than systemic disease (Oliver, 2006b). Wound infections caused by *V. vulnificus* are the predominant form of disease by this organism in Europe, with significantly increasing rates of infection being seen in Baltic countries (Baker-Austin et al., 2013). Considering the public health hazard presented by this pathogen, it is of extreme importance to be able to isolate and correctly identify this organism from its natural environment, particularly in countries where shellfish is consumed (Oliver and Kaper, 2007).

V. vulnificus strains are phenotypically and genetically diverse and can be categorized into 3 biotypes based on biochemical characteristics in which biotype 1 (BT1) strains are primarily responsible for the majority of human infections, whereas biotype 2 (BT2) strains are more frequently associated with disease in eels (Bisharat et al., 1999; Sanjuan et al., 2009; Tison et al., 1982). Biotype 3 (BT3) strains have distinct phenotypic and molecular patterns that indicate the occurrence of a recent genome hybridization event between BT1 and BT2 (Bisharat et al., 1999, 2005, 2007). To date, documented isolates of this biotype have been geographically limited to aquaculture facilities in Israel (Bisharat et al., 2005). Previous genetic analyses of BT1 strains have revealed the presence of genetic polymorphisms that highly correlate with the source of isolation (Chatzidaki-Livanis et al., 2006; Nilsson et al., 2003; Rosche et al., 2010; Rosche et al., 2005; Sanjuan et al., 2009; Senoh et al., 2005; Vickery et al., 2007). Thus, we further sub-type BT1 strains into two genotypes: C-genotypes (clinical)

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and E-genotypes (environmental), which can be differentiated by PCR analysis of the virulence-correlated gene (*vcg*) (Warner and Oliver, 2008). Additional genetic distinctions amongst these two genotypes have also been established (Cohen et al., 2007; Froelich and Oliver, 2011; Gulig et al., 2010; Morrison et al., 2012).

Various selective and/or differential media are employed to isolate *Vibrio* species from environmental samples (Oliver, 2012b). These media are typically useful in permitting the growth of select *Vibrio* species while excluding the growth of other vibrios and closely related genera. However, due to the phenotypic variability of *Vibrio* species, all of these media require an additional molecular step, such as PCR, to confirm the identification of the organism of interest (Harwood et al., 2004). Thiosulfate-citrate-bile salts-sucrose agar (TCBS) was one of the first selective media used for the isolation and purification of vibrios (Oliver, 2012b). On this medium, strains able to ferment sucrose form yellow colonies whereas sucrose non-fermenters, such as *V. vulnificus*, appear green (Thompson et al., 2004). This medium has been widely employed for isolation of *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio fluvialis*, and *V. vulnificus* from clinical specimens as well as from the aquatic environment, yet studies have demonstrated batch-to-batch and brand-to-brand variations of this medium, with the level of recovery of vibrios varying greatly (Bolinches et al., 1988; West et al., 1982). Additionally, despite the selectivity for vibrios, other genera such as *Staphylococcus*, *Flavobacterium*, *Pseudoaltermonas*, and *Shewanella* can grow on this medium as well (Thompson et al., 2004). Thus, TCBS is not typically used as the primary method for isolation of *V. vulnificus* from environmental samples.

Colistin/Polymyxin B/Cellobiose agar (CPC) and CPC+ (a modified version of CPC) have been used by labs worldwide to isolate *V. vulnificus* from environmental samples (Massad and Oliver, 1987; Sun and Oliver, 1995; Warner and Oliver, 2007). Due to its ability to ferment D-cellobiose, *V. vulnificus* yields characteristic yellow colonies with a darker center and yellow halo, whereas *V. cholerae* and other vibrios typically do not grow on this medium, or grow as green colonies with a purple halo. As demonstrated by Warner and Oliver (Warner and Oliver, 2007), CPC+ is highly effective in the isolation of *V. vulnificus*, without the need for enrichment, and does not exhibit genotypic bias. Presumptive *V. vulnificus* colonies on CPC+ can be confirmed by genetic testing (Rosche et al., 2005; Warner and Oliver, 2008).

CHROMagar *Vibrio* (CHROMagar; Paris, France), here denoted as CaV, is a proprietary medium that uses chromogenic technology to allow for isolation and detection of *V. vulnificus*, *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* based on colony color development. On this medium *V. vulnificus* colonies appear turquoise, likely due to β-galactosidase activity (Monget and Robichon, 2011). The ability to isolate and identify four pathogenic vibrios with one medium is highly advantageous, and such a medium provides a considerable amount of information about the population structure of an environment being examined. In the current study, we demonstrate that direct recovery of *V. vulnificus* on CaV is comparable to that of CPC+. However, this medium also must be used in conjunction with a molecular method in order to confirm the identity of presumptive colonies.

Despite the usefulness of CPC+ and CaV in isolating *V. vulnificus* from environmental samples, other organisms have the ability to grow on these media, sometimes appearing identical to colonies of *V. vulnificus*. This appears to be particularly the case when *V. vulnificus* populations are diminished, a phenomenon reported by several investigators (Arias et al., 1999; Froelich et al., 2012; Macian et al., 2000; Staley et al., 2013). Studies by our lab have indicated that certain environmental conditions, *e.g.* long-term drought leading to a significant increase in estuary salinity can, result in the ability of other *Vibrio* species (likely *V. coralliilyticus* and *V. mediterranei*) to outcompete *V. vulnificus* in the environment (Froelich et al., 2012). Staley et al. (2013) recently documented a similar phenomenon in which *V. vulnificus* appeared to be outcompeted by *V. sinaloensis* in warmer estuarine waters (Staley et al., 2013). Under these circumstances, the number of false positive isolates can greatly exceed the number of true *V. vulnificus* isolates using the aforementioned media. This can lead to a significant loss of time and resources when attempting to isolate and identify this species in an environmental sample. For example, a recent study in which 1041 *V. vulnificus* presumptive isolates from CPC+ were examined, reported only 0.6% could be confirmed to be this species (Froelich et al., 2012). That study resulted in an expense of over $1200 in order to positively identify only six isolates of *V. vulnificus*.

To better characterize the growth of *Vibrio* species and strains on CPC+, TCBS, and CaV, we examined colony color reactions for 60 *Vibrio* strains representing 17 *Vibrio* species and identified strains which appeared identical to *V. vulnificus*. We sought to eliminate these false positive isolates by developing an improved culture-based method utilizing all three media. In this case, presumptive colonies of *V. vulnificus* initially grown on CaV were subsequently plated onto both CPC+ and TCBS allowing for the identification of "triple-positive" isolates which were predicted to be *V. vulnificus*. We compared the performance of this new method to the conventional single-plating method, and validated its utility *in vitro* and *in situ* using molecular confirmation. Cross-plating *V. vulnificus* presumptive isolates on all three media was shown to significantly reduce the number of false positive isolates, subsequently reducing the number of isolates that require molecular confirmation. We suggest this new and simple triple-plating technique will provide a more accurate, more time efficient, and more cost effective method for the initial detection of *V. vulnificus*. This method would be particularly useful when molecular methods are not available or too costly.

2. Methods

2.1. Characterizing growth and colony color of Vibrio species/strains on each medium:

The twenty-five strains of *V. vulnificus* used in this study (including all three biotypes and both C- and E-genotypes) are listed in Table 1. Thirty-five additional *Vibrio* strains, representing 16 *Vibrio* species, were characterized on each medium, and are listed in Table 2. Each strain was grown overnight in Heart Infusion (HI) broth, diluted in phosphate buffered saline (PBS), and plated onto TCBS, CaV, CPC+, and HI agar plates. HI and TCBS were incubated at 30 °C, CaV at 37 °C and CPC+ at 37 °C (pure culture/laboratory studies) or 40 °C (environmental studies). Each medium was then evaluated for the ability of each organism to grow, along with colony color developments. Strains that grew identically to *V. vulnificus* on each medium were noted.

2.2. Comparing direct recovery of V. vulnificus on CaV and CPC+

To evaluate the use of CaV as an initial selective medium, overnight and starved cultures of *V. vulnificus* cells were plated onto CaV and compared to direct recovery on CPC+ and HI. *V. vulnificus* isolates (of both C- and E-genotypes) were prepared by growing strains from freezer stocks in HI for 24 h. Serial dilutions were made and plated onto HI, CPC+, and CaV and incubated at appropriate temperatures overnight. To demonstrate that physiologically starved cells could also be recovered on CaV, an aliquot of overnight cells were washed twice with PBS to remove residual nutrients, inoculated into a basal salt medium (BSM) lacking any carbon source at a final cell concentration of 2e8 CFU/ml, and incubated standing at room temperature for six months. These starved cultures were then diluted into PBS, plated onto HI and selective media and incubated at appropriate temperatures overnight. Growth on CaV and CPC+ was measured in CFU/ml and compared to growth on HI, with the resultant ratio calculated as percent recovery. *V. vulnificus* strains used for this study are specified in Table 1. Results were statistically analyzed using a 2-way ANOVA.

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