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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

Tiffany C. Williams, Brett Froelich [1], James D. Oliver *

The University of North Carolina at Charlotte, Biology Department, 9201 University City Blvd. Charlotte, NC 28223, USA

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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.

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)

* Corresponding author at: Biology Department, University of North Carolina at Charlotte, 9201 University City Blvd. Charlotte, NC 28223, USA. Tel.: +1 704 687 8716.
E-mail addresses: bafroeli@email.unc.edu (B. Froelich), jdoliver@uncc.edu (J.D. Oliver).
[1] Present address: The University of North Carolina at Chapel Hill Institute of Marine Sciences, 3431 Arendell St. Morehead City, NC 28557, USA. Tel.: +1 252 726 6841.

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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.

Table 1
V. vulnificus reference strains used in this study, along with biotype, genotype, and their respective colony color reactions on each medium.

Strain[a]	Biotype	Genotype	CPC+	CaV	TCBS
CMCP6[b,c,d]	1	C	Yellow	Turquoise	Green
YJ016[b,c]	1	C	Yellow	Turquoise	Green
M06-24	1	C	Yellow	Turquoise	Green
C7184k2[b,c]	1	C	Yellow	Turquoise	Green
LSU1866[c]	1	C	Yellow	Turquoise	Green
NR02-232[b,c]	1	C	Yellow	Turquoise	Green
UNCC913	1	C	Yellow	Turquoise	Green
UNCC1002	1	C	Yellow	Turquoise	Green
JY1701[b,c]	1	E	Yellow	Turquoise	Green
JY1305[b,c,d]	1	E	Yellow	Turquoise	Green
ENV1[b,c]	1	E	Yellow	Turquoise	Green
LSU549[c]	1	E	Yellow	Turquoise	Green
LSU2098[b]	1	E	Yellow	Turquoise	Green
BC478[b]	1	E	Yellow	Turquoise	Green
E64MW	1	E	Yellow	Turquoise	Green
CECT 4174[e]	2	E	Yellow	Turquoise	Green
CECT 4601[e]	2	E	Yellow	Turquoise	Green
CECT 4607[e]	2	E	Yellow	Turquoise	Green
33149[f]	2	E	Yellow	Turquoise	Green
CECT 4866[f]	2	E	Yellow	Turquoise	Green
RIU-2[f]	2	E	Yellow	Turquoise	Green
94-8-112[f]	2	E	Yellow	Turquoise	Green
32[f]	3	E	Green/Purple	White	Green
1033[f]	3	E	Green/Purple	White	Green
11028[f]	3	E	Green/Purple	White	Green

[a] Unless otherwise noted, all strains were from UNC Charlotte, Charlotte, NC.
[b] Strains used for comparing direct recovery of *V. vulnificus* cells on CaV and CPC+ (Section 2.2).
[c] Strains used for comparing direct recovery of starved *V. vulnificus* cells on CaV and CPC+ (Section 2.2).
[d] Strains used for *in vitro* evaluation of triple-plating method in artificially infected oysters (Section 2.4).
[e] Coleccion Espanola de Cultivos Tipo, Spain.
[f] Courtesy of Carmen Amaro, Universidad de Valencia, Valencia, Spain.

Table 2
Other *Vibrio* species and strains used in this study, along with their respective colony color reactions on each medium.

Species	Strain name	CPC+	CaV	TCBS
V. parahaemolyticus	NY477	NG	Purple	Green
V. parahaemolyticus	F11.3A	NG	Purple	Green
V. parahaemolyticus	SAK11	NG	Purple	Green
V. parahaemolyticus	SPRC1019	NG	Purple	Green
V. parahaemolyticus	AQ3815	Green/purple	Purple	Green
V. parahaemolyticus	WRI	Yellow	Purple	Green
V. cholerae	2633-78	NG	Light blue	Yellow
V. cholerae	2741-80	Green/purple	Light blue	Yellow
V. cholerae	2076-79	Green/purple	Light blue	Yellow
V. cholerae[a]	C6706	Green/purple	Light blue	Yellow
V. cholerae	2690-79	Green/purple	Light blue	Yellow
V. cholerae	924-79	Green/purple	Turquoise	Yellow
V. cholerae	Q2052313	Green/purple	Turquoise	Yellow
V. alginolyticus[a]	08653	Yellow	White	Yellow
V. alginolyticus[a]	2208-1B	NG	White	Yellow
V. alginolyticus[a]	0-04-01	Green/purple	White	Yellow
V. harveyi[a]	35084	Yellow	White	Yellow
V. harveyi[b]	BB120	Yellow	White	Green
V. anguillarum[c]	ATCC 19264	NG	White	Yellow
V. anguillarum	VIB1	NG	White	Yellow
V. anguillarum	VIB275	NG	White	Green
V. anguillarum	VIB2	NG	White	Yellow
V. fluvialis	UNK	Green/purple	Turquoise	Yellow
V. mimicus	UNK	NG	Turquoise	Green
V. hollisae[a]	8391	NG	Turquoise	Green
V. hollisae[a]	2039	NG	Purple	Green
V. furnissii[a]	1955-83	NG	Purple	Yellow
V. furnissii	8760	NG	White	Yellow
V. metschnikovii[c]	ATCC 7708	NG	White	Green
V. natriegens[c]	ATCC 14048	NG	Purple	Yellow
V. nigripulchritudo[c]	ATCC 27043	NG	White	Green
V. proteolyticus[c]	ATCC 15338	NG	White	Green
V. pelagius[c]	ATCC 25916	NG	White	Green
V. aestruianus	OSU-65	NG	Turquoise	Yellow
V. leiognathi	B474	NG	White	Green

[a] Courtesy of Food and Drug Administration, Gulf Coast Seafood Laboratory, Dauphin Island, AL.
[b] Courtesy of Bonnie Bassler, Princeton University, Princeton, NJ.
[c] ATCC — American Type Culture Collection, USA.

2.3. Triple-plating method

Environmental (water or oyster) samples were prepared as described by Froelich *et al.* (Froelich et al., 2012), plated onto CaV, and incubated at 37 °C overnight. Presumptive *V. vulnificus* (turquoise colored) colonies were isolated using sterile toothpicks, picked onto CPC+, TCBS, and HI using a 48-square colony grid template (Epicentre Biotechnologies, Madison, Wisconsin), and incubated overnight at the temperatures listed in Section 2.1. Each isolate's growth pattern (*i.e.* appearance on all three media) was documented. Isolates that produced the *V. vulnificus* growth pattern (turquoise on CaV, yellow on CPC+, and green on TCBS) were referred to as "triple positive isolates" and were predicted to be *V. vulnificus*. Isolates that did not produce the *V. vulnificus* growth pattern were considered to be false positives, but were still subjected to molecular testing for confirmation.

To validate the methodology, all isolates (triple positives and presumptive false positives) were picked from the HI plate and grown overnight in 1 ml of HI broth at 30 °C with shaking. DNA was extracted from these samples by boiling the liquid culture for 10 min followed by centrifugation at 10,000 ×*g* for 10 min and discarding the pellet. All samples were subsequently subjected to genetic testing using PCR, probing for *vvhA* (hemolysin) and *vcg* (virulence correlated) genes, as previously described (Warner and Oliver, 2008). The percentage of isolates correctly predicted to be *V. vulnificus* using one medium (CaV alone) was compared to the accuracy of using three media in tandem (the triple-plating method). A schematic illustration of the steps of the triple-plating method is provided in Fig. 2 for reference.

2.4. In vitro evaluation of triple-plating method in artificially infected oysters

To evaluate the performance of the triple-plating method, oysters held in aquaria with 20‰ artificial seawater [ASW, Instant Ocean, Aquarium Systems, Mentor, OH] at 23 °C were seeded with $5x10^4$ CFU/ml *V. vulnificus* (final concentration) for 24 h. Additionally, a set of control oysters not seeded with exogenous *V. vulnificus* were included in the study. Oysters were removed from tanks, then aseptically shucked and homogenized as previously described (Froelich et al., 2012). Oyster homogenates were diluted in PBS and 100 µl were spread onto CaV agar plates and incubated at 37 °C overnight. A total of 92 presumptive *V. vulnificus* isolates from CaV were selected and plated onto CPC+, TCBS, and HI and incubated overnight at appropriate temperatures. All 92 isolates were subjected to molecular testing using the methods described above to confirm *V. vulnificus* isolates. The accuracy of positively predicting *V. vulnificus* isolates using the new triple-plating method was then compared to the accuracy of using CaV alone. *V. vulnificus* strains used for this study are specified in Table 1.

2.5. In situ evaluation of triple-plating method for environmental isolates

To further validate the utility of the triple-plating method for environmental sampling, oysters and water samples were collected from several estuarine sites along eastern N.C. over a six month period. Each water and oyster sample was prepared as previously described (Froelich et al., 2012), spread onto CaV, and incubated at 37 °C overnight. Presumptive *V. vulnificus* isolates from CaV (turquoise colonies)

were selected and cross plated onto CPC+, TCBS, and HI followed by overnight incubation at appropriate temperatures. Colony color was documented on a total of 152 isolates which were then probed for *V. vulnificus* specific genes as outlined in Section 2.3. The accuracy of positively predicting *V. vulnificus* isolates using the new triple-plating method was compared to the accuracy of using CaV alone.

3. Results and discussion

3.1. Characterization of V. vulnificus strains on selective media

Considering the genotypic heterogeneity displayed by *V. vulnificus* strains, we characterized the growth and colony color development of a variety of *V. vulnificus* strains on CPC+, CaV and TCBS (Table 1). Of the 25 *V. vulnificus* strains examined, all BT1 and BT2 strains produced the expected *V. vulnificus* growth pattern, however BT3 strains appeared white on CaV, and green/purple on CPC+, indicating that the triple plating method is not suitable for this biotype. This finding is supported by a previous study which found BT3 strains to be incapable of sucrose and cellobiose fermentation, and negative for β-galactosidase activity (Bisharat et al., 1999). Thus, it is important to note that the triple-plating method developed in this study pertains specifically to BT1 and BT2 strains. Due to the current geographical isolation of BT3 strains (Bisharat et al., 1999, 2005; Paz et al., 2007) the inability to conform to the currently designed triple plating method does not present a significant limitation. Nonetheless, BT3 strains displayed a unique growth pattern (*i.e.* no other strain grew white on CaV, green/purple on CPC+, *and* green on TCBS), indicating that the triple-plating method could possibly be utilized to detect these strains in environmental samples when desired. Further studies are warranted to support this hypothesis.

3.2. Characterization of other Vibrio species on selective media

In this study, several *Vibrio* species were able to grow like *V. vulnificus,* consequently appearing as false-positive isolates on each medium (Tables 2 and 3). TCBS was the least selective of the three media, with all 60 strains tested being able to grow, 46% of which produced green (non-sucrose fermenting) colonies alike *V. vulnificus* (Tables 1 and 2). Previous studies have demonstrated the ability of CPC+ to inhibit or differentiate the growth of a number of non-*vulnificus Vibrio* species (Warner and Oliver, 2007). In the current study, CPC+ was the most selective media, with 63% of non-*V. vulnificus* strains being unable to grow however 11% of strains tested were able to ferment cellobiose resulting in yellow colonies similar to *V. vulnificus*. The selectivity of CaV was not exclusive to the four pathogenic vibrios for which it was developed as all *Vibrio* strains tested were able to grow on CaV, resulting in one of the four colors characteristic of this medium, of which 17% of non-*V. vulnificus* strains appeared similar to this species.

Table 3
Vibrio species which appear indistinguishable from *V. vulnificus* on each medium.

CPC+ (19%)[a]	CaV (31%)	TCBS (62.5%)
V. parahaemolyticus	*V. cholerae*	*V. parahaemolyticus*
V. alginolyticus	*V. hollisae*	*V. harveyi*
V. harveyi	*V. mimicus*	*V. anguillarum*
	V. fluvialis	*V. mimicus*
	V. aestruianus	*V. hollisae*
		V. metschnikovii
		V. nigripulchritudo
		V. proteolyticus
		V. pelagius
		V. leiognathi

[a] Percentage of non-*V. vulnificus Vibrio* species (total of 16) in which at least one strain appeared identical to *V. vulnificus*.

Table 3 lists *Vibrio* species of which at least one strain grew identical to *V. vulnificus* (BT1 and BT2) on each individual medium. Despite the presence of *V. vulnificus* false positives on a single medium, *V. vulnificus* strains displayed a unique growth pattern across all three media (*i.e.* no other strain appeared turquoise on CaV, yellow on CPC+, *and* green on TCBS). This led to the hypothesis that these media could be used in tandem to eliminate false-positive isolates and enhance the selectivity of culture-based methods for the initial detection of *V. vulnificus*.

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

The triple-plating method requires the use of an efficient initial selective medium to guarantee ample recovery of *V. vulnificus* from environmental samples when the bacterium is present. CPC+ was shown here to be the most selective *in vitro* (Table 2), and it has previously been demonstrated to permit greater recovery of physiologically starved *V. vulnificus* cells compared to other media (Warner and Oliver, 2007). However, a recent study found CaV to be slightly more selective compared to CPC+ when used for environmental sampling (Froelich et al., 2012). To compare the plating efficiencies of CaV and CPC+ we examined the percent recovery of *V. vulnificus* on these selective media relative to HI but found no significant difference between the media, as well as no selectivity between C- and E-genotypes ($p > 0.05$) (Fig. 1A).

To more closely simulate environmental conditions and demonstrate that physiologically starved cells could also be recovered on CaV, we examined the percent recovery of starved *V. vulnificus* cells

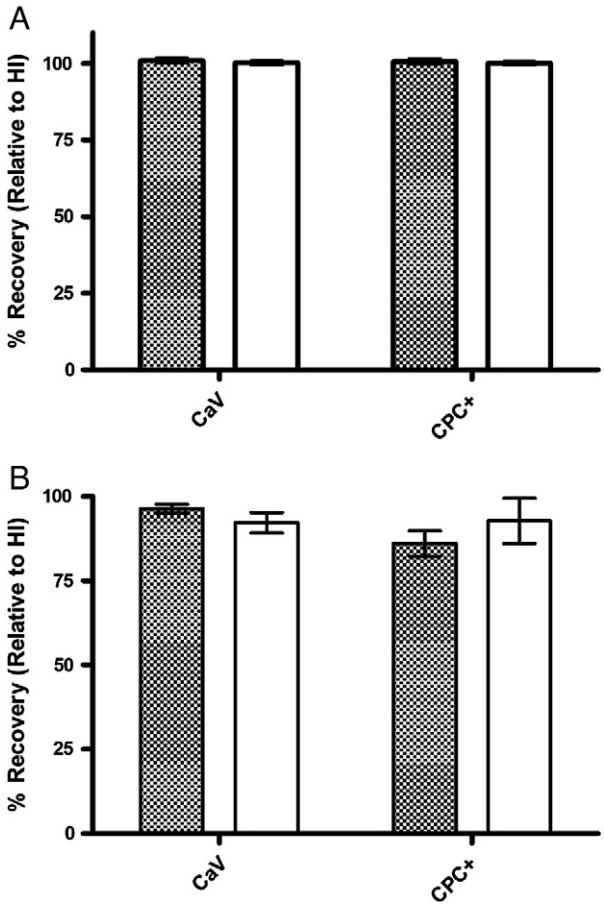

Fig. 1. Comparison of percent recovery of (A) overnight and (B) starved cultures of *V. vulnificus* on CaV, and CPC+ relative to HI (C-genotype, gray bar; E-genotype, white bar). No statistical difference ($p > 0.05$) was observed between media or C-genotype using a 2-way ANOVA.

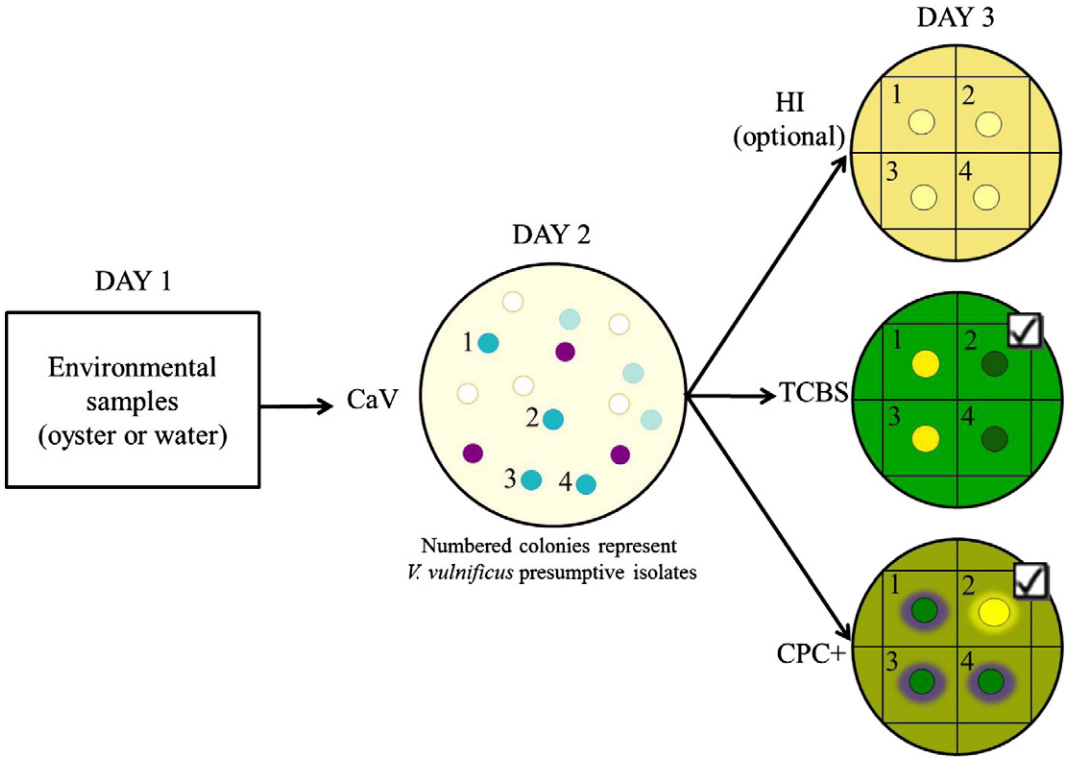

Fig. 2. Schematic illustration of the triple-plating method for the improved culture-based detection of *V. vulnificus*. On day 1, environmental samples are initially spread onto CaV and incubated overnight at 37 °C. On day 2, isolates that appear turquoise (presumptive for *V. vulnificus*; colonies numbered 1–4) are picked from CaV, plated onto HI (optional), TCBS, and CPC+ (using a grid template), and incubated at their appropriate temperatures (see Section 2.1). On day 3, isolates that appear green on TCBS and yellow on CPC+ are referred to as triple-positives and are predicted to be *V. vulnificus* (indicated by the checkmark on isolate #2), whereas all other isolates are non-*V. vulnificus* false-positives on CaV (colonies #1, 3, and 4). Optional molecular confirmation can be performed using isolates plated onto HI (see Section 2.3 for details). Note: figure has been simplified; grid templates can allow for up to 48 isolates per plate.

on CPC+ and CaV. Again, statistical analyses revealed no significant difference between the two media or genotypes ($p > 0.05$) (Fig. 1B). For the development of the triple-plating method, CaV was chosen as the initial selective agar due to its potential to differentiate between the four medically and economically relevant pathogens however results indicate that these media have comparable efficiencies for the recovery of *V. vulnificus*.

3.4. In vitro and in situ evaluation of triple-plating method

Considering the number of *Vibrio* species and strains that mimic *V. vulnificus* on routine culture media, we implemented a new triple-plating method to greatly reduce the number of false positive colonies that appear when attempting to isolate and identify this species from water and oysters (Section 2.3 and Fig. 2). We evaluated the efficiency of the new triple-plating method for detecting *V. vulnificus* by comparing it to the conventional single-plating method. To do this *in vitro*, we seeded laboratory-maintained oysters (determined to possess non-*V. vulnificus* endogenous flora that grew turquoise on CaV) with exogenous *V. vulnificus*. Using the single-plating method, 92 presumptive (turquoise) *V. vulnificus* isolates were subjected to PCR confirmation of which 43.5% were confirmed to be *V. vulnificus*.

Table 4
Comparison of culture-based methods for the accurate identification of *V. vulnificus* isolates in laboratory maintained oysters seeded with *V. vulnificus*.

	Single-plating method	Triple-plating method
No. of CaV turquoise isolates	92	92
No. predicted to be *V. vulnificus*	92	40
No. confirmed by PCR	40	40
% Correctly identified	43.5%	100%

Using the triple-plating method, 40 of these 92 isolates were identified as "triple positives" (presumptive *V. vulnificus* on all three media), of which 100% were confirmed to be this species using PCR (Table 4). The remaining 52 non-triple positive isolates were determined to not be *V. vulnificus* and were concluded to be false-positive isolates on CaV. Thus, the new triple-plating method was able to discriminate between CaV turquoise colonies present as the oyster's endogenous non-*V. vulnificus* population, and the population of *V. vulnificus* that was artificially added to these oysters. Compared to single plating, this new method reduced the amount of molecular testing needed by 56.5% and increased our ability to accurately identify *V. vulnificus* (using simply a culture-based method) by 2.3-fold.

To validate the performance of the triple-plating method using environmental samples, we collected 152 CaV presumptive isolates from water and oyster samples (Table 5). The triple-plating method reduced the number of presumptive *V. vulnificus* isolates from 152 to 49, of which 46 were confirmed to be *V. vulnificus* by PCR. As a

Table 5
Comparison of culture-based methods for the accurate identification of *V. vulnificus* isolates collected from environmental (oyster and water) samples.

	Single-plating method	Triple-plating method
No. of CaV turquoise isolates	152	152
No. predicted to be *V. vulnificus*	152	49
No. confirmed by PCR	54	46
% Correctly identified	35.5%	92.8%
No. false-positive isolates	98	3
No. false-negative isolates	0	8
Estimated cost of testing (with PCR)	$178	$60
Estimated cost of testing (without PCR)	Not applicable[a]	$8

[a] Not applicable — too inaccurate and unreliable to use this method without PCR.

result, the percentage of *V. vulnificus* accurately predicted on plating media increased from 35.5% to 92.8%, reducing the number of false-positive isolates from 64.5% to 2%.

Despite the apparent benefits of the triple-plating method, it is important to note the limitations of any such study, particularly regarding the existence of false-negative *V. vulnificus* isolates which would remain undetected using this method. In this study, eight *V. vulnificus* environmental isolates were sucrose positive (*i.e.* formed yellow colonies on TCBS), all of which were genetically confirmed to be E-genotypes. While *V. vulnificus* is referred to as a sucrose-negative species (Farmer, 1979; Oliver, 2012b; Tantillo et al., 2004), there has been previous documentation of sucrose-positive *V. vulnificus* isolates (Arias et al., 1998; Farmer et al., 1991; Wright et al., 1993). Although this phenotype is considered to represent a minority (~3–20%) of all *V. vulnificus* strains, they would inevitably be missed using the triple-plating method. Future studies are required to more thoroughly investigate the abundance of sucrose-positive *V. vulnificus* isolates in the environment.

We compared the cost expenditure to perform the single and triple-plating methods followed by confirmatory PCR. Cost analyses for each method were calculated by obtaining current (non-discounted) costs for culture-based and molecular-based methods. As shown in Table 5, the single-plating method required PCR confirmation of all 152 isolates, resulting in an estimated expense of $178, whereas the triple-plating method reduced the cost to approximately $60, representing a 66.3% decrease in cost. Considering the remarkable accuracy of the proposed triple-plating method, it is possible to remove the molecular component entirely when isolating and identifying *V. vulnificus* from environmental samples. In this case, testing 152 isolates would cost less than $8, representing a 95.5% decrease in cost. Thus, this new and simple cross-plating technique is more time efficient, more cost effective and could prove to be a suitable option for those who are not able to routinely employ molecular methods.

3.5. Conclusions

Elucidating the population structure of *V. vulnificus* in its natural environment is essential for our understanding of the ecology and prevalence of this opportunistic human pathogen. Currently used culture-based methods for the isolation and detection of *V. vulnificus* involve the use of a single medium in which *V. vulnificus* presumptive isolates are typically confirmed using molecular techniques. To date, there exists no single medium that possesses the specificity and selectivity required to accurately detect *V. vulnificus* in the environment, particularly when in low numbers (Harwood et al., 2004; Warner and Oliver, 2007). In our study three commonly used selective and differential media (CPC+, CHROMagar Vibrio, and TCBS) were all shown to permit growth of *V. vulnificus* false-positive isolates. This finding is supported by previous environmental studies in which *V. vulnificus* presumptive isolates from each medium sometimes failed confirmation using molecular-based methods (Arias et al., 1998; Froelich et al., 2012; Macian et al., 2000; Staley et al., 2013). This has the potential to confound results if *V. vulnificus* populations are low in an environment harboring other *V. vulnificus*-like species. Indeed, when *V. vulnificus* populations were diminished in North Carolina oysters and waters due to a period of prolonged and severe drought, the ability of CaV or CPC+ to accurately detect *V. vulnificus* was reduced to 4% and <1%, respectively. (Froelich et al., 2012).

From the results of this study, we suggest a new and simple cross-plating technique in which only isolates that phenotypically appear as *V. vulnificus* on all three media (referred to as triple-positive isolates) proceed to molecular testing (Fig. 2). While the triple-plating method is not completely exempt from false-positive and false-negative isolates, it considerably increases the efficiency of culture-based presumptive identification of *V. vulnificus* from environmental samples, while also reducing the time and resources required for confirmatory molecular methods. Indeed, our results indicate that molecular confirmation of *V. vulnificus* isolates may not be required following this new culture-based method. Furthermore, it is anticipated that a similar cross-plating method could be developed to improve culture-based identification of other vibrio pathogens such as *V. parahaemolyticus*. Substantially reducing false-positive isolates not only enhances the predictive power of culture-based methods, but also reduces the need for expensive laboratory equipment, costly molecular reagents, and complicated experimental procedures. Many laboratories may not have access to the required resources and in these cases this inexpensive, efficient, culture-based technique would be economically invaluable.

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References

Arias, C.R., Aznar, R., Pujalte, M.J., Garay, E., 1998. A comparison of strategies for the detection and recovery of *Vibrio vulnificus* from marine samples of the western Mediterranean coast. Syst. Appl. Microbiol. 21, 128–134.

Arias, C.R., Macián, M.C., Aznar, R., Garay, E., Pujalte, M.J., 1999. Low incidence of *Vibrio vulnificus* among *Vibrio* isolates from sea water and shellfish of the western Mediterranean coast. J. Appl. Microbiol. 86, 125–134.

Baker-Austin, C., Trinanes, J.A., Taylor, N.G.H., Hartnell, R., Siitonen, A., Martinez-Urtaza, J., 2013. Emerging *Vibrio* risk at high latitudes in response to ocean warming. Nat. Clim. Chang. 3, 73–77.

Bisharat, N., Agmon, V., Finkelstein, R., Raz, R., Ben-Dror, G., Lerner, L., Soboh, S., Colodner, R., Cameron, D.N., Wykstra, D.L., Swerdlow, D.L., Farmer, J.J., 1999. Clinical, epidemiological, and microbiological features of *Vibrio vulnificus* biogroup 3 causing outbreaks of wound infection and bacteraemia in Israel. Lancet 354, 1421–1424.

Bisharat, N., Cohen, D.I., Harding, R.M., Falush, D., Crook, D.W., Peto, T., Maiden, M.C., 2005. Hybrid *Vibrio vulnificus*. Emerg. Infect. Dis. 11, 30–35.

Bisharat, N., Cohen, D.I., Maiden, M.C., Crook, D.W., Peto, T., Harding, R.M., 2007. The evolution of genetic structure in the marine pathogen, *Vibrio vulnificus*. Infect. Genet. Evol. **7**, 685–693.

Bolinches, J., Romalde, J.L., Toranzo, A.E., 1988. Evaluation of selective media for isolation and enumeration of vibrios from estuarine waters. J. Microbiol. Methods 8, 151–160.

Chatzidaki-Livanis, M., Hubbard, M.A., Gordon, K., Harwood, V.J., Wright, A.C., 2006. Genetic distinctions among clinical and environmental strains of *Vibrio vulnificus*. Appl. Environ. Microbiol. 72, 6136–6141.

Cohen, A.L.V., Oliver, J.D., DePaola, A., Feil, E.J., Fidelma Boyd, E., 2007. Emergence of a virulent clade of *Vibrio vulnificus* and correlation with the presence of a 33-kilobase genomic island. Appl. Environ. Microbiol. 73, 5553–5565.

Daniels, N.A., 2011. *Vibrio vulnificus* oysters: pearls and perils. Clin. Infect. Dis. 52, 788–792.

Farmer III, J.J., 1979. *Vibrio* ("*Beneckea*") *vulnificus*, the bacterium associated with sepsis, septicaemia, and the sea. Lancet 2, 903.

Farmer, J.J., Hickman-Brenner, F.W., Kelly, M., 1991. *Vibrio*. In: Balows, A., Housler, W.A., Herrman, K., Isenberg, H., Shadomy, H. (Eds.), Manual of Clinical Microbiology. American Society of Microbiology, Washington, D.C.

Froelich, B.A., Oliver, J.D., 2011. Orientation of mannitol related genes can further differentiate strains of *Vibrio vulnificus* possessing the *vcgC* allele. Adv. Stud. Biol. 3, 151–160.

Froelich, B.A., Williams, T.C., Noble, R.T., Oliver, J.D., 2012. Apparent loss of *Vibrio vulnificus* from North Carolina oysters coincides with a drought-induced increase in salinity. Appl. Environ. Microbiol. 78, 3885–3889.

Gulig, P.A., de Crecy-Lagard, V., Wright, A.C., Walts, B., Telonis-Scott, M., McIntyre, L.M., 2010. SOLiD sequencing of four *Vibrio vulnificus* genomes enables comparative genomic analysis and identification of candidate clade-specific virulence genes. BMC Genomics 11, 512.

Harwood, V.J., Gandhi, J.P., Wright, A.C., 2004. Methods for isolation and confirmation of *Vibrio vulnificus* from oysters and environmental sources: a review. J. Microbiol. Methods 59, 301–316.

Jones, M.K., Oliver, J.D., 2009. *Vibrio vulnificus*: disease and pathogenesis. Infect. Immun. **77**, 1723–1733.

Macian, M.C., Arias, C.R., Aznar, R., Garay, E., Pujalte, M.J., 2000. Identification of *Vibrio* spp. (other than *V. vulnificus*) recovered on CPC agar from marine natural samples. Int. J. Microbiol. 3, 51–53.

Massad, G., Oliver, J.D., 1987. New selective and differential medium for *Vibrio cholerae* and *Vibrio vulnificus*. Appl. Environ. Microbiol. 53, 2262–2264.

Monget, D., Robichon, D., 2011. Culture medium for detecting and identifying *Vibrio* bacteria, Biomerieux (Marcy l'Etoile, FR), United States.

Morrison, S.S., Williams, T., Cain, A., Froelich, B., Taylor, C., Baker-Austin, C., Verner-Jeffreys, D., Hartnell, R., Oliver, J.D., Gibas, C.J., 2012. Pyrosequencing-based comparative genome analysis of *Vibrio vulnificus* environmental isolates. PLoS One **7**, e37553.

Nilsson, W.B., Paranjype, R.N., DePaola, A., Strom, M.S., 2003. Sequence polymorphism of the 16S rRNA Gene of *Vibrio vulnificus* is a possible indicator of strain virulence. J. Clin. Microbiol. 41, 442–446.

Oliver, J.D., 2006a. *Vibrio vulnificus*. In: Thompson, F.L., Austin, B., Swings, J. (Eds.), The Biology of Vibrios. American Society for Microbiology, Washington, D.C., pp. 349–366.

Oliver, J.D., 2006b. Vibrio vulnificus. In: Belkin, S., Colwell, R.R. (Eds.), Oceans and Health: Pathogens in the Marine Environment. Springer Science, New York, pp. 253–276.

Oliver, J.D., 2012a. *Vibrio vulnificus*: death on the half shell. A personal journey with the pathogen and its ecology. Microb. Ecol. http://dx.doi.org/10.1007/s00248-012-0140-9.

Oliver, J.D., 2012b. Culture media for the isolation and enumeration of pathogenic *Vibrio* species in foods and environmental samples. In: Corry, J.E.L., Curtis, G.D.W., Baird, R.M. (Eds.), Handbook of Culture Media for Food and Water Microbiology. The Royal Society of Chemistry, Cambridge, UK, pp. 377–394.

Oliver, J.D., Kaper, J., 2007. *Vibrio* species. In: Doyle, M.P., Beuchat, L.R. (Eds.), Food Microbiology: Fundamentals and Frontiers. American Society for Microbiology, Washington, D.C., pp. 343–379.

Paz, S., Bisharat, N., Paz, E., Kidar, O., Cohen, D., 2007. Climate change and the emergence of *Vibrio vulnificus* disease in Israel. Environ. Res. 103, 390–396.

Rosche, T.M., Yano, Y., Oliver, J.D., 2005. A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical or environmental isolation. Microbiol. Immunol. 49, 381–389.

Rosche, T.M., Binder, E.A., Oliver, J.D., 2010. *Vibrio vulnificus* genome suggests two distinct ecotypes. Environ. Microbiol. Rep. 2, 128–132.

Sanjuan, E., Fouz, B., Oliver, J.D., Amaro, C., 2009. Evaluation of genotypic and phenotypic methods to distinguish clinical from environmental *Vibrio vulnificus* strains. Appl. Environ. Microbiol. 75, 1604–1613.

Senoh, M., Miyoshi, S., Okamoto, K., Fouz, B., Amaro, C., Shinoda, S., 2005. The cytotoxin–hemolysin genes of human and eel pathogenic *Vibrio vulnificus* strains: comparison of nucleotide sequences and application to the genetic grouping. Microbiol. Immunol. 49, 513–519.

Staley, C., Chase, E., Harwood, V.J., 2013. Detection and differentiation of *Vibrio vulnificus* and *V. sinaloensis* in water and oysters of a Gulf of Mexico estuary. Environ. Microbiol. 15, 623–633.

Sun, Y., Oliver, J.D., 1995. Value of Cellobiose Polymyxin B Colistin Agar for Isolation of *Vibrio vulnificus* from Oysters. J. Food Prot. 58, 439–440.

Tantillo, G.M., Fontanarosa, M., Di Pinto, A., Musti, M., 2004. Updated perspectives on emerging vibrios associated with human infections. Lett. Appl. Microbiol. 39, 117–126.

Thompson, F.L., Iida, T., Swings, J., 2004. Biodiversity of *Vibrios*. Microbiol. Mol. Biol. Rev. 68, 403–431.

Tison, D.L., Nishibuchi, M., Greenwood, J.D., Seidler, R.J., 1982. *Vibrio vulnificus* biogroup 2: new biogroup pathogenic for eels. Appl. Environ. Microbiol. 44, 640–646.

Vickery, M.C., Nilsson, W.B., Strom, M.S., Nordstrom, J.L., DePaola, A., 2007. A real-time PCR assay for the rapid determination of 16S rRNA genotype in *Vibrio vulnificus*. J. Microbiol. Methods 68, 376–384.

Warner, E., Oliver, J.D., 2007. Refined medium for direct isolation of *Vibrio vulnificus* from oyster tissue and seawater. Appl. Environ. Microbiol. 73, 3098–3100.

Warner, E., Oliver, J.D., 2008. Multiplex PCR assay for detection and simultaneous differentiation of genotypes of *Vibrio vulnificus* biotype 1. Foodborne Pathog. Dis. 5, 691–693.

West, P.A., Russek, E., Brayton, P.R., Colwell, R.R., 1982. Statistical evaluation of a quality control method for isolation of pathogenic *Vibrio* species on selected thiosulfate-citrate-bile salts-sucrose agars. J. Clin. Microbiol. 16, 1110–1116.

Wright, A.C., Miceli, G.A., Landry, W.L., Christy, J.B., Watkins, W.D., Morris Jr., J.G., 1993. Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. Appl. Environ. Microbiol. 59, 541–546.