Characteristics of *Vibrio vulnificus* isolates from clinical and environmental sources

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ABSTRACT

Researchers have developed multiple methods to characterize clinical and environmental strains of *Vibrio vulnificus*. The aim of our study was to use four assays to detect virulence factors in strains from infected patients and those from surface waters/sediments/oysters of South Carolina and the Gulf of Mexico. *Vibrio vulnificus* strains from clinical (n=81) and environmental (n=171) sources were tested using three real-time PCR methods designed to detect polymorphisms in the 16S rRNA, *vcg* and *pilF* genes and a phenotypic method, the ability to ferment D-mannitol. Although none of the tests correctly categorized all isolates, the differentiation between clinical and environmental isolates was similar for the *pilF, vcgC/E* and 16S rRNA assays, with sensitivities of 74.1-79.2% and specificities of 77.4-82.7%. The *pilF* and *vcgC/E* assays are comparable in efficacy to the widely used 16S rRNA method, while the D-mannitol fermentation test is less discriminatory (sensitivity = 77.8%, specificity = 61.4%). Overall Percent Agreement for the D-mannitol fermentation method was also lower (66.7%) than Overall Percent Agreement for the 3 molecular assays (78.0% to 80.2%). This study demonstrated, using a large, diverse group of *Vibrio vulnificus* isolates, that three assays could be used to distinguish most clinical vs environmental isolates; however, additional assays are needed to increase accuracy.

Key words*: Vibrio vulnificus, clinical, environmental, genotyping, virulence.*

1. INTRODUCTION

Vibrio vulnificus is a halophilic, Gram negative bacterium, occurring naturally in coastal and brackish waters worldwide [1]. Primarily an opportunistic pathogen, *V. vulnificus* can cause skin infections through the exposure of open wounds to contaminated water, and is the leading cause of seafood-related deaths in the United States [2, 3]. Uncommon, but potentially deadly infections can occur from the consumption of raw shellfish, with onset of symptoms, including severe gastroenteritis and primary septicemia, within 24 hours. *Vibrio vulnificus* infections have a 50% or greater fatality rate, with greatest risk among immuno-compromised individuals, or persons with elevated serum iron, characteristic of liver disease [4].

While most frequently associated with filter-feeding oysters, *V. vulnificus* has also been detected in plankton, fish, eels, crabs, and lobsters [5]. Increases in environmental counts and human infections correlate well with warmer, summer temperatures [6-9], and a report citing significant increases in *Vibrio* spp., including *V. vulnificus*, infections from 1996-2010 is a cause for concern for public health officials [10]. More recent studies provide evidence that global increases in ocean water temperatures may generate conditions which allow *Vibrio* spp. and their accompanying diseases to proliferate in formerly less hospitable waters, exposing previously unaffected populations to increased health risks [6, 11, 12].

Although *V. vulnificus* concentrations in oysters can exceed 10,000 cells per gram of oyster meat during warmer, summer months [13], and high numbers of *V. vulnificus* can be found in warm estuarine waters, including those in the Northern Gulf of Mexico, incidence of *V. vulnificus* illnesses as a result of raw oyster consumption or wound infections [14] are relatively rare compared with levels that might be expected if all *V. vulnificus* strains were virulent. As a result of public health concerns, researchers have

78 developed numerous methods with the goal of distinguishing between clinical (virulent) and
79 environmental strains (presumed to be less virulent) [15].

80 An early study using terminal restriction fragment length polymorphisms (T-RFLP) by Nilsson et al.
81 (2003) [16] described a link between two variations in the 16S rRNA region of *V. vulnificus* and the
82 clinical or environmental origin of strains. Later modified by Vickery et al. (2007) [17] as a real-time PCR
83 assay, isolates identified as Type A were found to be associated with environmentally-collected strains,
84 type B isolates were consistent with those of clinical origin, and type AB (positive for both type A and B),
85 a mix of clinical and environmental. Frequently used to characterize the pathogenicity of
86 environmentally collected samples [18, 19], the 16S rRNA method has been used as a standard in some
87 method comparison studies [20]. As an alternative to molecular-based detection, Drake et al. (2010)
88 [20] reported a positive correlation between the fermentation of D-mannitol and 16S rRNA type B
89 isolates, suggesting its application as a simple screening tool for virulence potential. Most recently, an
90 assay was reported utilizing a polymorphism in the *pilF* gene of *V. vulnificus* to identify clinical strains
91 [21, 22]. Since *pilF* encodes a protein necessary for the assembly of a Type IV Pilus, an essential
92 virulence factor in many Gram negative bacteria, early studies suggest that detection of the *pilF*
93 polymorphism could serve as a marker for human pathogenic strains of *V. vulnificus* [23]. Based upon
94 Roig et al's study (2010) [22], another group of researchers, Baker-Austin et al. (2012) [21], developed a
95 real-time PCR assay, utilizing *pilF,* to differentiate strains capable of causing infections in humans from
96 non-pathogenic strains in different matrices.
97
98 An additional method of distinguishing pathogenic potential is the virulence correlated gene (*vcgC*/*vcgE*)
99 assay, which has been reported to differentiate between virulent and non-virulent strains of *V. vulnificus*
100 in a manner similar to the 16S rRNA assay discussed above. Application of the *vcg* gene model has
101 demonstrated a strong discriminatory capability of clinical vs environmental in biotype I strains [24, 25].

Research indicates that two primary lineages may exist within biotype I strains; one, the *vcgC* allele and the other, *vcgE* [26, 27]. While both may express virulence in murine models, *vcgC* positive strains are more likely to be associated with systemic infection and mortality [27]. Recent research suggests that some C type strains of environmental origin (not recovered from human patients) demonstrate growth inhibition by human serum [28], and many *E* type strains have shown virulence in iron dextran treated mouse models [27]. Thus, the *vcgC/vcgE* assay is particularly useful in identifying strains more likely to be associated with clinical cases than not.

The goal of this study was to characterize clinical and environmental strains of *V. vulnificus* isolated from sources in the Gulf of Mexico and Southeast U.S. using one phenotypic and three genotypic methods. We examined 252 previously collected clinical and environmental isolates using the 16S rRNA gene assay, the *pilF* gene assay, the *vcgC*/*vcgE* assay, and D-mannitol fermentation to compare the virulence potential of each isolate, as well as the agreement between the 16S rRNA method and the three other techniques.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

A total of 252 previously-isolated strains of *V. vulnificus* were used in this study. Environmental strains (n=171) were collected between 2005 and 2012 from oysters, sediment, or water samples in Florida, Texas, Louisiana, or South Carolina, and clinical strains (n=81) were provided by the Texas Department of Health Lab (n=28), the U.S. Food and Drug Administration (n=32), the Florida Department of Health (n=19), and the Alabama Department of Health (n=2).

Environmental strains from FL, TX, LA, and the 2005 SC strains were isolated on *Vibrio vulnificus* Agar (VVA) and confirmed as *V. vulnificus* using colony blot hybridization with an alkaline phosphatase labeled DNA probe targeting the species-specific *vvhA* gene in *V. vulnificus* (VVAP) [29-32]. Positive (*Vibrio vulnificus* ATCC 27562) and negative (*Vibrio parahaemolyticus* ATCC 17802) controls were used. After color development, colonies that hybridized with the probe were purple-brown in color. The limit of detection is 10 CFU/g oyster, sediment or mL of water. The 2012 SC environmental isolates were collected from sites along the Waccamaw River and in three shellfish growing water areas in Winyah Bay, and plated onto CPC+ and CHROMagar Vibrio (CAV) plates. DNA was extracted from individual *V. vulnificus* isolates using the Qiagen DNeasy protocol and stored at -80°C. A TaqMan based real-time PCR assay targeting the hemolysin *A* gene (*vvhA*) [33] was used to confirm the *V. vulnificus* isolates. *V. vulnificus* strains M-06 and Env1, were provided by Rachel Noble (University of North Carolina, Institute of Marine Sciences) and used as positive and negative controls for clinical and environmental strains, respectively in the 16S rRNA, and *pilF* real-time PCR assays and the D-mannitol fermentation test. All strains were maintained at -80°C in tryptic soy broth with 2.5% NaCl and 20% (vol/vol) glycerol.

2.2 Bacterial Growth and DNA isolation

Bacterial strains were grown from frozen stocks on Tryptic Soy Agar (TSA) with 2.5% NaCl at 35°C for 24 hours prior to DNA extraction or D-mannitol inoculation. Bias was minimized during sample analyses by numerically coding individual strains. Strains were analyzed and typed without the knowledge of strain origin. For DNA extractions, a loopful (~1µL) of cells was added to a 1.5 mL micro-centrifuge tube of phosphate buffered saline (PBS) [7.65 g NaCl, 0.724 g anhydrous Na_2HPO_4, 0.21 g KH_2PO_4 L^{-1} of D.I. water, pH 7.4] (APHA 1970) [34] and extracted using the DNeasy Blood and Tissue kit following the manufacturer protocol for bacterial cells (Qiagen). Recovered DNA was checked for purity by spectrophotometry using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Extractions

147 showing very low DNA readings were discarded, and the procedure was repeated beginning with the
148 frozen stock cultures. D-mannitol tubes were inoculated directly with a loop (~1µL) of cells from the TSA
149 with 2.5% NaCl plates.

150 2.3 16S rRNA Assay

151 The characterizations of each isolate as type A (environmental), type B (clinical) or type AB was
152 performed using the previously described qPCR protocol (Vickery et al. 2007) [17] on an iQ5
153 thermocycler (BioRad). Small modifications were made to the procedure to reflect a difference in
154 premade reagents. Each 25µl reaction contained: 12.5µl of 2X BioRad iQ Supermix, 2µl of 25µM MgCl,
155 0.5 µl of 10µM forward primer (Vvu16S51-F; **Table 1**), 0.5 µl of 10 µM reverse primer (Vvu16S221-R),
156 0.1875 µl of 10µM Type A probe (Vvu16SA-P), 0.1875 µl of 10µM Type B probe (Vvu16SB-P), 7.125 µl of
157 nuclease-free water, and 2µl of extracted DNA. Cycle conditions were consistent with those reported in
158 Vickery et al. (2007) [17].

159 2.4 *vcgC/vcgE* Polymorphism Assay

160 A species specific primer/probe combination was employed for the PCR detection of the C type allele of
161 the virulence correlated gene (*vcgC*) [35]. Primers and probes are shown in **Table 1**. The mastermix was
162 comprised of 1 µm PCR Buffer, 2.5 µm MgCl, 0.2 µm DNTP's, 0.25 µm forward and reverse primers, 0.18
163 µm probe, 0.3 µm Qiagen Top Taq (Qiagen, Venlo, Limburg), and water to volume for 25µL reactions
164 using 3 µL template DNA. Amplification and product detection were performed on a Bio Rad CFX 96
165 Real Time PCR System under the following conditions: Initial denaturation at 95°C for 180 s, followed by
166 45 cycles of denaturation at 95°C for 15 s and combined annealing and extension at 60° C for 90 s.

167 Primer pairs previously published [36, 37] were used for PCR detection of the Environmental type (E)
168 allele of the virulence correlated gene (*vcg*). Primers and probes are shown in **Table 1**. The mastermix

was comprised of 12.5 µL Sso Fast Evagreen® Supermix (Bio-Rad Laboratories, Inc., Hercules, CA), 0.3 um of each primer, and water to volume for 25µL reactions using 5µl template DNA. Amplification and product detection was performed on a Bio-Rad CFX 96 Real Time PCR System under the following conditions: a 3 minute initial denaturation at 94° C, followed by 40 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72° C for 45 seconds. Products were visualized in real time using the SYBR green channel and evaluated post-hoc using melt curve analysis.

2.5 *pilF* Polymorphism Assay

Amplification of the *pilF* polymorphism was performed by modifying a previously described method [21]. Optimal results were achieved on an iQ5 thermocycler (BioRad) using 2x iQ5 Supermix and by reducing the primer and probe (**Table 1**) to a final concentration of 0.1µM.

2.6 D-mannitol Fermentation

All isolates were tested for the ability to ferment the carbohydrate D-mannitol. Actively growing cells of each isolate were transferred to a culture tube containing 5mL of D-mannitol fermentation broth with phenol red. Inoculated tubes were incubated at 35°C for 48 hours and each strain was evaluated for the ability to ferment D-mannitol as indicated by a color change from a neutral red to an acidic yellow. For each set of samples, a positive and negative control, and an un-inoculated blank were used for color comparison.

2.7 Data Analysis

Each isolate was identified as positive or negative for the *pilF* gene and ability to ferment D-mannitol, and typed as C or E by the *vcg* assay and as A, B, or AB using the 16S rRNA method (also called the rrn system by several researchers) (**Tables 2, 3**). While isolates of clinical origin (blood or other samples from infected patient) can be assumed with reasonable confidence to be virulent, all that is known of

those of environmental origin is that they were collected from water/sediment/oysters. The presence of the *pilF* gene, *vcgC*, ability to ferment D-mannitol and B typing have all been suggested as characteristics of clinical strains.

Classification of isolates identified as 16S rRNA type AB is generally considered ambiguous; therefore all strains identified as such (n=20; **Tables 2, 3**) were excluded from the analyses of the 16S rRNA assay. Additionally 15 environmental isolates that could not be typed as *vcg C* or *E* were omitted from the calculations which included this assay.

The ability of each assay to correctly characterize isolates as clinical or environmental in origin was assessed by calculating: a) the positive predictive value (PPV) for clinical isolates (proportion of isolates that tested as *vcgC*, D-mannitol fermentation positive, 16S rRNA type B or *pilF* positive that were of clinical origin); b) the negative predictive value (NPV) (proportion of isolates that were *vcgE*, D-mannitol fermentation negative, 16S rRNA type A or *pilF* negative that were of environmental origin); c) the sensitivity (proportion of clinical isolates correctly identified by each test [38] (- *vcgC*, D-mannitol positive, 16S rRNA type B or *pilF* positive); d) specificity (proportion of environmental isolates that were correctly identified as *vcgE*, D-mannitol negative, 16S rRNA type A or *pilF* negative) [37], [38]; and e) the overall percent agreement of each assay for all isolates (all correctly predicted isolates, both environmental and clinical, divided by total number of isolates). All values were calculated using Fisher's Exact Test and significance of results was assessed at the p-value < 0.05 level.

While the pathogenicity of all clinical isolates is presumed known, the pathogenicity of the environmentally collected strains is not clear. For this reason, we also calculated agreement between the D-mannitol, *vcg* and *pilF* individual tests with the 16S rRNA assay.

Finally, Pearson's correlation coefficients for each assay and source were calculated to determine the strength of the relationships between assays and between each assay and origin.

3.0 RESULTS

Our study examined 252 *V. vulnificus* isolates, 171 collected from environmental sources (water, sediment, or oyster samples), and 81 recovered from infected patients, here described as 'clinical' (**Tables 2 and 3**).

The results of each of the four assays (16S rRNA gene, the *vcg C* and *E* alleles, the *pilF* gene, and the ability to ferment D-mannitol) for all isolates are shown in **Tables 2 and 3**.

The 16S rRNA method, developed by Vickery et al. (2007) [17], uses a polymorphism in the 16S rRNA gene to type isolates into A (environmental), B (clinical) or AB (having characteristics of both). Twenty of the 252 isolates tested were identified as type AB (7.9%) in the present study, and these were excluded from any comparative analysis that included the 16S rRNA results. Of the 81 clinical isolates, four were characterized as type AB (4.9%), while 75.3% were type B and 19.8% were type A. Distribution of the 171 environmental isolates was 9.4% AB, 20.5% B and 70.1% A. Thus, 19.8 % of the clinical isolates typed as environmental isolates, and 20.5% of the environmental isolates typed as clinical isolates with the 16S rRNA marker.

The *vcgC/vcgE* assay utilizes sequence variations in the virulence correlated gene as a means of differentiating source and potential virulence. For our isolates 74.1% of the clinical group had the *vcgC* sequence, 25.9% *vcgE*. For environmental isolates the proportions were 75.4% with *vcgE*, 15.8% *vcgC*, and neither variant could be detected in 8.8% of the strains. For the *vcgC/vcgE* marker, 25.9 % of the clinical isolates typed as environmental isolates, and 15.8% of the environmental strains typed as clinical ones.

The *pilF* method differentiates between clinical and environmental isolates through the positive amplification of a polymorphism in the *pilF* gene frequently present in clinical isolates [21, 22]. A

236 negative result in the *pilF* test, no amplification, is associated with an isolate of environmental origin. Of
237 our clinical isolates 76.5% were *pilF* positive, while 81.9% of the environmental isolates were *pilF*
238 negative, and only 18.1% were positive for *pilF.* Overall, for the *pilF* assay, 23.5% of the clinical strains
239 were negative (typed as environmental strains), and 18.1 % of the environmental isolates were positive
240 (typed as clinical strains).

241 The final method examined the ability of a *V. vulnificus* isolate to ferment the carbohydrate, D-mannitol.
242 A positive D-mannitol test has been associated with clinical isolates [20] and a negative result with
243 environmental source isolates. While 77.8% of the clinical isolates were able to ferment D-mannitol,
244 almost 40% of the environmental isolates were also D-mannitol fermentation positive.

245 Predictive values, sensitivity and specificity [38] were examined for each of the assays to evaluate their
246 potential use in differentiating clinical from environmental *V. vulnificus* isolates using the characteristics
247 positive for *pilF,* ability to ferment D-mannitol, *vcgC* and type B as 'virulence' characteristics. Results are
248 summarized in **Table 4**. Positive predictive values (PPV; **Table 4**) i.e. proportion of isolates that tested
249 positive (clinical) that were clinical, for each method ranged between 63.5 and 68.9% for the *pilF*, *vcg*
250 and 16S rRNA assays; the ability to ferment D-mannitol had a significantly lower positive predictive value
251 (48.8%) ($p<0.05$), due to a large number of environmental isolates that tested positive for this assay, i.e.
252 were false positives. Negative predictive values (NPV) were all greater than 85%; i.e. over 85% of
253 isolates testing *pilF* negative, *vcgE*, type A or D-mannitol negative were from environmental sources.

254 The proportion of clinical isolates that were identified as such by each assay (sensitivity) were similar for
255 all the assays, ranging from 74% (*vcgC*) to 79% (type B), showing each method was able to identify
256 approximately three quarters of the clinical isolates as clinical. However, when comparing specificity, the
257 proportion of environmental isolates that were identified as such (16S rRNA type A, *pilF* negative, *vcgE*
258 or did not ferment D-mannitol) was significantly higher for *pilF* and *vcg* assays (~82%). D-mannitol

fermentation (61.4%) was significantly inferior ($p < 0.01$) to each of the other methods for specificity, as the result of the large number of environmental isolates (66 of 171) incorrectly identified as clinical in origin (i.e. D-mannitol fermentation positive).

Overall percentage agreement for all isolates was high, and similar, for the three molecular assays (78.0-80.2%) but was significantly lower for D-mannitol fermentation (66.7%). Many of the environmental isolates tested D-mannitol positive (38.5%) i.e. there were many false positives for clinical origin isolates.

The overall percent agreement (both positives and negatives) between each test and the 16S rRNA assay were high – for mannitol fermentation 78.9% (183/232), *pilF* 85.3% (198/232) and for *vcg* 95.4% (207/217).

Pearson's correlation coefficients between each assay and between each assay and isolate source showed moderate to strong relationships and all were highly significant ($p<0.001$). D-mannitol fermentation and source had the weakest relationship ($r=0.3661$) with D-mannitol and *pilF* also having a weaker relationship ($r=0.4013$). *Vcg* type and 16S rRNA were strongly correlated ($r=0.89$) while *pilF* and D-mannitol were also correlated with 16S rRNA ($r=0.6960$ and 0.6057 respectively).

4.0 DISCUSSION

Public health concerns over *V. vulnificus* outbreaks have motivated the development of multiple procedures aimed at rapidly and effectively identifying potentially pathogenic strains [20]. Although numerous systems have been reported, none has proven to be accurate in all cases. Previous studies have demonstrated the ability of methods utilizing polymorphisms of the 16S rRNA gene, the *vcg C* and *E* alleles, the *pilF* gene, and the ability to ferment D-mannitol, as ways to distinguish between clinical and environmental strains of *V. vulnificus*. The focus of our study was the validation of these four

281 methods using a large collection of previously isolated *V. vulnificus* strains of both clinical and
282 environmental origin.

283 The application of the 16S rRNA gene for *V. vulnificus* clinical/environmental discrimination has been
284 used historically in comparative studies and environmental detection applications [15, 18, 19, 27, 39,
285 40]. Vickery et al. (2007) [17] found that type B isolates were associated with 76% of the clinical strains
286 tested. Our examination provided similar results, with 61/81 (75.3%) of clinical isolates identified as
287 type B. Vickery et al. (2007) [17] also found that 20/23 (87.0%) of non-clinical strains, not identified as
288 type AB, were type A. Again, we found fairly similar results, 77.4% of environmental isolates not
289 identified as AB were identified as type A. Vickery et al. (2007) [17] determined that 22% of 86 *V.*
290 *vulnificus* strains tested in their study originally appeared as type A, but when reanalyzed, were type 16S
291 rRNA type AB.

292 A study by Nigro et al. (2011) [41] examined *V. vulnificus* in Lake Pontchartrain water after Hurricanes
293 Katrina and Rita (2005-2006) and found 80% of the isolates were type A and 20% were type B. Gordon et
294 al. (2008) [42] identified 30% of oyster isolates and 40% of water isolates as type B in a Florida study.
295 The *vcgC/E* assay has been used in several studies to distinguish clinical from environmental strains.
296 Jones et al. (2013) [39] found 32% (153/471) of isolates were *rrn*B*/vcgC* and 61% (289/471) were
297 *rrn*A/*vcgE* or *rrn*AB/*vcgE*. In Jones et al. (2013) [39] study, six isolates were untypeable by the *rrn*
298 system (16s rRNA) and four isolates were untypeable by the *vcg* system, while in the present study, 15
299 *V. vulnificus* isolates were untypeable by the *vcg* method but all isolates could be typed with the 16S
300 rRNA system (now being called the *rrn* system by some researchers). Arias et al. (2010) [43] had 11
301 isolates that were untypeable by the *rrn* system. Previous studies report that the *vcg* type corresponds
302 closely with source: *vcgC* type corresponds 90% to clinical isolation and the *vcgE* type corresponds 93%
303 to environmental isolation [28, 36]. According to Warner and Oliver (2007) [44] estuarine water

304 samples contain almost equal percentages of *C*-type and *E*-type cells, while oysters contain 13% *C*-type
305 and 87% *E*-type cells. In this study only 18 oyster isolates were included and of these 17 were *vcgE* type.
306 Overall, three quarters of our 171 environmental isolates were *vcgE* type, and conversely 74% of the
307 clinical isolates were *vcgC,* suggesting that this assay has some discriminatory value. However, the fact
308 that 26% of clinical isolates (from infected patients) were *vcgE* type, clearly demonstrates the difficulty
309 in differentiating clinical strains of this species. A study by Kim and Cho (2015) [45] using BOX-PCR,
310 isolated 52 unique genotypes in *V. vulnificus* leading them to conclude that genotype assays may not be
311 reliable for either virulence or isolate source.

312 Although the *pilF* assay has only been subjected to limited testing, Roig et al. (2010) [22] found that
313 unlike other genotypic methods, the *pilF* polymorphism appears to be present in human-pathogenic
314 strains of all three biotypes. Although we only tested this with isolates collected from the Gulf and
315 Southeastern U.S. , the *pilF* polymorphism was detected by Roig et al. (2010) [22] utilizing isolates
316 collected from the U.S., Europe, Asia and Australia. Challenging the classification ability of the *pilF*
317 polymorphism against isolates confirmed to be pathogenic (resistant to human serum), the real-time
318 PCR assay developed and tested by Baker-Austin et al. (2012) [21] correctly identified 91% (16/17) of *V.*
319 *vulnificus* biotype 1. Only 76.5% (62/81) of clinical isolates were correctly identified, using the *pilF* assay
320 by itself in our study, although overall percentage agreement, i.e. clinical isolates *pilF* (+), environmental
321 *pilF* (-), was 80.2%. Roig et al. (2018) [46] identified five new lineages within 80 separate *V. vulnificus*
322 strains that were different from the three standard biotypes. Two other papers suggest the difference
323 between virulent and avirulent *V. vulnificus* strains may be either a strain's ability to colonize and
324 survive in a human host or the host's susceptibility [46] rather than which virulence genes are
325 present/not present [48].

D-mannitol fermentation was first reported as an effective means of categorizing isolates by Drake et al. (2010) [20]. In their study of 469 *V. vulnificus* isolates collected from oyster and water samples in the Gulf of Mexico, 91% of those identified as type B were also positive for D-mannitol fermentation. Bier et al. (2013) [49] identified positive mannitol fermentation in 15 of 19 (79%) of isolates from clinical cases, but in only 5 of 28 (18%) of environmental isolates. Our results were similar to those of Bier et al. (2013) [49] for clinical isolates - approximately 77.8% were able to ferment D-mannitol (sensitivity); however we found a much higher proportion of environmental isolates were D-mannitol fermentation positive (38.5%) (specificity). The mannitol phenotypic method was the same between the current study and Bier's 2013 assay. Differences in results between the two studies may be attributed to differences in the specific environmental sources (water, sediment, oysters) or specific locations. Bier et al. (2015) [50] developed and validated a multiplex PCR assay for the detection of 5 potential virulence genes in *V. vulnificus*: 16S rRNA type B allele, *vcgC* allele, the *nanA* gene, pathogenicity region XII and the mannitol *manIIA* gene. They could detect the *nanA* gene and the *vcgC* allele with a sensitivity and specificity of 100% [50].

Froelich and Oliver (2011) [28] found that *vcgC* strains can be further separated into 2 subtypes (based upon isolate source and whether the individual strain could ferment mannitol or not). These researchers stated that all mannitol positive *Vibrio vulnificus* strains contain the 3 genes of the mannitol fermentation operon located next to each other. However, if the gene-spanning fragment is missing, the strain was mannitol negative [28]. As 16S rRNA identification has been widely applied, the agreement between the individual tests and the A/B designation was calculated and found to be relatively high, especially for the *vcgC/E* assay, where agreement was 95.4%. Agreement with the ability to ferment D-mannitol was significantly less, at 78.9%.

As noted earlier, the type AB identified isolates (7.9% of 252 isolates) were removed from the analyses which included the 16S rRNA assay due to uncertainty in classification. Sixteen out of 20 originated from environmental sources (**Table 3**). However, as 67.9% (171/252) of our isolates were of environmental origin, if 20 type AB designations were selected randomly from our 252 isolates, statistically 13.6 of those 20 would be associated with environmentally collected isolates. Therefore our study provides no additional insight into the relationship between type AB isolates and source. Also of note, all four of the type AB clinical isolates in our study were identified as negative by the *pilF* and D-mannitol assays, and six of the 18 *V. vulnificus* strains isolated from oyster samples (33%) were identified as type AB, a much higher percentage than the expected 6.0% (14/234) ratio of all other strains in our collection not isolated from oysters. This increased prevalence of type AB in oysters has been observed in studies conducted in Alabama [19], Louisiana [20, 51], and New Zealand [40]. Gordon et al. (2008) [42] also found a substantial proportion of oyster isolates from restricted waters (19.4%) were 16S rRNA type AB. In a study of *V. vulnificus* isolates collected from the dorsal fin of fishes in the Gulf of Mexico, Tao et al. (2012) [18] identified 9% of isolates as type AB. Givens et al. (2014) [52] examined *V. vulnificus* in fish intestines, oysters, sediment and water and determined that overall 60% of 123 isolates were type A, 7.3% were type B, 32% were type AB, and 0.8% were untypeable.

Classifying strains of environmental origin only on the source of collection may not indicate an avirulent strain. Thiaville et al. (2011) [27] reported virulent strains of the E genotype. Bier et al. (2013) [49] reported clinical E genotypes from wound infections in the Baltic. Roig et al. (2010) [22] found several environmentally collected isolates that were resistant to human serum, and thus would be categorized as pathogenic. The evidence Roig et al. (2018) [46] found in their later lineage study supports their theory that all *V. vulnificus*, regardless of biotype, origin, or year collected, can infect humans. Reynaud et al (2013) [26] stated “No available molecular markers with sufficient resolving power to categorize with absolute certainty the pathogenicity of *V. vulnificus* strains” (from [53]). Baker-Austin and Oliver

(2018) [47] also stated that no single virulence gene correlates with observed virulence. Several studies utilizing the 16S rRNA assay have found type B isolates in non-clinical samples. Wood and Arias (2012) [19], noted that more than 50% (n=154) of 294 isolates collected from oysters in coastal Alabama, were type B, although it is unknown how many of these isolates would be considered pathogenic. Tao et al. (2012) [18] observed that 72 of 244 *V. vulnificus* isolates collected from the surfaces of fish were type B (29%), 22 were type AB (9%), and 157 were type A (62%). In 10 samples collected from Pacific oysters in New Zealand, Kirs et al. (2011) [40] found an 8:1:1 ratio of Type A:B:AB. Again, questions remain as to how many of these environmentally collected type B isolates are potentially pathogenic to humans. Conversely, Gordon et al. (2008) [42] detected approximately equal numbers of type A and B in a collection of clinical strains from Florida and suggested a geographical variation in *V. vulnificus* populations and further calling into question the effectiveness of the 16S rRNA assay when used as the sole method of strain characterization. As many of our clinical isolates were provided by the Florida Department of Health, we would like to note that 16 of the 19 isolates collected in Florida in 2012 were identified as 16S rRNA type B (**Table 3**), suggesting a possible anomaly in the previous data. Only two of the five 1994-1996 Florida-collected clinical strains in our collection were found to be type B, which is far too small of a sample to draw any conclusions about Florida isolates. In addition, the isolates examined in this study are limited to the Southeast U.S., and thus unable to address geographical variation in *V. vulnificus* populations.

Overall this study contributes to the literature on distinguishing between clinical and environmental *Vibrio vulnificus* isolates of known origin, using several assays currently being investigated to clarify virulence mechanisms in this pathogen. Based on our set of 252 *V. vulnificus* isolates, collected in the Gulf of Mexico and Southeast U.S., we can recommend not using the phenotypic D-mannitol

fermentation test as the sole means of identifying virulent strains. The *vcgC/E* typing and the *pilF* assay are each similar in classification ability to the 16S rRNA method, suggesting they are valid alternatives.. In addition, an advantage of using *pilF* is that it provides a dichotomous classification, removing ambiguous identifications such as the 16S rRNA type AB, or isolates that are *vcg* untypeable. The story of virulence in *V. vulnificus* is continuously evolving. Recent advances using Whole Genome Sequencing and other 'omics' based approaches [54, 55] are rapidly expanding our knowledge of this opportunistic pathogen.

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624 **Table 1** Primers, probes and oligonucleotides used in the study to characterize *Vibrio vulnificus* isolates

Primer, Probe, or Oligonucleotide	Sequence	Reference
Vvu16S51-F	5'-CAAGTCGAGCGGCAGCA-3'	Vickery et al. 2007
Vvu16S221-R	5'-TCCTGACGCGAGAGAGGCC-3'	Vickery et al. 2007
Vvu16SA-P	5'-6FAM-TGATAGCTTCGGCTCAA-MGBNFQ -3'	Vickery et al. 2007
Vvu16SB-P	5'-TET-CCCGTAGGCATCATGC-MGBNFQ -3'	Vickery et al. 2007
PilF F	5'-GATTGACTACGAYCCACACCG-3'	Baker-Austin et al. 2012
PilF R	5'-GRCGCGCTTGGGTGTAG-3'	Baker-Austin et al. 2012
PilF Probe	5'-FAM-TGCTCAACCTCGCTAAGTTGGAAATCGATAC-TAMRA-3’	Baker-Austin et al. 2012
vcgC-F	5'-AAAACTCATTGARCAGTAACGAAA-3'	Baker-Austin et al. 2010
vcgC-R	5'AGCTGGATCTAAKCCCAATGC-3'	Baker-Austin et al. 2010
vcgC-P	5'/TAMRA/AATTAAAGCCGTCAAGCCACTTGACTGTAAAGAA/FAM/-3'	Baker-Austin et al. 2010
vcgE-F	5'-CTCAGAAAGGCTCAATTGAC-3'	Warner and Oliver 2008*b*
vcgE-R	5'-GATTAACGCTGTAAGGCCG-3'	Warner and Oliver 2008*b*

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635 **Table 2** Summary of characteristics of environmental *Vibrio vulnificus* isolates from Alabama (AL), Florida
636 (FL), Louisiana (LA), Maryland (MD), Mississippi (MS), Oregon (OR), Rhode Island (RI), South Carolina (SC)
637 or Texas (TX).

# of Isolates	States	Matrix	Years	*vcgC/vcgE*	16S rRNA	*pilF*	Mannitol
22	TX	Water	2006, 2007	E	A	-	-
6	TX	Water	2006, 2007	E	A	-	+
2	TX	Water	2007	E	A	+	-
3	TX	Water	2006, 2007	C	B	-	+
6	TX	Water	2006, 2007	C	B	+	+
33	SC	Water	2005, 2012	E	A	-	-
15	SC	Water	2005, 2012	E	A	-	+
2	SC	Water	2005, 2012	E	A	+	-
2	SC	Water	2005, 2012	E	B	-	-
1	SC	Water	2012	E	B	-	+
5	SC	Water	2005, 2012	C	A	-	+
1	SC	Water	2005	C	A	+	+
1	SC	Water	2012	C	B	-	-
2	SC	Water	2012	C	B	-	+
4	SC	Water	2005, 2012	C	B	+	+
12	SC	Sediment	2005, 2012	E	A	-	-
8	SC	Sediment	2005, 2012	E	A	-	+
1	SC	Sediment	2005	C	A	-	-
1	SC	Sediment	2012	C	B	-	+
1	SC	Sediment	2012	C	B	+	-
1	SC	Sediment	2005	C	B	+	+
7	TX, LA, FL, MS, OR, RI	Oyster	1998, 1999	E	A	-	-
3	TX, FL	Oyster	1999	E	A	-	+
2	LA	Oyster	1998, 1999	E	A	+	-
	Vv *vcgC/vcgE* untyp strains						
5	SC	Water	2012	Untyp	B	-	+
5	SC	Water	2012	Untyp	B	+	-
1	SC	Water	2012	Untyp	B	+	+
1	SC	Sediment	2012	Untyp	A	+	-
2	SC	Sediment	2012	Untyp	B	-	+

1	SC	Water	2005	Untyp	AB	+	-
	Vv 16S rRNA type AB strains						
4	TX, SC	Water	2007, 2012	E	AB	-	-
1	SC	Water	2012	E	AB	-	+
2	TX, SC	Water	2007, 2012	E	AB	+	-
4	LA, MD	Oyster	1998	E	AB	-	-
1	LA	Oyster	1998	E	AB	+	-
1	TX	Oyster	1999	C	AB	+	+
2	SC	Sediment	2012	E	AB	-	-

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646 **Table 3**. Summary of characteristics of clinical *Vibrio vulnificus,* isolates from Alabama (AL), Florida (FL),
647 Louisiana (LA) or Texas (TX).

# of Isolates	States	Years	16S rRNA	*pilF*	Mannitol	*vcgC/vcgE*
10	TX, LA,FL	1995, 1997, 2008, 2012	A	-	-	E
1	TX	2009	A	-	+	E
4	TX, LA,FL	1996, 2009, 2012	A	+	-	E
1	FL	1996	A	+	+	E
4	TX	2012	B	-	+	C
56	TX,LA,AL,FL	1994,1995,1996,1996,2008, 2009,2012	B	+	+	C
1	LA	1995	B	+	+	E
4	TX,LA/FL	1994, 1995, 2008	AB	-	-	E

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Table 4. Predictive values, sensitivity and specificity of each assay for clinical and environmental *Vibrio vulnificus* isolates.

	16S RNA B/A	D-mannitol fermentation +/-	*pilF* +/-	*vcg* C/E
PPV*	63.5% (61/96)	48.8% (63/129)	66.7% (62/93)	68.9% (60/87)
NPV†	88.2% (120/136)	85.4% (105/123)	88.1% (140/159)	86.0% (129/150)
Sensitivity	79.2% (61/77)	77.8% (63/81)	76.5% (62/81)	74.1% (60/81)
Specificity	77.4% (120/155)	61.4% (105/171)	81.9% (140/171)	82.7% (129/156)
Overall agreement	78.0% (181/232)	66.7% (168/252)	80.2% (202/252)	79.8% (189/237)

*PPV=Positive Predictive Value; † NPV=Negative Predictive Value