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The evaluation of four recent culture-based methods for the isolation and enumeration of *Vibrio vulnificus* bacteria from oyster meat

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ABSTRACT

The most common cause of seafood-borne death in the United States is the bacterium *Vibrio vulnificus* which can be concentrated into high numbers in the tissues of oysters or other shellfish. The ability to quickly, accurately, and inexpensively isolate living strains of this organism from oyster tissues is crucial for effective research on this pathogen. In this report, we evaluate four methods for isolating and quantifying *V. vulnificus* from oyster tissues, the solid media CPC+ (a refined version of cellobiose–polymyxin B-colistin medium), CHROMagar *Vibrio*, VVX (*Vibrio vulnificus* X-gal), and a method termed "Triple plating". Up to 1225 presumptive isolates were detected by each method, and 335 were subjected to molecular typing. The selectivity and sensitivity of each method was examined and VVX was found to be the most accurate method, with each of the other methods being recommended for task-specific uses. CHROMagar *Vibrio* is recommended for ease of use and relative accuracy, CPC+ is best used to differentiate between clinically associated and environmental strains.

1. Introduction

The bacterium, *Vibrio vulnificus*, is a food-borne and wound-associated human pathogen that is capable of causing a gamut of infection including gastroenteritis, necrotizing fasciitis (aka flesh eating disease), and septicemia (Johnston et al., 1985; Oliver, 1989, 2006; Baker-Austin et al., 2010a). These infections often require hospitalization and have extremely high fatality rates, even with aggressive antibiotic treatment (Oliver, 2006; Centers for Disease Control, Prevention, 2013). This bacterium is responsible for 95% of seafood-borne deaths, with 95% of those deaths resulting from the consumption of raw or undercooked oysters, which as filter feeders are capable of concentrating *V. vulnificus* from the surrounding water and serve as an environmental reservoir (Oliver, 2006; Oliver et al., 1983; Froelich and Oliver, 2013).

V. vulnificus strains are divided into three biotypes, of which biotype 1 is the most important in oysters, as this is the biotype that is the cause of the greatest number of human infections (Oliver, 1989; Bisharat et al., 1999; Tison et al., 1982). The biotype 1 strains can exhibit two different alleles in the virulence correlated gene, *vcg*. The strains with allele *vcgE* (E-genotype) are the majority of the strains found in the environment, while the *vcgC* strains (C-genotype) are correlated with clinical isolation (Warner and Oliver, 1999; Rosche et al., 2005). Other methods of differentiating strains based on virulence potential include comparing the 16s rDNA sequences of strains, and identify a clinical "B-type" and an environmental "A-type" (Aznar et al., 1994; Kim and Jeong, 2001; Nilsson et al., 2003). The spacer regions found between the 16s and 23s rDNA sequences could also be used to separate strains (Gonzalez-Escalona et al., 2007). The work presented here focused on the *vcg* gene. Additionally there have been several recent advances in the ability to detect viable *V. vulnificus* bacteria in oyster meats using quantitative PCR techniques. These techniques include the targeting of the *vvhA* gene for total *V. vulnificus* quantification, which is the same gene used in the multiplex conventional PCR technique used in this study (Garrido-Maestu et al., 2014; Panicker et al., 2004; Panicker and Bej, 2005). Pathogenicity potential is quantified by probes targeting either the *vcgC* gene (Garrido-Maestu et al., 2014; Baker-Austin et al., 2010b), similar to this study, or by the use of polymorphism within the *pilF* gene of *V. vulnificus* (Baker-Austin et al., 2012).

Because of the virulence of this bacterium coupled with the increased exposure that comes with purposely eating raw oysters, there is much research effort expended on examining the relationships between oysters and *V. vulnificus*. Thus, the ability to easily, cheaply, and accurately enumerate these bacteria from oyster and water samples, and to isolate individual colonies for additional studies, such as bioaccumulation experiments in oysters, is critical for successfully reducing the number of infections.

While there are several published methods of culturing *V. vulnificus* from environmental samples, there have recently been a few additions to the vibriologist's repertoire. These new methods include the media CPC+, CHROMagar *Vibrio*, and VVX, and a technique termed "triple plating" (Panicker and Bej, 2005; Tamplin et al., 1991; Warner and Oliver, 2007). None of these media or techniques requires an

Abbreviations: CPC, cellobiose–polymyxin B-colistin medium; CPC+, refined cellobiose–polymyxin B-colistin medium; VVX, *Vibrio vulnificus* X-gal medium; TCBS, thiosulfate–citrate–bile salts–sucrose.

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enrichment step to isolate and quantify *V. vulnificus* from oyster tissues but all differ in their cost, difficulty to perform or create, and their accuracy. To determine the best medium or media to use when conducting research on environmental *V. vulnificus* in oyster meats, a total of 330 presumptive *V. vulnificus* isolates from oysters collected at various sites along coastal North Carolina were used to determine the ability of the above mentioned methods to correctly identify *V. vulnificus* colonies. These presumptive isolates were all confirmed or rejected, via PCR analysis, as *V. vulnificus*. Furthermore, the genotype of each isolate, based on the virulence-correlated gene (*vcg*) was identified. The benefits and drawbacks of each technique are discussed herein.

2. Material and methods

2.1. Oyster collection and processing

Oysters were collected from May to July, 2013 from sites along eastern North Carolina including North River, South River, Hoop Pole Creek, Harlowe Creek, and Calico Creek (Fig. 1). Oysters were collected by hand, by dredge, by rake, or by tongs and were placed in plastic bags, and kept on ice during transport to the laboratory. Oysters were processed within 3 h of collection.

The oysters were cleaned of mud, and rinsed with 70% ethanol and patted dry with paper towels before being aseptically shucked with ethanol and flame sterilized instruments. Ten oysters were collected from each site per sampling date, with the meats from groups of five oysters being combined, drained of mantle fluid and hemolymph, and the wet tissue weighed. An equal amount (w:v) of sterile phosphate buffered saline (PBS) was added to each batch of combed tissues to facilitate homogenization, with a minimum of 25 ml PBS being added. Oyster meats were homogenized in a blender (Waring, Stamford, CT) by blending for 15 s followed by 5 s of rest, repeated two additional times. Homogenates were serially diluted with PBS and 100 μl was used for plating.

2.2. Media testing of oyster tissue

CHROMagar *Vibrio* (CHROMagar, Paris, France) solid medium was prepared as per manufacturer's instructions. *V. vulnificus* presumptive isolates appear dark blue, and were differentiated from the lighter blue presumptive *Vibrio cholerae* isolates. The refined version of cellobiose–polymyxin B-colistin (CPC) medium (Massad and Oliver, 1987) termed "CPC+" was prepared as described by Warner and Oliver (2007). Presumptive *V. vulnificus* isolates on this medium appeared dark yellow with a white center and a yellow halo. Thiosulfate–citrate–bile salts–sucrose (TCBS) agar and heart infusion (HI) agar were prepared as instructed by the manufacturer (BD, Franklin Lakes, NJ). The medium *Vibrio vulnificus* X-Gal (VVX) was prepared as described by Griffitt and Grimes (Panicker and Bej, 2005). Presumptive *V. vulnificus* isolates on this medium appear blue. The triple plating method was performed as described by Williams et al. (2013), with colonies that appeared green on TCBS, dark yellow with yellow halo on CPC+, and dark blue on CHROMAgar *Vibrio*, being presumptive for *V. vulnificus*.

Oyster homogenates were plated onto TCBS, CHROMagar *Vibrio*, CPC+, and VVX and all media were incubated at 37 °C for 24 h. After incubation, five colonies that were presumptive for *V. vulnificus* were selected per oyster batch, with two batches used per sample, for each of the media or methods used for a total of 335 tested colonies. These colonies were transferred to an HI plate using sterile toothpicks, and were individually labeled using gridded plate labels. The HI plates were used as a non-stressful maintenance medium for the bacterial isolates while experiments were being performed. HI plates were incubated overnight at 37 °C. Sterile squares of velveteen were used to replica plate the colonies from the HI plate onto CHROMagar *Vibrio*, VVX, CPC+, and TCBS media. In this way, each colony that had been

Fig. 1. Map of oyster sampling sites near Morehead City, North Carolina USA.

identified as presumptive *V. vulnificus* on one medium was then grown and examined on all other media, with TCBS included for the triple plating method. These replica plates generated from the HI master plate were grown at 37 °C for 24 h, and the morphology of each colony on each medium was recorded. A total of 335 colonies were examined in this fashion.

2.3. Confirmation of presumptive V. vulnificus

Each colony from HI plates was transferred to a tube containing 0.3 ml of HI broth, and allowed to grow overnight. The tubes were boiled for 10 min, and centrifuged at 10,000 ×*g* for 10 min to pellet the cellular material. The supernatant was used as the template in PCR analysis. Conventional PCR analysis was performed, as described by Rosche et al. (2005) with modifications suggested by Warner and Oliver (2008). This technique allows for the simultaneous confirmation and genotype identification of presumptive *V. vulnificus* colonies.

2.4. Calculating assumed V. vulnificus abundance

After counting the number of presumptive *V. vulnificus* colonies on a medium that selects for *V. vulnificus*, this number is multiplied by the ratio of colonies picked from that medium that was confirmed via PCR to the total number of colonies picked for confirmatory PCR analysis. The resulting value is the number of assumed *V. vulnificus* in the sample. For the triple plating method, the percentage of colonies that are "triple positive" are multiplied by the number of presumptive colonies on CHROMagar *Vibrio* to generate the nPCR umber of assumed colonies. In this way, the actual number of *V. vulnificus* cells in an oyster samples can be estimated even when using media that permit the growth of other species.

2.5. Statistics

Data were analyzed using SigmaPlot statistical analysis software version 12.5 (Systat Software, Chicago, IL). The average discrepancy between presumed and assumed values was compared for all media/methods using a Kruskal–Wallis test followed by a Tukey multiple comparison test to determine significant differences between the methods. The average presumed and assumed values were compared using a two-way repeated measures ANOVA with a post-test for multiple comparisons using the Holm–Sidak method. An alpha of 0.05 was used in all analyses.

3. Results

3.1. Confirmed V. vulnificus isolates

A total of 335 colonies subjected to PCR analysis confirmed that 125 were *V. vulnificus*. There were 118 (94%) E-genotype and 7 (6%) C-genotype strains. A summary of the results for all media is presented in Table 1.

3.2. Triple plating method results

The triple plating method uses CHROMagar *Vibrio* as the initial isolation medium. That medium detected an average of 559 presumptive *V. vulnificus* colony forming units (CFU) per gram of oyster tissue, average from all sites and dates. The triple plating method correctly identified (positively or negatively) 229 (68%) of the 335 tested isolates (Fig. 2). This method resulted in 23 (7%) false positive reports, where the method presumed an isolate to be *V. vulnificus* but PCR confirmation revealed it was not (Fig. 3). Triple plating also generated 83 (25%) false negative reports, where a PCR confirmed *V. vulnificus* isolate was presumed to be some other species. This method correctly detected 40 (34%) of E-types and 2 (29%) of C-types. The average discrepancy between presumed and assumed abundance of *V. vulnificus* was 451 CFU/g oyster tissue (Fig. 4). The number of presumed *V. vulnificus* was not significantly different from the final number of assumed *V. vulnificus* after the PCR step ($p = 0.097$, Fig. 5). The presumed versus assumed values for each site and date are presented in Supplementary Fig. 1A.

3.3. CPC + plating results

The medium, CPC+, detected a mean of 1225 presumptive *V. vulnificus* (CFU) per gram of oyster tissue, averaged from all sites and dates. CPC+ correctly identified 146 (44%) of the 335 tested isolates (Fig. 2). This medium reported 157 (47%) false positive and 32 (10%) false negative reports (Fig. 3). CPC+ correctly identified 86 (73%) of the E-genotype strains and 7 (100%) of the C-genotype strains. The average discrepancy between the presumed and assumed values was 1175 CFU/g of oyster tissue, which was significantly higher than the other methods ($p < 0.05$, Fig. 4). The number of presumed *V. vulnificus* was significantly greater than the assumed *V. vulnificus* ($p < 0.001$, Fig. 5). The presumed versus assumed values for each site and date are presented in Supplementary Fig. 1B.

3.4. CHROMagar Vibrio plating results

As stated previously, CHROMagar *Vibrio* detected an average of 559 presumptive *V. vulnificus* colony forming units (CFU) per gram of oyster tissue, average from all sites and dates. This medium correctly identified 247 (74%) of the 335 tested isolates (Fig. 2). Sixty-three (19%) false positive and 25 (7%) false negative reports were generated by using this medium (Fig. 3). Discrepancy between the initial presumed and final assumed *V. vulnificus* values averaged 340 CFU/g of oyster tissue (Fig. 4) and the presumed values were significantly greater than the assumed values ($p = 0.012$, Fig. 5). The presumed versus assumed values for each site and date are presented in Supplementary Fig. 1C.

3.5. VVX plating results

The use of VVX resulted in an average of 521 presumptive *V. vulnificus* isolates from oyster tissues and this medium correctly identified 272 (81%) of the 335 tested isolates (Fig. 2). This medium generated 50 (15%) false positive and 13 (4%) false negative results (Fig. 3). The average difference between the presumed and assumed

Table 1
The number of PCR tested isolates correctly or incorrectly identified by each medium or method.

	CHROMagar	VVX	Triple plating	CPC+
Correctly identified	247/335 (74%)	272/335 (81%)	229/335 (68%)	146/335 (44%)
Correct E-types	98/118 (83%)	110/118 (93%)	40/118 (34%)	86/118(73%)
Correct C-types	2/7 (29%)	2/7 (29%)	2/7 (29%)	7/7 (100%)
Incorrectly identified	88/335 (26%)	63/335 (19%)	106/335 (32%)	189/335 (56%)
False positive results	63/335 (19%)	50/335 (15%)	23/335(7%)	157/335 (47%)
False negative results	25/335 (7%)	13/335 (4%)	83/335 (25%)	32/335 (10%)

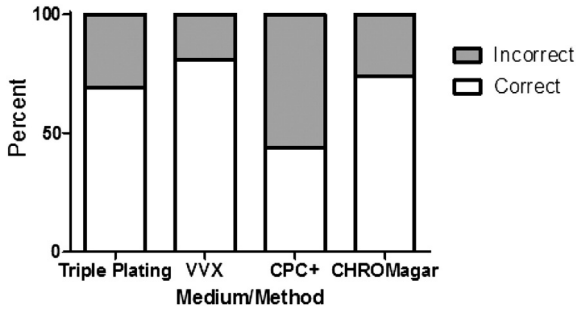

Fig. 2. Percentage of PCR tested presumptive *V. vulnificus* isolates that were correctly (white bars) or incorrectly (gray bars) identified by each medium or method.

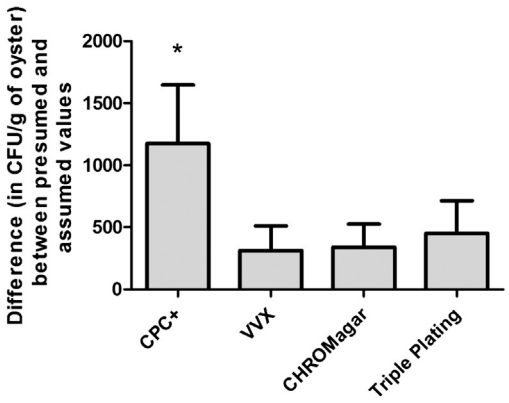

Fig. 4. The difference between the numbers of presumed and assumed *V. vulnificus* bacteria from oysters for each medium/method averaged among all sites and dates. See the Material and methods section for calculation of assumed *V. vulnificus*. Asterisk indicates value that is significantly ($p > 0.05$) different. Error bars indicated standard error of the mean.

V. vulnificus abundance was 313 CFU/g of oyster tissue (Fig. 4) and presumed values were significantly greater than the assumed values ($p = 0.006$, Fig. 5). The presumed versus assumed values for each site and date are presented in Supplementary Fig. 1D.

4. Discussion

Each of these culture-based methods for isolating and enumerating living *V. vulnificus* bacteria from oyster tissue has their advantages and disadvantages. None of the individual media alone is sufficient for accurately assuming the concentration of *V. vulnificus* bacteria in oysters, with CPC+, VVX, and CHROMagar *Vibiro* all having significantly different presumed and assumed values ($p < 0.001$, $p = 0.006$, and $p = 0.012$, respectively, Fig. 5). Each of these media requires a confirmatory follow up step to determine the ratio of actual *V. vulnificus* isolates to the number of false positives. After performing a confirmatory follow-up step, PCR of the *vcg* gene in the case of this study, all three media generated statistically similar calculated assumed values for the concentration of *V. vulnificus* in oysters ($p > 0.7$ in all cases, Fig. 5).

The triple plating method was able to achieve statistically similar results ($p > 0.76$ in all cases) in the number of assumed CFU/g (Fig. 5). Thus, even though this method only correctly identified 68% (Fig. 2) of the isolates tested in this study, the extremely low rate of false positive results (Fig. 3) without the use of a molecular confirmatory step makes it an attractive alternative to PCR to enumerate *V. vulnificus* in oysters. One reason for the lower accuracy of the triple plating method was the unusual morphology of many *V. vulnificus* colonies on TCBS agar. All 135 of the isolates in this study that were confirmed to be *V. vulnificus* via PCR analysis were plated onto TCBS agar plates. Of these confirmed isolates, 67 (54%) appeared as green colonies, a required for being assumed as *V. vulnificus* using the triple plating method. Twenty-one (17%) of the confirmed *V. vulnificus* colonies exhibited a yellow color on TCBS, a result comparable to previous reports (Tamplin et al., 1991; Wright et al., 1993; Harwood et al., 2004), which is a known limitation of the triple plating technique (Williams et al., 2013). The remarkable finding is that 29 (23%) of the PCR confirmed *V. vulnificus* bacteria failed to grow on TCBS. This was unexpected as TCBS is typically a permissive medium that grows most pathogenic vibrios with ease. These unusual isolates have been archived and sequencing should uncover the differences in these strains.

As mentioned earlier, after a confirmatory step, CPC+ performed as well as the other media and methods to quantify *V. vulnificus*. This was the least accurate method used in this study, with 44% (Fig. 2) of isolates identified correctly. This percentage is similar to previous reports on the selectivity of this medium (Froelich et al., 2012). It is important to note that this medium was the only one tested that identified all of the C-genotype strains of *V. vulnificus* in oyster tissues, with the other three methods finding only 29% of these strains. The use of CPC+ could provide a critical increase in sensitivity to these strains that are correlated with infection through ingestion of oysters.

CHROMagar *Vibrio* and VVX were the best performing of the four methods in identifying the 335 tested strains (74% and 81% correct, respectively, Fig. 2). Thus, other factors should be considered in the evaluation of these media. VVX was the most sensitive and second most selective method used in this study, making it the best performing overall (Fig. 3). Furthermore, it is relatively inexpensive when compared to CHROMagar *Vibrio*. It is, however, more complicated to create than CHROMagar *Vibrio*, which is available commercially as a pre-mixed powder. VVX requires the mixing of two solutions, autoclaving, and filtering, while CHROMagar *Vibrio* can be made on a hotplate or in a microwave in as little as 5 min. Additionally, CHROMagar *Vibrio* is more versatile, as it is indicated for the identification of *V. vulnificus*, *V. cholerae*, and *Vibrio parahaemolyticus*.

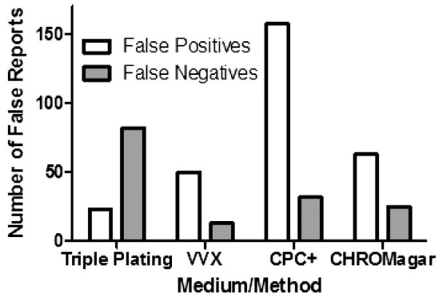

Fig. 3. The total number (out of 335 tested isolates) of false positive (white bars) and false negative (gray bars) reports from each medium or method.

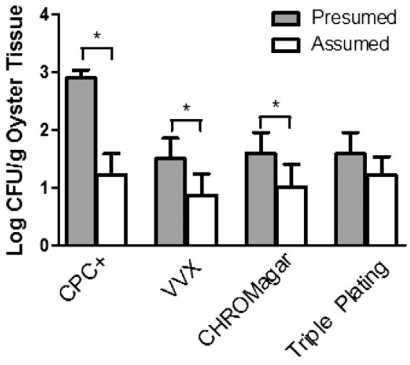

Fig. 5. Total presumed (gray bars) and assumed (white bars) *V. vulnificus* concentrations as determined by each medium or method averaged over all sites and dates. See the Material and methods section for calculation of assumed *V. vulnificus*. Asterisks indicated pairs of columns with significant ($p < 0.05$) differences. Error bars are significant error of the mean.

5. Conclusions

There has yet to be created an easy, inexpensive method for the isolation, enumeration, and culture of *V. vulnificus* bacteria in oysters that is ideal for every situation, but each of the methods presented here is fit for their particular task. The triple plating method provides an alternative to PCR for laboratories or field research sites that do not have molecular capabilities. CPC+ agar is the preferred choice for the accurate quantification of the clinically associated C-genotypes strains of *V. vulnificus*. CHROMagar *Vibrio* is an easy, albeit expensive, medium that has the versatility of differentiating between different pathogenic vibrios, while VVX is the most accurate and inexpensive way to study *V. vulnificus* total populations specifically.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2013.12.004.

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