Development of Selective Enrichment Medium for Clinical Isolates of *Vibrio vulnificus* Based upon Virulence Correlating Genes

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Abstract: The objective of this study was to find out if different enrichment media would influence the selection of clinical and environmental strains of *Vibrio vulnificus*. Therefore, traditional enrichment and maintenance medium for *V. vulnificus* were tested for selective preference for clinical or environmental strains. In the laboratory, Alkaline Peptone Water (APW) and Brain Heart Infusion Broth (BHIB), with adjusted salinity to that of APW, were inoculated with equal concentrations of clinical and environmental strains and incubated for 20 hr at 35 °C. BHIB selected for clinical strains (85%) as indicated by percentage of *vcgC* to *vvhA* genes, enumerated with quantitative PCR. In APW, the ratio of clinical (47%) to environmental strains (53%) was roughly equal. Enrichments of seawater with BHIB, APW, and Luria Bertani broth (LB) resulted in higher percentage of clinical strains in BHIB but not in APW or LB, in most samples. However seasonal differences in predominance of clinical or environmental strains in the seawater samples influenced the enrichment process. Our study suggests that BHIB10 might be advantageous for studying virulence factors and APW10 could be suitable for isolation and MPN enumeration in *V. vulnificus*. The selective preference of the BHIB10 medium for clinical strains indicates that iron plays an important role in the infection of the human host.
Keywords: *Vibrio vulnificus, Selective enrichment, vcgC, vvhA.*

Vibrio vulnificus Klinik İzolatlarının Virülens Genlerine Dayalı Olarak Selektif Zenginleştirme Ortamının Geliştirilmesi

Özet: Bu çalışmanın amacı, farklı zenginleştirme besiyerlerinin *V. vulnificus*'un klinik ve çevresel suşlarının seçimini etkileyip etkilemediğini bulmaktı. Bu nedenle, geleneksel zenginleştirme ve koruyucu besiyerlerinin selektif özellikleri, *V. vulnificus*' un klinik ve çevresel suşları için test edildi. Laboratuarda, aynı tuz konsantrasyonuna sahip Alkali Peptonlu Su (APW) ve Beyin Kalp İnfüzyon Broth (BHIB) eşit konsantrasyonlarda klinik ve çevresel suşlar ile ekimi yapıldı ve 35 °C' de 20 saat boyunca inkübe edildi. Kantitatif Polimeraz Zincir Reaksiyon (PZR) sayım sonuçları; BHIB' de geliştirilen suşların %85' ini klinik suşlar olduğunu göstermiştir. Bu oran *vcgC* geninin *vvhA* genine yüzdelik hesaplamalarıyla belirlenmiştir. APW' de klinik suşların (47%) çevresel suşlara (53%) oranı neredeyse eşitti. BHIB, APW ve Luria Bertani (LB) broth ile zenginleştirilen deniz suyu numunelerinin büyük kısmında, klinik suşlarının toplam suşlara oranı BHIB'de yüksek çıkarken, APW ve LB'de çıkmamıştır. Ancak, deniz suyu örneklerinde klinik veya çevresel suşların birbirine oranında mevsimsel farklılıklar zenginleşme sürecini etkilemiştir. Çalışmamız BHIB10'un virülans faktörlerini çalışmak için avantajlı olabileceğini ve APW10'un *V. vulnificus*'ta izolasyon ve MPN sayımı için uygun olabileceğini göstermektedir. Tuzluluk oranı %10 olan BHIB besiyerinin klinik suşlar için selektif özelliği, demirin insanlarda oluşturduğu infeksiyonlarda önemli bir rol oynadığını gösterir.
Anahtar Kelimeler: *Vibrio vulnificus, Selektif zenginleştirme, vcgC, vvhA.*

Introduction

Life-threatening, halophilic, Gram-negative marine bacterium *Vibrio vulnificus* was first isolated by the Centers for Disease Control (CDC) in 1964 (Strom and Paranjpye, 2000), since then, tremendous threats to human health as well as seafood industry have been documented (Jones and Oliver, 2009; Strom and Paranjpye, 2000). This opportunistic human pathogen, among the most severe of all foodborne infections, causes primary septicemia and severe necrotizing wound infections with a mortality rate exceeding 50%, mostly in susceptible individuals having an underlying disease such as hemochromatosis, immune disorders, and diabetes. Infection is mainly caused by ingestion of raw or undercooked oysters but can also be the result of exposure of pre-existing wounds to seawater or seafood products (Gulig et al., 2005; Strom and Paranjpye, 2000). Death occurs in one or two days after the onset of disease unless an effective treatment can be implemented (Gholami et al., 1998; Strom and Paranjpye, 2000).

V. vulnificus biotype 1, primarily infecting humans, has been demonstrated to have degrees of virulence. Strains isolated from hospital patients, referred to as 'clinical,' are highly virulent, whereas strains isolated from seawater, oysters, fish, and sediments, referred to as 'environmental,' are typically less virulent with some exceptions (DePaola et al., 1994; Hlady, 1997; Oliver et al., 1983; Potasman et al., 2002; Starks et al., 2000; Strom and Paranjpye, 2000; Wright et al., 1996). Human tissue appears to select for more virulent clinical strains of *V. vulnificus* while those isolated from oysters exhibit a high level of virulence diversity (Jackson et al., 1997).

Most studies of the virulence of *V. vulnificus* have relied upon isolate cultures. In the clinical environment *V. vulnificus* from tissues and blood are isolated on blood agars (Abbott et al., 2011). Isolation methods for environmental samples involve an overnight enrichment in alkaline peptone water (APW), having a salinity of 10 PSU, that selects for halophilic bacteria (Elliot et al., 1995). Enrichment is followed by streaking onto differential-selective agars. For several decades, thiosulfate-citrate-bile salts-sucrose (TCBS) agar was used to select for and isolate pathogenic *Vibrio* species (Kobayashi et al., 1963). Superior differential-selective media agars specific for *V. vulnificus* have since been developed and are now standard method. The first, cellobiose-polymyxin B-colistin (CPC) agar utilizes the resistance of *V. vulnificus* to colistin and polymyxin B for selection (Masad and Oliver, 1987). Other standard agars are modifications of CPC. Tamplin et al. (1991) modified CPC (mCPC) by reducing the amount of colistin to improve isolation success from environmental samples. Høi et al. (1998) was able to significantly increase the isolation rates of *V. vulnficus* from seawater and sediments by removing polymyxin B from CPC to make CC agar.

Because of the discovery of clinical and environmental strains it is important to know if standard methods for isolation (i.e. enrichment in liquid media followed by selective/differential agars) select for one strain over the other. Warner and Oliver (2007) determined through genotyping of the virulence correlating gene (*vcgC*) that CPC and mCPC agars select preferentially for clinical strains. We were interested in knowing if the prior overnight enrichment step with liquid media could preferentially select for clinical versus environmental strains of *V. vulnificus*. We compared APW, the traditional enrichment broth for *V. vulnificus* and other *Vibrios* (Kaysner and DePaola, 2004), to media employed for maintenance of *V. vulnificus* isolate cultures in the laboratory such as brain heart infusion broth (BHIB) (Rosche et al., 2005; Warner and Oliver, 1999), and Luria Bertani broth (Campbell and Wright, 2003; Randa et al., 2004; Sanjuán et al., 2009).

Since standard isolation methods for *V. vulnificus* utilizes agars (i.e. CPC and mCPC) that select for clinical strains, we opted to use quantitative PCR (qPCR) assays to detect clinical strains directly in the enrichment media. QPCR allows for rapid detection of targeted sequences without the requirement of time-intensive isolation and has been used to examine *V. vulnificus* in the environment and in experimental settings (Campbell and Wright, 2003; Chase and Harwood, 2011; Gordon et al., 2008). Moreover, qPCR can quantify cells that might be excluded during isolation methods. We quantified the hemolysin/cytolysin gene-*vvhA* that is present in all *V. vulnificus* for total cell counts. Panicker et al. (2004), who developed the assay, found it to have absolute specificity for *V. vulnificus* and high correlation (R^2 = 0.98) with plate counts. The clinical variant of the virulence correlated gene (*vcgC*) was used to quantify clinical strains of *V. vulnificus* (Han et al., 2011). The *vcgC* gene clinical variant has a 90% detection rate in clinical isolates (Rosche et al., 2005). Two studies observed a high correlation (R^2=0.98; R^2=0.99, respectively) with the 16S rRNA type B gene (Çam et al., 2019; Han et al., 2009) that is also present in clinical isolates.

We hypothesized that different enrichment media will influence the selection for *V. vulnificus* clinical and environmental strains. Furthermore, we hypothesized that BHIB, which contains whole cells of calf brains and heart and has a higher iron concentration (24.5 µM) than other media we tested (~7 µM) (Cam, 2016a), is most like human blood that selects for more virulent clinical strains. Normal iron concentrations of human serum are 14-32 µM in males and 10-28 µM in females and in persons with hemochromatosis 40 to 50 µM (Huebers et al., 1987; Nwafia et al., 2006). The purpose of the current study was to determine if the standard enrichment media APW is selective and would consequently influence the ratio of environmental versus clinical strains on differential-selective agars.

Materials and Methods

Isolate enrichments: This experiment was designed to test if clinical or environmental isolates were preferentially selected in enrichment media. Ten ml (3 replicates) of APW broth having a salinity of 10 PSU ('APW10') and BHIB with an adjusted salinity of 10 PSU ('BHIB10') were inoculated with equal volumes (10 µl) of log phase (OD_{600} = 0.6) clinical (BUF7211) and environmental (98-640)

strains of *V. vulnificus* (obtained from the U.S. FDA Gulf Coast Seafood Laboratory) and incubated for 20 hr at 35 °C with shaking at 100 rpm. After incubation, a 2 ml aliquot was transferred into 2 ml tubes, centrifuged for 10 min at 10.000 rpm (4 °C) to obtain a bacterial cell pellet and the supernatant was removed.

Seawater enrichments: This experiment was designed to determine if enrichment media preferentially enriched clinical versus environmental *V. vulnificus* from environmental samples. Five ml (3 replicates) of APW having a salinity of 10 PSU, APW with 1/2 concentration of NaCl ('APW5'; 5 PSU), BHIB ('BHIB5'; 5 PSU), BHIB modified to a salinity of 10 PSU, and Luria Bertani broth ('LB10'; 10 PSU), were inoculated with five ml of seawater, collected from six locations in Galveston Bay (GB4 lat 29.576801°/lon -94.934458°; GB6 lat 29.407004°/lon -94.806573°; GB12 lat 29.478671°/lon -94.757890°; GB17 lat 29.231857°/lon -94.989873°; GB25 lat 29.697525°/lon -94.783926°; GB29 lat 29.649323°/lon -94.811337°) in March, September, and November 2012 and vortexed then incubated for 20 hr at 35 °C with shaking at 100 rpm. After incubation, a 2 ml aliquot was transferred into 2 ml tubes, centrifuged for 10 min at 10.000 rpm (4 °C) to obtain a bacterial cell pellet and the supernatant was removed.

DNA extraction: CTAB (Cetyltrimethylammonium Bromide, 3%) method was followed for DNA extraction modified by Çam et al. (2019).

Quantitative PCR: To determine total *V. vulnificus*, we quantified *V. vulnificus* specific hemoylysin (*VvhA*) genes with a SYBR Green quantitative PCR (qPCR) assay (Panicker et al., 2004). Total volume was 25 µL which contained 12.5 µL SYBR green Supermix (BioRad), 1 µM *vvh*-L primer (5'-TTCCAACTTCAAACCGAACTATGA-3'), 1 µM *vvh*-R primer (5'- ATTCCAGTCGATGCGAATACGTTG-3'), 2.5 mM $MgCl_2$, 1 X BSA, and 2 µL of template DNA. The PCR reaction was performed in a SmartCycler (Cepheid) at 94 °C for 2 minutes, followed by 45 cycles of at 94 °C for 15 seconds, at 56 °C for 15 seconds and at 72 °C for 25 seconds. Melt curve analysis was conducted from 60 °C to 94°C at increments of 0.2 °C per second. PCR positive control for qPCR runs and construction of standard curve was clinical strain BUF7211 obtained from the U.S. FDA Gulf Coast Seafood Laboratory.

To determine clinical strains, we used a SYBR Green qPCR assay that quantifies the clinical variant of the virulence correlating gene (*vcg*) (Han et al., 2011). Total volume was 20 µL which contained 10 µL SYBR green Supermix (BioRad), 1 µM of each primers; VCGF3 (5'-CGCCTTTGTCAGTGTTGCA-3') and VCGB3 (5'-TAACGCGAGTAGTGAGCCG-3'), 2.5 mM $MgCl_2$, 1 X BSA, and 2 µL of DNA template. The PCR reaction was performed in a SmartCycler (Cepheid) at 95 °C for 2 minutes for initial denaturation, followed by 45 cycles at 95 °C for 20 seconds and 60 °C for 30 seconds and 72 °C for 25 seconds. Melt curve analysis was conducted from 60 °C to 94°C at increments of 0.2 °C per second. PCR positive control for qPCR and for construction of standard curve was clinical strain BUF7211 obtained from the U.S. FDA Gulf Coast Seafood Laboratory.

Two sample T-test and One-way ANOVA ($P<0.05$) was conducted with STATA 13.1 (StataCorp). For ANOVA Bartlett's test was conducted *a priori* to ensure equal variances. Bonferroni *post hoc* test was used to determine differences in means ($P<0.05$).

Results

Isolate enrichments: *VvhA* concentration, as a proxy for *V. vulnificus* cells, was the same in enrichment media APW10 ($1.5\times10^6\pm3.7\times10^5$ CFU/ml) and BHIB10 ($1.6\times10^6\pm3.6\times10^5$) (Figure 1) (Two-sample T-test; P=0.8695). Percent *vcgC* genes were higher in the BHIB10 medium (85.6±3.4%) than in the APW10 medium (47.3±7.6%) (Two-sample T-test; P=0.0234) (Figure 1).

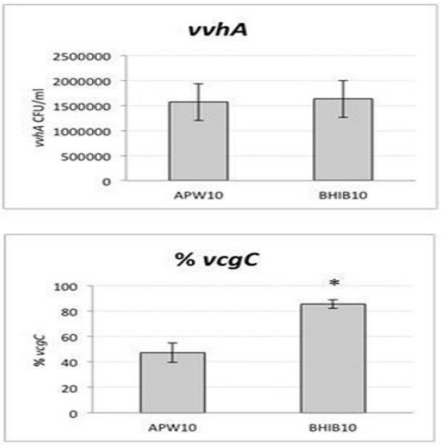

Figure 1. Concentration of *vvhA* (CFU/ml) and % *vcgC* genes in enrichment media APW10 and BHIB10. Asterisk denotes significant difference P<0.05.

Seawater enrichments: *VvhA* concentrations (CFU/ml) are presented in Figure 2 according to sampling date and station numbers. For samples collected on 3.22.12, no significant differences were observed between enrichments in samples GB12 and GB25 (ANOVA; P=0.748, P=0.245, respectively). Concentrations in sample GB17 enrichments APW5 and BHIB5 were significantly lower than APW10, BHIB10, and LB10 (ANOVA; P=0.0097). Total *V. vulnificus* (i.e. *vvhA* CFU/ml) in all samples ranged from 76±11 to 275±44 CFU/ml.

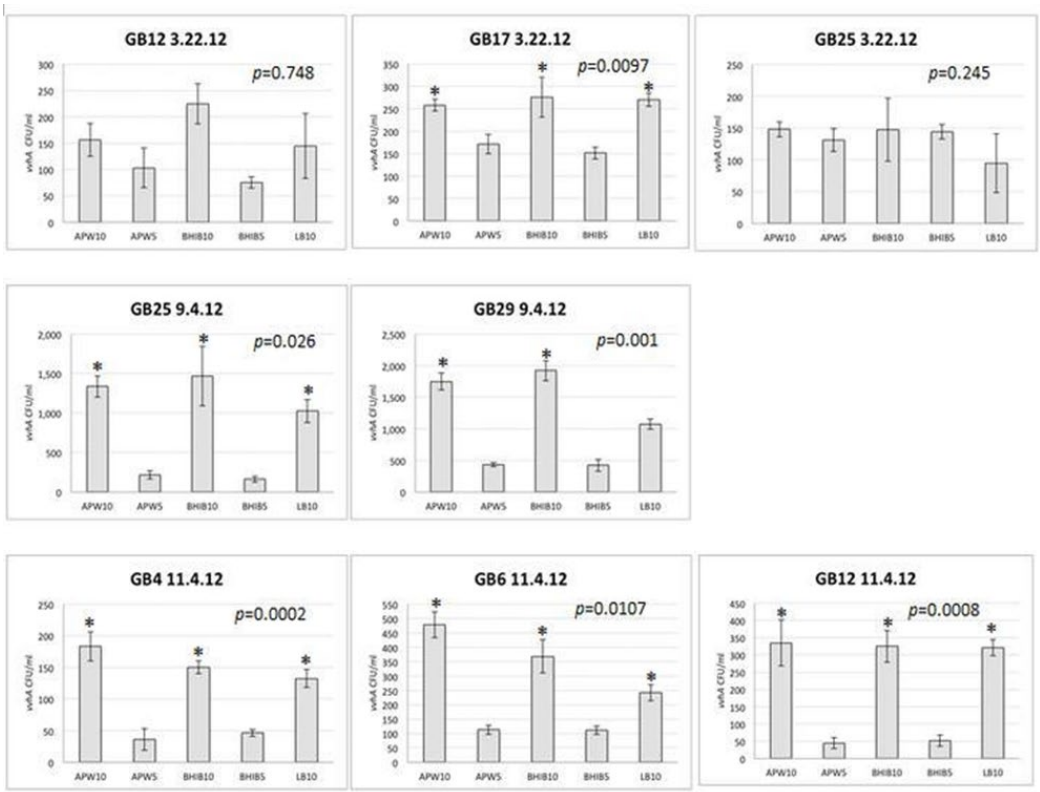

Figure 2. *VvhA* CFU/ml in seawater enriched with APW10, APW5, BHIB10, BHIB5, and LB10 for samples collected 3.22.12, 9.4.12, and 11.4.12 analyzed with one-way ANOVA, P<0.05. Asterisk denotes significance of P<0.05 in Bonferroni *post hoc* test.

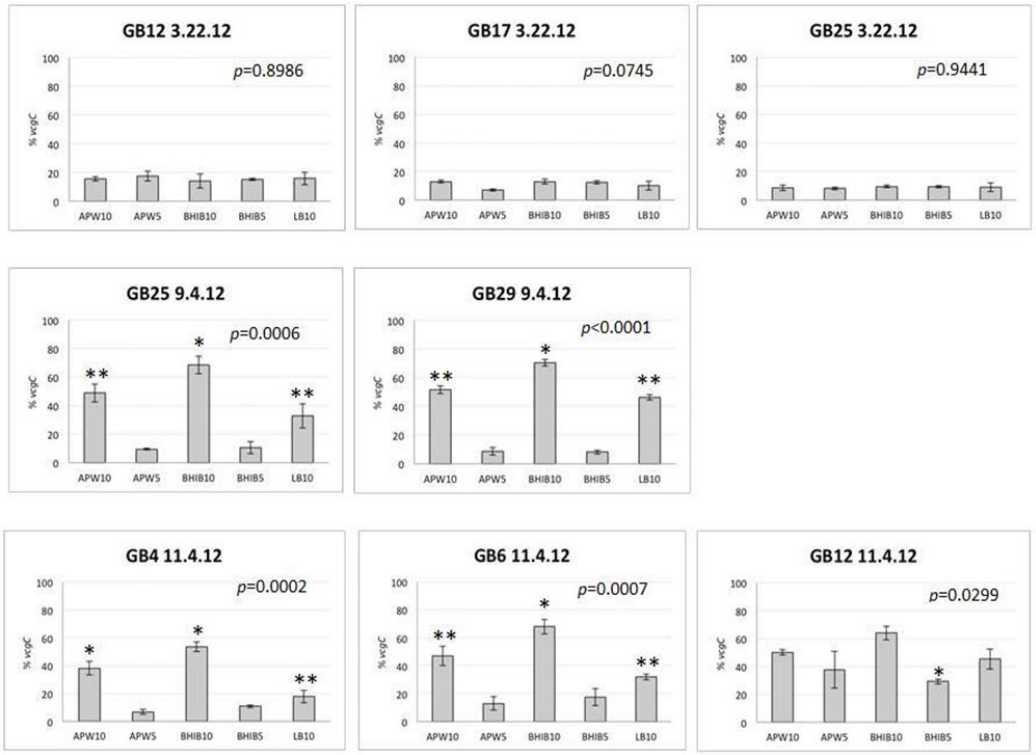

Figure 3. Percent *vcgC* in seawater enriched with APW10, APW5, BHIB10, BHIB5, and LB10 for samples collected 3.22.12, 9.4.12, and 11.4.12 analyzed with one-way ANOVA, P<0.05. Asterisk denotes significance of P<0.01 and double asterisk P<0.05 in Bonferroni *post hoc* test.

For sample GB25, collected on 9.4.12, *vvhA* concentrations in enrichments APW10 (1,335±133 CFU/ml), BHIB10 (1,469±376 CFU/ml) and LB10 (1,026±145 CFU/ml) were not significantly different from each other however they were approximately 4 to 6 fold higher (ANOVA; P=0.0026) than the other enrichments with APW5 and BHIB5. Enrichments APW10 (1,747±134 CFU/ml) and BHIB10

(1,920±158) in sample GB29, collected on the same day, had significantly higher *vvhA* concentrations than all other enrichments (ANOVA; P=0.0001). Enrichment LB10 (1,076±81) had significantly higher concentrations than APW5 (435±32 CFU/ml) and BHIB5 (422±92 CFU/ml) (Bonferroni *post hoc* test; P=0.020 and P=0.018, respectively).

For samples collected on 11.4.12, GB4, GB6, and GB12, *vvhA* concentrations in the APW10 (183±23, 335±66, 478±45 CFU/ml, respectively), BHIB10 (150±10, 368±57, 324±46 CFU/ml, respectively), and LB10 (132±14, 242±27, and 321±13 CFU/ml, respectively) enrichments were not significantly different from each other but were approximately 3 to 4 fold higher (P=0.0002, P=0.0107, and P=0.0008, respectively) than the other enrichments, APW5 and BHIB5. The percentage of *vcgC* gene counts out of total *V. vulnificus* cell counts (i.e. *vvhA*) are presented in Figure 3. according to sampling date and station numbers. All samples collected on 3.22.12 had low percentages (<20%) of the *vcgC* gene with no significant differences between enrichments.

Sample GB25 collected on 9.4.12 had significantly higher *vcgC* percentages in enrichment BHIB10 (68±6; P<0.01 Bonferroni *post hoc* test) than APW10 and LB10 (49±6 and 33±9%, respectively), that were higher (P<0.05, Bonferroni *post hoc* test) than the other enrichments having ∼10% (ANOVA; P=0.0006). Percent *vcgC* genes in the BHIB10 enrichment (70±3%; P<0.01 Bonferroni *post hoc* test) from sample GB29 was significantly greater than all other enrichments APW10 (52±3%), APW5 (9±3%), BHIB5 (8±2%) and LB10 (46±2%) (ANOVA; P<0.0001).

Percent *vcgC* genes in enrichment BHIB5 from sample GB12 collected on 11.4.12 were significantly lower (ANOVA; P=0.03) than the other enrichments, which were equal to each other (P<0.05 Bonferroni *post hoc* test). While in samples GB4 and GB6, no differences in % *vcgC* was determined between enrichments APW10 and BHIB10 (P<0.01 Bonferroni *post hoc* test), but they were significantly greater than LB10 (P<0.05) that was higher than APW5 and BHIB5 (ANOVA; P=0.0002 and P=0.0007, respectively).

Discussion and Conclusions

In literature, liquid media typically used to maintain *V. vulnificus* isolate cultures or to enrich for *V. vulnificus* prior to plating on selective-differential agars for selective enrichment of clinical versus environmental strains. Standard isolation methods for *V. vulnificus* involve a prior enrichment step with Alkaline Peptone Water (APW) followed by streaking onto selective-differential agars. The question is whether APW selects preferentially for clinical or environmental strains and whether Brain Heart Infusion Broth with its high iron content selects preferentially for clinical strains similar to the human host. As hypothesized, the BHIB10 medium selected for clinical strains of *V. vulnificus* likely due to the high iron content. Percent vcgC genes were higher in the BHIB10 medium (85%) in isolate enrichments as well as in seawater enrichments up to 70%. This selection could be advantageous for studying virulence factors in *V. vulnificus*. Similar to our findings, selective-differential agars have also been found to enrich clinical over environmental strains. Warner and Oliver (2007) determined through genotyping of the virulence correlating gene (*vcgC*) that CPC and mCPC preferentially select for clinical strains. But, there has been no study stating the preference of clinical over environmental strains in liquid media until the current study. The ratio of clinical (47%) to environmental strains (53%) in APW10 was roughly equal in isolate enrichments. Similar results were also obtained from seawater enrichments, indicating that APW10 could be suitable for isolation and MPN enumeration. Based upon the isolate enrichment results (Figure 1), we can conclude that ratios of clinical to environmental strains in the seawater enrichments were reflective of the actual starting concentrations when using the APW10 medium.

For seawater, the lack of significant differences between enrichments having different salinities observed in samples collected in March (Figure 1) might be explained by seasonal strain predominance differences (i.e. clinical versus environmental). Previous studies of Galveston Bay (Lin and Schwarz, 2003) have found that 'environmental' strains (i.e. 16S rRNA type A) predominate (up to 100%) in seawater and oysters from the early spring (March) to early summer (June) as water temperatures rise from below 10 °C up to ∼25 °C and salinities are low (<10 PSU). Concentrations of *V. vulnificus* are also low in March (non-detectable to <10) and increase gradually with increasing water temperatures. Between late June and October, as temperatures increase to >25 °C and salinities increase up to 30 PSU, there is a shift to a predominance (>70%) of 'clinical' strains (i.e. 16S rRNA type B, *vgcC* positive). Concentrations of *V. vulnificus* are also highest in oysters (10^3-10^4 CFU/g) and seawater (∼10^2 CFU/ml) during this time. Then as temperatures begin to decrease again in early November (<20 °C) but salinities are still >20 PSU, the ratio of clinical to environmental strains is roughly even. In November, concentrations of *V. vulnificus* also decline rapidly with decreasing temperatures. The

low concentration of *V. vulnificus* and the low percentage of clinical strains (i.e. % *vcgC*) in the March enrichments agree with the findings of these studies and we detected <20% *vcgC* genes for all enrichment types (Figure 3) indicating a predominance (>80%) of environmental strains.

Salinity appeared to be the key factor that influenced the concentration of *V. vulnificus* in the enrichments from seawater samples collected in September and November. These samples when enriched with the higher salinity broths (APW10, BHIB10, and LB10), had higher *vcgC* gene percentages than the broths having 5 PSU. The question is whether salinity is selecting for clinical strains or if clinical strains were already predominant in the original sample. The answer may be both. Ambient salinities in the seawater used for these enrichments were 10 to 18 PSU higher than in the March samples (∼10 PSU). Moreover, Lin and Schwarz (2003), who examined 16S rRNA types (A/B) in Galveston Bay seawater and oysters, found a predominance of 16S rRNA type B strains in August to November with salinities ranging from 21 to 29 PSU and temperatures ranging from 23 °C to 30 °C. Similar observations were also made when examining clinical versus environmental strains in Galveston Bay oysters (Çam et al., 2019). The 5 PSU salinity in the enrichment broths APW5 and BHIB5 may have selected for environmental strains (i.e. 16S rRNA type A) or may have limited growth of clinical strains as percent *vcgC* genes were lower in these treatments. Chase and Harwood (2011), who examined the influence of temperature and salinity on clinical strains of *V. vulnificus,* found that highest growth rate (3.97 generations/hr) was achieved at temperature of 37 °C and salinity of 25 PSU. Growth rate at the same temperature but at a lower salinity of 5 PSU was significantly lower (2.03 generations/hr). At 10 PSU, growth rate (3.24 generations/hr) was still significantly higher than at 5 PSU.

Enrichment with BHIB10 selected for a higher percentage of virulent clinical strains (i.e. *vcgC* positive) than the other enrichment media in the September samples. Percent clinical strains in the BHIB10 medium was ∼20% higher than in the APW10 medium and ∼30% higher than in the LB10 medium. All of these media contain 1% oligopeptides derived from digests of protein, but BHIB also contains 50% infusions of calf brains and beef hearts that increase the iron content by 17.5 µM. The high iron content in BHIB stimulates cytolysin/hemolysin activity of *V. vulnificus* (Kim et al., 2009; Tison and Kelly, 1984) and induces production of flagellar proteins that are integral for adhesion and biofilm formation in *V. vulnificus* (Alice et al., 2008). This trend was weakly continued in the November samples with only one sample (GB6; Figure 2) having significantly higher percent clinical strains in the BHIB10 medium. It is apparent that salinity also plays a role in selecting for clinical strains since the BHIB5 medium with lower salinity had 50-60% lower percent clinical strains than BHIB10.

Different enrichment media selected for different ratios of clinical to environmental strains. Seasonality of *Vibrio vulnificus* clinical and environmental isolates influenced the starting inoculum and ultimately the selective enrichment for clinical strains in all media tested. The enrichment of seawater results indicated that not only does the starting ratio matter, so does the salinity of the seawater inoculum. In other words, seasonal differences in predominance of clinical or environmental strains in seawater influence the enrichment process. The selective preference of the BHIB10 medium for clinical strains indicates that iron plays an important role in the infection of the human host.

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