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Differential specificity of selective culture media for enumeration of pathogenic vibrios: Advantages and limitations of multi-plating methods

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ABSTRACT

Plating environmental samples on vibrio-selective chromogenic media is a commonly used technique that allows one to quickly estimate concentrations of putative vibrio pathogens or to isolate them for further study. Although this approach is convenient, its usefulness depends directly on how well the procedure selects against false positives. We tested whether a chromogenic medium, CHROMagar Vibrio (CaV), used alone (single-plating) or in combination (double-plating) with a traditional medium thiosulfate-citrate-bile-salts (TCBS), could improve the discrimination among three pathogenic vibrio species (*Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*) and thereby decrease the number of false-positive colonies that must be screened by molecular methods. Assays were conducted on water samples from two estuarine environments (one subtropical, one tropical) in a variety of seasonal conditions. The results of the double-plating method were confirmed by PCR and 16S rRNA sequencing. Our data indicate that there is no significant difference in the false-positive rate between CaV and TCBS when using a single-plating technique, but determining color changes on the two media sequentially (double-plating) reduced the rate of false positive identification in most cases. The improvement achieved was about two-fold on average, but varied greatly (from 0- to 5-fold) and depended on the sampling time and location. The double-plating method was most effective for *V. vulnificus* in warm months, when overall *V. vulnificus* abundance is high (false positive rates as low as 2%, n = 178). Similar results were obtained for *V. cholerae* (minimum false positive rate of 16%, n = 146). In contrast, the false positive rate for *V. parahaemolyticus* was always high (minimum of 59%, n = 109). Sequence analysis of false-positive isolates indicated that the majority of confounding isolates are from the Vibrionaceae family, however, members of distantly related bacterial groups were also able to grow on vibrio-selective media, even when using the double-plating method. In conclusion, the double-plating assay is a simple means to increase the efficiency of identifying pathogenic vibrios in aquatic environments and to reduce the number of molecular assays required for identity confirmation. However, the high spatial and temporal variability in the performance of the media mean that molecular approaches are still essential to obtain the most accurate vibrio abundance estimates from environmental samples.

1. Introduction

Members of the bacterial genus *Vibrio* are important constituents of coastal microbial communities. Some vibrio species, such as *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*, are also opportunistic human pathogens. Infections with these species may occur by exposure of open wounds to marine or estuarine water, or by ingestion of contaminated water or raw or poorly cooked seafood (Pacini, 1854; Blake et al., 1979; Colwell and Huq, 1994; Wittman and Flick, 1995; Oliver, 2005). The resulting infections can be mild to severe, with the worst cases being fatal (Blake et al., 1979; Faruque et al., 1998). The ability to quickly isolate and identify pathogenic vibrios is important to environmental microbiologists trying to assess the risk of vibrio infection from coastal waters, and to clinicians trying to diagnose infections that have already occurred.

Vibrio-selective media have been developed (Oliver, 2012) and several are commercially available. Although pathogenic vibrio species are the primary target of these media, the growth of other vibrios and even non-vibrio species has been reported (Gomez-Gil and Roque, 2006). Thiosulfate-citrate-bile salts-sucrose (TCBS) is primarily used to culture pathogenic vibrio strains and it is the most widely used medium for vibrio isolation (Gomez-Gil and Roque, 2006). The medium contains sucrose as well as a pH indicator that is green at alkaline pH and turns yellow in the presence of acid (Table 1). Vibrios that grow on TCBS and do not ferment sucrose (sucrose negative) form green colonies, while those that do ferment sucrose (sucrose positive) produce local areas of reduced pH and form yellow colonies (Fig. 1). This medium does not allow discrimination between many vibrio species. For example, *V. cholerae* and *V. alginolyticus* are both predominantly sucrose positive, and *V. parahaemolyticus* and *V. vulnificus* are both predominantly

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Table 1
Composition of TCBS and CaV media.

TCBS (g l^{-1})		CaV (g l^{-1})	
Agar	15	Agar	15
Proteose peptone	10	Peptone and yeast extract	8
Yeast extract	5	Salts	51.4
Sodium citrate	10	Chromogenic mix	0.3
Sodium thiosulfate	10		
Ox gall	8		
Saccharose	20		
Sodium chloride	10		
Ferric ammonium citrate	1		
Bromothymol blue	0.04		
Thymol blue	0.04		

sucrose negative. Non-Vibrio genera reported to grow on TCBS include *Staphylococcus*, *Flavobaterium*, *Pseudoalteromonas*, *Streptococcus*, *Aeromonas*, and *Shewanella* (Nicholls et al., 1976).

The medium CHROMagar Vibrio (CaV) selects for vibrios using a high pH (9.0) and discriminates among strains based on differences in their ability to metabolize chromogenic substrates (Fig. 1). Some of the medium ingredients are known (Table 1), but the chromogenic mixture that causes the color change is proprietary. Two studies investigating the accuracy and specificity of TCBS and CaV for isolating *V. parahaemolyticus* from seafood samples have both indicated that CaV is more accurate and specific than TCBS (Hara-Kudo et al., 2001; Di Pinto et al., 2011). This is expected, because *V. parahaemolyticus* is not distinguishable from *V. vulnificus* on TCBS (most strains of each species form green colonies), but these species form different colors on CaV. Conversely, *V. vulnificus* and *V. cholerae* are not discriminated from one another on CaV, but can be discriminated on TCBS.

We took advantage of the complementary capabilities of CaV and TCBS in studies of vibrios in Lake Pontchartrain, LA (Nigro et al., 2011) and in the Ala Wai Canal, HI (Nigro, 2012) by employing a double-plating procedure to obtain isolates.

We reasoned that streaking colonies on these two chromogenic media sequentially and using the color change information from each would significantly decrease the percentage of false positive colonies for all three of the species in which we were interested (*V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*), both by discriminating among them and by eliminating a greater proportion of non-target species. A similar approach using three media was later described as a means to minimize false positives when isolating *V. vulnificus* (Williams et al., 2013), but the method described is specific to that species and was tested in one location over a limited period. Our objectives with this study were 1) quantify the false positive identification rates of three pathogenic vibrio species based on color changes on TCBS and CaV when used alone and together, and 2) identify, by 16S rRNA sequencing, some of the species that present as false positives for *V. cholera*e, *V. parahaemolyticus*, or *V. vulnificus* using the double-plating method.

2. Materials and methods

2.1. Sampling

Whole water samples were collected from each of two environments, one subtropical (Lake Pontchartrain, New Orleans, LA) and one tropical (Ala Wai Canal, Honolulu, HI). Whole water samples were taken from 15 stations in Lake Pontchartrain on four occasions (October 2005, January, March, and September 2006) as previously described (Sinigalliano et al., 2007; Nigro et al., 2011). Water was collected from the Ala Wai Canal on five occasions (March, June, September, and December 2008, and March 2009) at 15 stations (Nigro, 2012). In all cases, whole water samples were filtered in duplicate or triplicate through sterile 0.45 μm filters. Duplicate or triplicate filters from each station were placed face up on each of two different, chromogenic, vibrio-selective media (TCBS and CaV) immediately after filtering (four to six replicates in total). Putative *Vibrio* spp. were enumerated as colony-forming units (CFUs) after 12–18 h of incubation at 37 °C. Selected colonies picked from one medium were streaked onto plates of the other medium and incubated for 12–18 h at 37 °C and color changes on the two media were used to make preliminary species assignments.

2.2. Isolate identification: DNA extraction and PCR

Following preliminary identification, colonies were streak purified three times, alternating between TCBS and CaV agar plates for each streaking. Template DNA was prepared by dispersing a single colony in TE buffer (10 mmol l^{-1}Tris, 1 mmol l^{-1} EDTA, pH 8), heating the suspension to 100 °C in a thermal cycler with a heated lid for 10 min, followed by centrifugation at 5000 ×*g* for 10 min.

PCR analysis was performed on DNA from isolated colonies from Lake Pontchartrain that were presumed to be *V. cholerae* (n = 591), *V. parahaemolyticus* (n = 401), or *V. vulnificus* (n = 570). From the Ala Wai Canal, only putative *V. vulnificus* isolates (n = 1084) were collected. The Ala Wai isolates were used to determine only the false positive rate of the double-plating method. For each PCR reaction, 1 μl

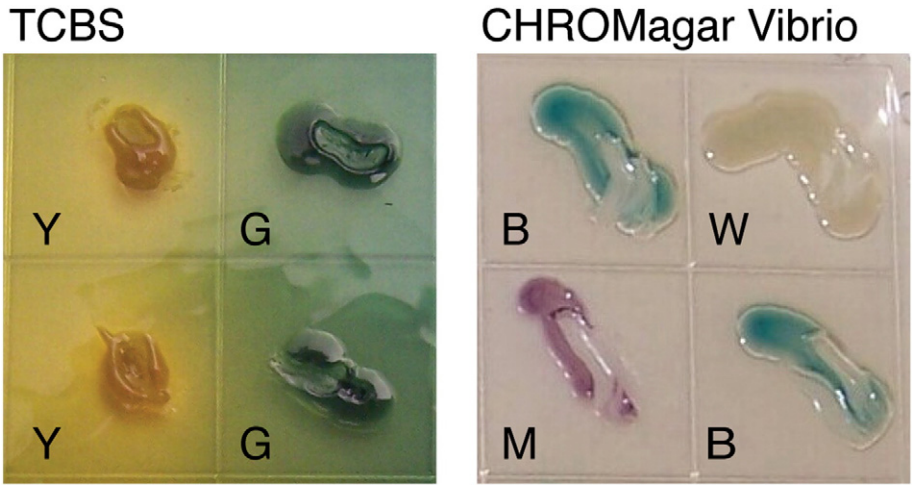

Fig. 1. Appearance of Vibrio isolates on TCBS and CaV. On TCBS, *V. cholerae* appears yellow (Y). *V. parahaemolyticus* and *V. vulnificus* appear green (G). On CaV, *V. parahaemolyticus* appears mauve (M), while *V. cholerae* and *V. vulnificus* appear blue (B). Non-target species may appear white (W) or a variety of other colors.