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Characterization of *Vibrio vulnificus* strains using phenotypic and genotypic assays

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Abstract

In this study 143 *V. vulnificus* isolates of clinical and environmental origin, were examined for growth on differential media, identified to species and tested for antibiotic resistance. A multiplex PCR was created and optimized, and phylogenetic analysis was conducted. The first objective was to compare phenotypic methods to identify *V. vulnificus*. Colony colors of confirmed *V. vulnificus* isolates on selective media (*Vibrio vulnificus* agar, thiosulfate citrate bilesalts sucrose agar, CHROMAgar Vibrio (CAV), and colistin polymyxin B cellobiose agar), mostly matched those characteristic of *V. vulnificus*. To test the ability of these media to select for *V. vulnificus*, new presumptive *V. vulnificus* isolates were collected and grown on the four media. Most of the tested media had very high false positive rates, with isolates not confirmed as *V. vulnificus* through PCR of the *vvhA* gene, growing with characteristic *V. vulnificus* colony colors. CAV was determined to be the most differential. The Biolog Microbial Identification System was used to identify *V. vulnificus* isolates and had a 96% correct identification rate. Antibiotic resistance of *V. vulnificus* isolates was assessed and compared with isolate origin. Almost all the *V. vulnificus* isolates were susceptible to all the antibiotics used. Only five isolates displayed any type of resistance and thus no relationship with origin could be established. A multiplex PCR protocol using amplification of *vvhA* and the 16S gene was developed, optimized, and tested to identify an isolate as *V. vulnificus*. Buffer optimization was simultaneously conducted and TBE was determined to be the most effective buffer for gel electrophoresis with the produced amplicons. Finally, a fragment of the *vvhA* gene from each isolate was sequenced. Analysis of the fragment resulted in phylogenetic trees with two distinct branches related to clinical or

environmental isolates. Virulence factors associated with the constructed phylogeny but isolate origin did not. This study was conducted to further characterize a group of well-studied *V. vulnificus* isolates and to find methods to differentiate clinical from environmental isolates. Continued analysis of these and additional isolates may further our knowledge of the species and reveal more characteristics indicative of pathogenicity.

INTRODUCTION

V. vulnificus is a Gram negative, halophilic bacterium that ferments lactose (Hollis *et al.* 1976). Among vibrios, the ability to ferment lactose is unique to *V. vulnificus* and led to its speciation. However, a sub population (~15%) of *V. vulnificus* lacks the ability to ferment lactose (Oliver 2005), including all biotype III strains (Zaldenstein *et al.* 2008). The first isolate recovered from an infected patient in 1976 (Hollis *et al.* 1976) was not named and was referred to as a lactose-positive *Vibrio*. Later the same year it was named *Beneckea vulnificus* (Reichelt *et al.* 1976) and in 1979 it was renamed and classified as *V. vulnificus* (Farmer 1979). Isolates are now identified as *V. vulnificus* by the presence of the *Vibrio vulnificus* hemolysin A (*vvhA*) gene and are placed into one of three biotypes (I, II, or III) (Johnson *et al.* 2012).

Vibrio vulnificus is a human pathogen with ~50% mortality rate from food borne, and ~22% mortality rate from cutaneous infections (Oliver 2013). *V. vulnificus* is found in warm brackish waters around the world. Isolates have been recovered from coastal areas along the Atlantic, Pacific and Gulf Coasts of the U. S. (Oliver 2005), Europe (Dalsgaard *et al.* 1999), Israel (Zaldenstein *et al.* 2008), and several East Asian countries (Oliver 2005). The threat to humans is compounded by the fact that *V. vulnificus* is often associated with invertebrates (oysters, shrimp, etc.) that are consumed by humans (Hollis *et al.* 1976; CDC 2013a). Vibrios, such as *V. vulnificus*, also pose a threat to commercial aquaculture as they are fish and crustacean pathogens (Molina-Aja *et al.* 2002; Horseman and Surani 2010; Kitiyodom *et al.* 2010). Several invertebrates, including shrimp and oysters, that are farmed commercially, are at risk of infection (Molina-Aja *et al.* 2002), which can result in high crop mortality and economic losses for the industry. Human

health is also at risk through consumption of infected products. Of all seafood borne illnesses in the U. S., *V. vulnificus* causes the most infections and has the highest mortality (Oliver 2005). Most human infections result from eating contaminated seafood; primarily oysters and other shellfish. Despite the widespread occurrence of this pathogen in water and seafood, less than 100 infections occur per year in the U. S. In 2012 there were 35 deaths nationwide, with the majority of cases occurring along the Gulf Coast (CDC 2013a). However, the incidence of infections has been increasing since 1996 (CDC 2013b). A study on vibriosis was conducted by Newton *et al.* (2012) in which data from the Centers for Disease Control and Prevention (CDC), Cholera and Other Vibrio Illness Surveillance, and Foodborne Diseases Active Surveillance Network were compiled. They concluded that from 1996 to 2010 the overall incidence of vibriosis has approximately tripled while *V. vulnificus* infections increased from 0.01 to 0.05 per 100,000 people (Newton *et al.* 2012).

In addition to genotypic differences, specific phenotypic assays have been used to differentiate strains. Distinct requirements for growth, such as temperature, salinity, and pH ranges, and substrate utilization vary between biotypes (Bisharat *et al.* 1999; Chase and Harwood 2011). With the severity and rapid progression of infections caused by this organism, antibiotic resistance is another important phenotypic characteristic. Biotypes I and III are known to cause human infections, while biotype II is primarily an eel pathogen (Horseman and Surani 2010). Most cases of human infections result from biotype I strains and infections caused by biotype III have been limited to Israel (Zaldenstein *et al.* 2008). Each biotype is distinguished by different metabolic profiles (for example, a characteristic of biotype I strains is mannitol fermentation) and growth

parameters (for example, biotype 2 strains cannot grow at 42°C) (Oliver 2005). Chase

and Harwood (2011) reported that *V. vulnificus* biotype I isolates grew rapidly, and

preferred a temperature of 37°C, salinity of 2.5% and pH of 7.0 which are close to human

physiological conditions, whereas biotypes II and III grew significantly more slowly,

under all conditions, compared to biotype I. Oliver (2005) described differences between

biotypes including the ability of biotype 1 to grow at 42°C. Biotype III isolates differ

from the other two in that they lack the ability to ferment lactose and cellobiose (Bisharat

et al. 1999). However, identifying definitive characteristics, that can be used to

differentiate pathogenic and non-pathogenic strains, has met with little success, due to the

variability within the species (Sanjuán *et al.* 2009).

      V. vulnificus strains can also be grouped into clinical (C) and environmental (E)

genotypes based on several housekeeping genes which are involved in basic cellular

maintenance (chaperonin, uridine monophosphate kinase, RNA polymerase σ^{70} factor,

glutamine synthetase, and gyrase B and DNA repair (*recA*) (Rosche *et al.* 2010; Oliver

2013) or the virulence correlated gene (*vcg*) (Rosche *et al.* 2010; Bier *et al.* 2013).

Polymorphisms in the 16S rRNA gene were used by Nilsson *et al.* (2003) to correlate

pathogenic isolates with a genotype of B and non-pathogenic with A and AB.

VIRULENCE OF *V. VULNIFICUS*

      Not all *V. vulnificus* isolates cause disease and much attention has been focused

on identifying virulence factors associated with human infections. High iron

concentration in blood, possibly due to liver dysfunction/alcoholism, has been identified

as a risk factor for infections (Oliver 2005; Zaldenstein *et al.* 2008). The presence of iron

in blood combined with the ferrophilic nature of *V. vulnificus* contribute to the virulence

of the species. Biotype I isolates, in particular, take advantage of the replete nutrients

(such as iron) and when combined with rapid doubling time, can overwhelm the host

(Kim *et al.* 2006; Chase and Harwood 2011).

There are a number of other characteristics that have been linked with virulence

of *V. vulnificus* such as the ability to ferment mannitol (Drake *et al.* 2010). Sialic acid is

abundant in the human digestive tract and the ability to metabolize it may also be linked

with virulence (Bier *et al.* 2013); strains not capable of metabolizing sialic acid exhibited

decreased virulence in mice (Jeong *et al.* 2009). Type IV secretion systems have been

identified as virulence factors in several bacteria (Cascales and Christie 2003) and a

polymorphism in the type IV secretion system has been identified as a putative virulence

factor in *V. vulnificus* (Roig *et al.* 2010). Bier (2013) clustered *V. vulnificus* isolates based

on several genes including putative virulence factors and markers and observed that

mannitol fermentation, *pilF*, 16S type B, *vcg* C, and the sialic acid metabolizing gene

nanA clustered together in group IIA. However, there was much variation in the

presence/ absence of virulence factors across all groups. Capsules have been associated

with virulence in some bacteria including *V. vulnificus*. Typically, capsule positive

colonies of *V. vulnificus* appear opaque, with a smooth or rugose surface (Garrison-

Shilling *et al.* 2011), while capsule negative strains appear translucent and smooth on

laboratory media. In addition to capsule presences, phase variation of capsules in *V.

vulnificus* is common, resulting in numerous different types of capsules that can be

expressed within a population (Hilton *et al.* 2006). Hilton *et al.* (2006) discovered a

correlation between capsule phase variation and C and E genotypes; C genotypes took

significantly longer in the lab to lose their capsules than E genotypes. Other phenotypic differences between clinical and environmental isolates could be used for rapid identification of potentially pathogenic *V. vulnificus*.

PHENOTYPIC IDENTIFICATION AND DIFFERENTIATION OF *V. VULNIFICUS* STRAINS

Substrate Utilization

Identification of *V. vulnificus* from environmental samples is often conducted using selective and differential agars from which colonies with appropriate color and morphology are isolated. *Vibrio vulnificus* agar (BAM Media M190: *Vibrio vulnificus* Agar (VVA) 2013) selects for *V. vulnificus* through an elevated pH and a single carbon source: cellobiose. *V. vulnificus* has the ability to ferment cellobiose and colonies appear on VVA as a yellow "fried egg" colony with a yellow halo (Kaysner and DePaola 2004). CHROMagar™ *Vibrio* (CAV), colistin polymyxin B cellobiose plus (CPC+), and thiosulfate citrate bile salts sucrose agar (TCBS) are also commonly used for rapid isolation and phenotyping of *V. vulnificus* (Williams *et al.* 2013). Both VVA and CPC+ contain cellobiose as the sole carbon source which when fermented results in a color change of the medium, differentiating potential *V. vulnificus* isolates from other bacteria. CPC+ also contains the antibiotics colistin and polymyxin B which inhibit the growth of non-target organisms. CAV uses a blend of proprietary chromogenic compounds to differentiate species based on color; *V. vulnificus* turns turquoise. TCBS contains inhibitory salts and is selective for *Vibrio* spp. but has limited species differentiation capabilities (Williams *et al.* 2013). A triple plating method was developed by Williams *et*

al. (2013) in which environmental samples are initially isolated on CAV and turquoise colonies are then spotted on CPC+ and TCBS. Colonies of the appropriate color on both types of media (yellow and green respectively) can then be presumptively identified as *V. vulnificus* with a 93% confirmation level by PCR (Williams *et al.* 2013).

Additional selective and differential media for *V. vulnificus* have been proposed and compared in an attempt to simplify and optimize recovery of isolates. Cerdá-Cuéllar (2001) compared cellobiose colistin agar (CC), *Vibrio vulnificus* medium (VVM), VVMc (VVM without polymyxin), and TCBS for positive identification of *V. vulnificus*. VVMc and VVM had 53% and 49% positive identification rates while CC and TCBS had 47% and 42% positive identification rates respectively (Cerdá-Cuéllar *et al.* 2001). *Vibrio vulnificus* X-Gal (VVX) has also been proposed as a selective and differential medium in which the antibiotics colistin and polymyxin B select for *V. vulnificus* and lactose fermentation causes *V. vulnificus* colonies to turn blue (Griffitt and Grimes 2013).

Various media, alone or in combination, have been tested for quick screening of *V. vulnificus* in environmental samples. Froelich *et al.* (2014) tested the efficiency of CPC+, CAV, VVX, and the triple plating method, developed by Williams *et al.* (2013), for recovering *V. vulnificus* from environmental samples and eliminating false positives and negatives. VVX had the highest correct identification percentage at 81% of presumptive *V. vulnificus* colonies (confirmed by PCR amplification of *vvhA*), followed by CAV at 74% and CPC+ at 44%. The triple plating method had a 68% correct identification rate; lower than the 93% reported by Williams *et al.* 2013. The discrepancy was due to a high false negative rate because of isolates that fermented sucrose on TCBS (which was a disqualification in the triple plating method). However, the false positive

rate was 7% with the triple plating method and the next lowest rate was 15% with VVX

(Froelich *et al.* 2014). Despite the usefulness of the media for isolating *V. vulnificus* from

environmental samples, the problem of differentiating potentially pathogenic populations

remains. Further characterization of carbon sources that can be used by certain strains of

V. vulnificus may lead to the creation of new differential media for clinical and

environmental isolates.

Antimicrobial Resistance

Antibiotic resistance has important ramifications for both the medical community

and industry. With growing use of antibiotics to treat infections in aquaculture, antibiotic

resistance in *V. vulnificus* has increased (Li *et al.* 1999; Kitiyodom *et al.* 2010).

Currently the Centers for Disease Control and Prevention recommend doxycycline and a

third generation cephalosporin or a fluoroquinolone to treat human infections (CDC

2013a) while oxytetracycline, enrofloxacin, sarafloxacin, trimethoprim sulfamethoxazole,

chloramphenicol, gentamicin, and florfenicol are used in aquaculture (Roque *et al.* 2001;

Cabello *et al.* 2013). Antibiotic susceptibility profiles can be developed for isolates and

may be able to be used to differentiate between clinical and environmental strains as well

as origin. A survey of literature has found that many isolates were either susceptible to

most antibiotics tested or were resistant to multiple antibiotics (Han *et al.* 2007; Kim *et*

al. 2011). Such variability may be due to regional differences, time of the year, tested

antibiotics, or sample size. However, some large studies (n>100) have failed to find

widespread antibiotic resistance at the tested location (Han *et al.* 2007; Roig *et al.* 2009).

Another challenge in using this parameter for differentiating isolates is the variability in

selected antibiotics and the number included in studies. Researchers in different countries, as well as in smaller regions, have chosen antibiotics that may be relevant only for their area, such as those used nearby in agriculture. Such variability complicates comparison of antibiotic resistance in the *V. vulnificus* population. Additionally, variability in drug panel size limits results comparison when the drug panel is small or not balanced. Currently CLSI M45-A2 lists a recommended drug panel for *V. vulnificus* that includes frequently used drugs and those used to treat infected humans. However, a larger common drug panel would aid between-study comparisons.

GENOTYPIC IDENTIFICATION AND DIFFERENTIATION OF *V.* *VULNIFICUS* STRAINS

Genetic analyses have been useful for differentiating bacterial species as well as strains. Typically the 16S gene is used to distinguish species of bacteria and group them based on their relatedness. However, differences in species specific genes have enabled subspecies classifications. For example, Nilsson *et al.* (2003) discovered that clinical and nonclinical *V. vulnificus* strains could be differentiated by a 16S polymorphism. Type A and AB isolates were typically not of clinical origin while type B were recovered from patients (Nilsson *et al*. 2003). The virulence correlated gene E (*vcgE*) (environmental) and C (*vcg*C clinical) have also been identified as markers for non-pathogenic (E) and pathogenic (C) *V. vulnificus* (Rosche *et al.* 2005). The *vcgE* and *vcgC* polymorphisms were later correlated with other distinct differences in housekeeping genes between isolates of environmental and clinical origin (Rosche *et al.* 2010). Multilocus sequence typing has also yielded clusters for environmental and clinical isolates (Rosche *et al.*

2005). Mahmud *et al.* (2010) recently used Rep PCR to group isolates recovered from aquaculture into clinical and environmental classes.

PHYLOGENETIC DIFFERENTIATION OF *V. VULNIFICUS* STRAINS

The advent of DNA sequencing and polymerase chain reaction (PCR) has enabled researchers to identify bacterial species without the need for culturing. Amplification and sequencing of 16S genes has led to differentiation of species and has been used to determine species relatedness. However, obtaining sequence data is a two-step process, amplification and then sequencing, which can take days to complete. For faster identification of a species, amplification of unique species specific genes, such as *vvhA* in *V. vulnificus*, can be performed. PCR product can be visualized by gel electrophoresis (or fluorescence for real time PCR) and presence of a product of the appropriate size, indicates a positive reaction. After identification, PCR products can then be sequenced and compared to one another using many different phylogenetic techniques.

An important first step in phylogenetic analysis is sequence alignment. Multiple sequence alignments can be performed by tools such as Clustal Omega, Clustal X, MUSCLE, etc. (www.ebi.ac.uk/Tools/msa). Each program utilizes a different alignment strategy, potentially resulting in different phylogenies. It has been shown that alignment strategy has an effect on tree topology (Wong *et al.* 2008) and therefore, careful choice of alignment program is necessary.

Step two for constructing phylogenetic trees is to choose a tree building strategy, such as neighbor joining, maximum likelihood analysis etc. Neighbor joining was developed in 1987 by Saitou and Nei (1987). The basic strategy is to build a tree with

minimal evolutionary steps but it does not attempt to make the most parsimonious tree as in maximum parsimony (Saitou and Nei 1987). Neighbor joining has the advantage of speed compared to other programs (maximum parsimony and maximum likelihood) and usually produces a tree with the correct topology (Saitou and Nei 1987; Tateno *et al.*1994). Maximum likelihood analysis of DNA sequences began in 1981 when Joseph Felsenstein developed a program to process DNA sequences (Felsenstein 1981). At its most basic the maximum likelihood method uses all sequence data to find the most probable phylogenetic tree. To do this, the algorithm assumes that all changes to DNA are independent of one another. The first sequence in the alignment file is used as the starting point and each subsequent sequence is added and placed on the tree. Before each new sequence is added, the algorithm maximizes the likelihood of placement and branch length. As the tree grows, sequences are rearranged after each addition and the tree with the highest likelihood is selected and the process repeats until all sequences have been added (Felsenstein 1981). Due to the algorithm constantly modifying the tree and calculating the maximum likelihood of each addition the process is time and computationally intensive. However, the maximum likelihood method can produce more accurate trees than other methods and includes information that can be used for statistics. Some drawbacks to the maximum likelihood are that the order of sequences in the alignment file can have an effect on tree topology, as they are read in the order they appear, and the algorithm considers all changes to be independent of one another which is known to be false (Felsenstein 1981).

After a phylogenetic tree has been constructed, the next step is to determine how much confidence can be placed in it. Bootstrapping is a technique for resampling data to

determine confidence in phylogenetic trees and was first used by Felsenstein (1985) to determine the confidence of species relatedness. The process involves replicating the original set of sequences in an alignment file and modifying characters in each sequence (bases for DNA) at random. This process is repeated a large number of times (as specified by the user) where each modified sequence represents a new "sample." By increasing the number of "samples" a more accurate estimate of relatedness can be determined (ie 2000 resampling events would be more accurate than 100). Each modified sample set is used to construct a phylogenetic tree (using any of the previously described methods). All of the constructed trees can then be combined into a consensus tree where the arrangement is determined by the most common arrangements in the group of trees.

OBJECTIVES OF THIS STUDY

1. Compare several phenotypic methods to identify *V. vulnificus*, including use of selective media and the Biolog Microbial Identification System.

2. Assess several methods for their ability to differentiate the origin of *V. vulnificus* isolates (clinical vs. environmental), including antibiotic resistance profiles, appearance on selective media, and phylogeny.

3. Use phylogenetic analysis of a fragment of the *vvhA* gene to determine if putative virulence factors associate with phylogenetic relatedness.

METHODS

V. VULNIFICUS ISOLATES

One hundred and forty two confirmed (by detection of the *vvhA* gene using PCR) *V. vulnificus* cultures were provided by the National Oceanic and Atmospheric Administration (NOAA), from multiple sources (including state health laboratories, FDA etc.) and including both clinical and environmental source isolates (Table 1). Clinical isolates were originally obtained from state and federal agencies while environmental isolates were collected from water, sediment, and oysters (Supplementary table 1) (Moore and Mott 2013). Some phenotyping and genotyping analyses had been previously conducted on these isolates (including but not limited to: *pilF*, mannitol fermentation, and heavy metal resistance) (Diaz and Mott 2012; Moore and Mott 2013; Shi *et al.* 2012). One additional confirmed *V. vulnificus* isolate collected during this study was included, to make a total of 143 confirmed *V. vulnificus* isolates. An additional ~80 isolates, presumptively identified as *V. vulnificus* by selective media, were collected from residential and commercial aquaria, and coastal Virginia sites: Colonial Beach, VA, and Virginia Beach, VA (below).

Table 1. Origin of clinical and environmental *V. vulnificus* isolates used in this study.

	Alabama	Florida	Louisiana	Texas	South Carolina	North Carolina	Oregon	Virginia	Total
Clinical	3	14	4	37	0	0	0	0	58
Environmental	0	0	2	31	49	1	1	1	85
									143

Sample Collection

Water samples were collected on August 25, 2013 from Virginia Beach, VA at a depth of ~10cm with a water temperature of 24.4°C for sample one, 24.0°C for sample two, and 27.5°C for sample three. A sample of water from Colonial Beach, VA was collected on September 7, 2013 with a water temperature of 26.5°C and depth of ~10cm. Water from local saltwater aquaria was collected at three different locations: a home aquarium (sample collected on September 9, 2013 with a water temperature of 26.5°C), a commercial aquarium at a pet store (water from a single tank collected on September 24, 2013), and two water samples were collected from two separate aquaria at a different commercial pet store (on September 24, 2103). At the first commercial pet store, all aquaria were linked by a re-circulating water filtration system. Temperature of water samples could not be measured at the time of collection at the pet stores. Water samples at Virginia Beach, Colonial Beach, and home aquaria were collected by opening a sterile plastic bottle below the surface of the water, allowing the bottle to fill, and then capping the bottle under water. Water samples from local pet stores were acquired by staff that scooped out water from tanks and poured it into bags. Samples were transported to the lab at ambient temperature and stored at room temperature until they could be processed.

Sample Processing

Water samples were processed as follows: sterile vacuum apparati were set up
with 0.45um nitrocellulose filters (Sartorius Stedim, Bohemia, New York); ~4ml of
sterile phosphate buffered saline (PBS) was used to wet each filter. Volumes of 0.5, 1,
and 2ml of sample were filtered. The sides of each vacuum funnel were then rinsed with
the PBS solution (~3ml sprayed onto the sides in a circular motion) three times. Using
sterile forceps, each filter was removed and placed onto *Vibrio vulnificus* agar (VVA)
plates. The plates were incubated at 35°C overnight. Yellow colonies were transferred to
a new VVA plate and incubated. Individual yellow colonies on these plates were selected
and used to inoculate tryptic soy broth (TSB) (Becton, Dickinson and Company, Franklin
Lakes, New Jersey) supplemented with NaCl (Merck, Whitehouse Station, New Jersey)
to a final concentration of 2% NaCl; these tubes were incubated at 35°C. Newly collected
isolates were identified by numbers starting at 269.

Culture Maintenance

All cultures were maintained on Tryptic Soy Agar (TSA) (Becton, Dickinson and
Company) supplemented with NaCl to a final concentration of 2% NaCl. Slants were
prepared by adding 6ml of TSA to capped culture tubes (~15ml volume). After
solidifying, they were inoculated with a loop full of broth culture, incubated, and stored
at room temperature (~22°C) for up to one month. In addition, isolates were stored (up to
six isolates per TSA plate, depending on the growth characteristic of the isolate) at room
temperature. Each culture was transferred, by selecting an individual colony, every six to
eight weeks to ensure that viability was maintained. If abnormal growth or

contamination was observed, isolates were streaked for re-isolation on CHROMAgar

Vibrio (CHROMAgar^{TM}, Paris, France) (CAV)/ colistin cellobiose polymyxin B (CPC+)/

or thiosulfate citrate bile salts sucrose (TCBS) agar.

Culture Preservation

All isolates were preserved for long term storage by preparing broth cultures and

immediately cryogenically preserving them. Broth cultures were prepared by inoculating

5ml of TSB supplemented with NaCl to 2%. Cultures were placed in a rotary incubator at

35°C overnight. To each cryo vial, 0.5ml of a fresh overnight broth culture was added

together with 0.5ml of a 70% TSB/30% glycerol (Amresco, Solon, Ohio) solution. Tubes

were mixed briefly and then placed into a -80°C freezer.

Culture Activation from Cryogenically Frozen Stock

Isolates that could not be recovered from room temperature slants were grown

from cryogenically frozen stock cultures. Up to a maximum of ten isolates were removed

from the freezer at one time (to ensure cultures did not thaw). A sterile 100ul pipette tip

was used to scrape off a portion of frozen bacteria, which was then used to inoculate a

CAV or TCBS plate (NOAA isolates $\leq$268) or VVA or TCBS plate (isolates 269$\leq$) or

TSA plate (isolates that failed to grow on CAV, TCBS, or VVA), and incubated. Isolates

were checked for abnormalities in colony morphology and streaked for isolation onto

CAV, VVA, TCBS, or CPC+ plates. Each individual colony was then streaked onto TSA

for use as a working culture.

PHENOTYPIC ANALYSIS

Growth on Selective Media

The following media were prepared: VVA (BAM Media M190: *Vibrio vulnificus* Agar (VVA) 2013), CPC+ modified from Warner and Oliver (2007), CAV (CHROMagar^{TM}), and TCBS (Becton, Dickinson and Company). CPC+ was prepared according to Warner and Oliver (2007) except CPC base (Sigma-Aldrich) was used. The media was comprised of CPC base medium (Sigma-Aldrich) and 900ml distilled water (solution A). Solution B contained: 4g $MgCl_2$ $6H_2O$ (Amresco), 4g KCl (Merck), 0.0781g colistin methanosulfonate (MP Biomedicals, Santa Ana, California) ($1*10^6$ units), and 0.0667g polymyxin B (Enzo Life Sciences, Farmingdale, New York) ($4*10^5$ units) all dissolved, by gentle heating, in 100ml distilled water. Solution B was filter sterilized before being added to solution A at a ratio of 100ml solution B per 900ml of solution A. VVA (BAM Media M190: *Vibrio vulnificus* Agar (VVA) 2013) consisted of solution one and two. Solution one consisted of: 20g peptone (Becton, Dickinson and Company), 30g NaCl, 10ml dye stock (0.6g bromothymol blue (Harleco, Hartman-Leddon Company) and 100ml 70% ethanol), and agar 25g (Becton, Dickinson and Company). Solution two consisted of 10g of cellobiose (Alfa Aesar, Ward Hill, Massachusetts) dissolved in 100ml distilled water with gentle heat. Solution one was brought to a boil and 10M NaOH was added to bring the pH up to 8.2$\pm$ 0.2. After being autoclaved for 15min at 121°C, solution one was allowed to cool and solution two was filter sterilized and added to solution one and plated. CAV and TCBS (Becton, Dickinson and Company) were prepared according to the manufacturer's instructions. Isolates were streaked onto each type of medium and incubated at 35°C overnight. Growth and color of isolates and media were observed

visually and imaged with a Biomic V3™ system (Giles Scientific, Santa Ana, California). Some color changes were only visible when examined manually and were not present or were distorted when imaged. Incomplete color changes (some yellow or green) were subjectively determined to be more yellow or green. Control cultures of *V. vulnificus* (PCR confirmed isolate) and *V. parahaemolyticus* (ATCC 17802) were used to test all chromogenic media for colony color.

Biolog Microbial Identification System (Biolog MIS)

All isolates were analyzed using the Biolog MIS (Biolog, Hayward California) to confirm their identification by a phenotypic method. Procedures followed the manufacturer's instructions for Biolog GEN III Microplate™ (Biolog, Hayward California). Briefly, isolates were first grown on TSA plates overnight at 35°C and then were streaked on BUG+B agar and incubated at 33°C for 18-24h. Isolates that were taken from long term storage were streaked on TSA twice before analysis, as per manufacturer's instructions. Using Inoculatorz™ (Biolog, Hayward California), bacteria were suspended in inoculating fluid-B (IF-B) (presumptive *V. vulnificus*) or IF-A for control cultures and *V. parahaemolyticus* ATCC 17802) to a turbidity in the range of 0.90-0.98. The inoculating fluid was then transferred to a reservoir and, using an eight lane pipettor, 100ul of sample was pipetted into each well of a 96 well plate. Plates were incubated at 33-35°C for 20-26h and then read using the Biolog Microplate™ reader (Biolog, Hayward California). The lid of each 96 well plate was removed and the bottom cleaned with a Kim Wipe before being read. For isolates that were not identified to a species, based on the automatic reader, the plates were read manually. Isolates for which

there was no identification or were not identified as *V. vulnificus* were re-analyzed to confirm the results. *Staphylococcus epidermidis* ATCC 12228, *E. coli* ATCC 11775, *Paenibacillus polymyxa* ATCC 842, and *Stenotrophomonas maltophilia* ATCC 13637 were used as controls.

Antibiotic Resistance Analysis (ARA)

One hundred forty two previously collected isolates (Moore and Mott 2013) and one additional isolate, collected during this study, were confirmed to be *V. vulnificus* by PCR amplification of the *vvhA* gene (Panicker and Bej 2005) and then subjected to antibiotic resistance analysis. A comprehensive literature survey was conducted to determine the most common antibiotics used when screening *V. vulnificus* for antibiotic resistance. 'Instances of resistance' was calculated based on the number of studies that found at least one isolate that was resistant to the specified antibiotic. To determine how frequently antibiotic resistance to a particular drug occurred, 'Instances of use' was included; in which the number of studies including the drug were totaled. A twelve drug panel for testing the *V. vulnificus* isolates was constructed based on the findings of the literature search, including CLSI M45-A2 recommendations (CLSI 2010) (Table 2). Ten classes of antibiotics were included along with a beta-lactam and beta-lactamase inhibitor combination. The drugs selected have been used frequently in *V. vulnificus* antibiotic resistance analysis tests which allows cross study comparisons. Additionally, drugs used for the treatment of *Vibrio* infections were chosen.

Table 2. Antibiotic drug panel used for antibiotic resistance analysis.

Antibiotic	Abbreviation	Class
Ampicillin	AM	Penicillin
Piperacillin-tazobactam	TZP	Beta-lactam+beta-lactamase inhibitor
Gentamicin	GM	Aminoglycoside
Tetracycline	TE	Tetracycline
Cefepime	FEP	Cephalosporin 4th gen
Trimethoprim-sulfamethoxazole	SXT	Folate pathway inhibitor
Meropenem	MEM	Carbapenem
Imipenem	IPM	Carbapenem
Ceftriaxone	CRO	Cephalosporin 3rd gen
Chloramphenicol	C	Amphenicol
Amikacin	AN	Aminoglycoside
Ciprofloxacin	CIP	Fluoroquinolone

Antibiotic resistance analysis was conducted strictly according to the Clinical and Laboratory Standards Institute's guidelines in M45-A2 (2010). Briefly, for each isolate, TSA was inoculated and incubated overnight at 35°C, and then enough bacteria were suspended in 0.85% saline to reach an OD600 between 0.08-0.1. In a biosafety cabinet, a sterile cotton tipped applicator was dipped in the saline solution and used to inoculate a 150mm plate containing 60ml Mueller Hinton agar (MHA). Twelve commercial antibiotic disks (Becton, Dickinson, and Company) (Table 2) were applied to each plate using a disk dispenser. Plates were allowed to sit for 15min before being inverted and incubated for 16-18h at 35C. Control cultures of *E. coli* ATCC 25922 and 35218 were used for antibiotic disk quality control for zone diameter of typical antibiotics and ß-lactam+ ß-lactamase inhibitor combinations respectively. Plates were read with a

BIOMIC® V3 image analysis system and interpreted using CLSI standards for *V. vulnificus,* with the exception that ceftriaxone susceptibility was interpreted according to enteric bacteria standards (as there were no guidelines for zone diameter with *V. vulnificus*).

POLYMERASE CHAIN REACTION (PCR) BASED ANALYSES

Species Identification and PCR Verification

To confirm that all isolates were *V. vulnificus,* PCR of the *vvhA* gene was conducted. Amplicons were also sequenced for phylogenetic analysis. Amplification of the 16S gene was included for some isolates for sequencing and as a positive control for DNA extraction and PCR. Also, a multiplex PCR was designed to both identify an isolate as *V. vulnificus* and confirm that the reaction was not inhibited.

vvhA

PCR to amplify the *vvhA* gene was conducted for all isolates utilized in this study. *vvhA* was selected as it can be used to identify *V. vulnificus* because it is present in no other species of bacteria (Wright *et al.* 1985). Procedures followed Panicker and Bej (2005) except fluorescent probes were not used, number of cycles was reduced to 30, and $MgCl_2$ concentration was reduced to 15mM. A master mix consisting of 1x PCR buffer, 15mM $MgCl_2$, 200uM dNTPs, 1.5U Taq polymerase (Amresco and Fisher Scientific, Waltham, Massachusetts), PCR grade nuclease free water (Amresco and Geno Technology Inc., St Louis, Missouri), and 0.4uM of each primer (F-vvh785 (5' TTC CAA CTT CAA ACC GAA CTA TGAC 3') and R-vvh990 (5' ATT CCA GTC GAT

GCG AAT ACG TTG 3')) (Invitrogen) was prepared. The primers chosen were selected

due to specificity and previous usage (Panicker and Bej 2005). Template DNA was

extracted from 1ml of an overnight culture grown in TSB by using an UltraClean®

Microbial DNA Isolation Kit (MO BIO, Carisbad, California) and a Biospec Mini-

Beadbeater (Bio Spec Products Inc., Bartlesville, Oklahoma). To each PCR reaction tube

24ul of master mix was added followed by 1ul of template DNA. PCR with crude DNA

extraction was utilized for routine analysis. For DNA extraction, a colony was suspended

in de-ionized water, boiled for 10min, centrifuged at 16000 x g and supernatant was used

as the DNA template. PCR reactions with crude DNA extract utilized 22.5ul of master

mix and 2.5ul template. More template was used due to lower concentrations of DNA.

Cycling parameters were as follows: 94°C for 120s and 30 cycles of: 94°C for 15s, 56°C

for 15s, and 72°C for 25s followed by an infinite hold at 4°C.

Two percent agarose gels were prepared with 50ml of tris-borate EDTA (TBE)

(Amresco) and 1g molecular grade agarose (Fisher Scientific). Five microliters of product

was mixed with 2ul of 6x loading dye, placed in a well of a 2% agarose gel and run at

6.7V/cm until the leading dye band was at 75% of the length of the gel (~30min). A

100bp ladder (Thermo Fisher Scientific) was included along with a negative control. An

isolate that had previously been identified a *V. vulnificus* was included as a positive

control in each run and no template DNA served as the negative control. Gels were

stained in a 0.5% ethidium bromide solution for 5min, then de-stained in distilled water

for 1min before being imaged. A 205bp product indicated the presence of the *vvhA* gene

and confirmed the identity of the species as *V. vulnificus.*

16S PCR

If no PCR product was obtained from amplification of *vvhA,* the DNA was subjected to 16S PCR using BAC-8f (5'AGA GTT TGA TCC TGG CTC AG 3') and Univ-1492r (5' GGT TAC CTT GTT ACG ACT T 3') primers (Invitrogen) (Fierer and Jackson 2006). PCR conditions were as follows: 1x buffer with 15mM magnesium chloride, 0.2mM dNTPs, 0.5uM each primer, 1.25U taq polymerase (Amresco or Fisher Scientific), 1ul template DNA (2.5 for crude DNA extract), and sufficient PCR grade water to reach 25ul final volume. Cycling parameters were as follows: 95°C 300s followed by 35 cycles of 95°C 60s, 50°C 30s, 72°C 90s, and a final elongation of 72°C for 300s followed by an infinite hold at 4°C. Gels were prepared as before except a 1kb+ (Affymetrix, Santa Clara, California) ladder was included. A band at 1500bp indicated a successful reaction.

DNA Quantification

DNA was quantified for samples in which no *vvhA* product was obtained or had a band present on a gel that was not ~200bp. A NanoVue (General Electric Company, Schenectady, New York) spectrophotometer was used to quantify DNA in ng/ul based on its 260/280nm ratio. Approximately 1ul of DNA extract was placed in the NanoVue and measured, the sample was then wiped away and a new 1ul of sample was measured. If the measured values differed by more than 60ng/ul the procedure was repeated a third time. A threshold difference of 60ng/ul was used as most repeated measurements were within this range. Values were averaged to give a DNA concentration. DNA was diluted in sterile PCR water to 10-60ng/ul for use in *vvhA* and 16S PCR.

Multiplex PCR

A multiplex PCR was created to quickly and simultaneously determine if an isolate was *V. vulnificus*, by amplification of the *vvhA* gene, and to confirm that the reaction was not inhibited by amplification of the 16S gene. Primers were screened for compatibility using AutoDimer (Vallone and Butler 2004) (http://www.basic.northwestern.edu/biotools/oligocalc.html) and no primer dimers were reported (Supplementary Figure 17). Next, the reaction was optimized and tested.

DNA extracted from *V. vulnificus* isolates as well as *V. parahaemolyticus* and *E. coli* was adjusted to 50-75ng/ul using sterile PCR grade water (Geno Technology Inc.) and used for all multiplex PCR reactions unless stated otherwise. Initial PCR conditions were as follows for 35ul reactions: 1x buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.5uM of primer BAC-8f, 0.5uM of primer UNIV-1492r, 0.4uM of primer vvh785f, 0.4uM of primer vvh990r, 1.25U taq, 1.0ul template DNA, and PCR grade water to bring up to volume. Cycling conditions were set to: 94°C for 300s, followed by 30 cycles of : 94°C for 60s, 55°C for 30s, and 72°C for 90s with a final extension of 300s at 72°C and an infinite hold at 4°C. Presence of 1500 and 205bp bands indicated a successful reaction and confirmed the species as *V. vulnificus*.

To get more equal amounts of both the 16S and *vvhA* amplicons, the *vvhA* primer concentration was reduced from 0.4uM to 0.2uM. $MgCl_2$ concentration was optimized to increase yields. PCR reactions using the optimized primer concentration had $MgCl_2$ concentrations increased from 1.5 to 2.5, 3.5, and 4.5mM.

To simulate a real world application of the proposed multiplex reaction, a crude DNA extraction of overnight presumptive *V. vulnificus* isolates was conducted and

multiplex PCR was performed. The final multiplex PCR conditions used are as follows: 1x buffer, 2.5mM $MgCl_2$, 0.2mM dNTPs, 0.5uM of primer BAC-8f, 0.5uM of primer UNIV-1492r, 0.2uM of primer vvh785f, 0.2uM of primer vvh990r, 1.25U taq, 1.0ul template DNA, and PCR grade water to bring up to volume. Cycling conditions were set to: 94°C for 300s, followed by 30 cycles of: 94°C for 60s, 55°C for 30s, and 72°C for 90s with a final extension of 300s at 72°C and an infinite hold at 4°C.

Buffer Optimization

While conducting gel electrophoresis under normal conditions, band resolution of the *vvhA* PCR product was not adequate and an exact size was difficult to resolve due to poor DNA ladder separation at low molecular weights. In an attempt to improve band resolution, increase band separation, and reduce gel running times a comparison of three common gel electrophoresis buffers was performed. Additionally, the multiplex PCR protocol was optimized while conducting the buffer comparison to compare the effects of each buffer on high (1500bp) and low (205bp) molecular weight PCR products.

Three buffers were prepared. For Tris acetate EDTA (TAE) one liter of 1x TAE was prepared with 980ml de ionized water (DI) combined with 20ml of a 50x stock solution (Amresco) and mixed thoroughly. Tris borate EDTA (TBE) (1x) was prepared by combining 900ml DI with 100ml of 10x TBE stock solution (Amresco) which was then mixed thoroughly. One liter of 20x Sodium borate (SB) (Brody and Kern 2004) was prepared by combining 200ml 1N NaOH and 600ml DI with enough boric acid (Fisher Scientific) until the pH reached 8.3$\pm$ 0.2 (note: 40.7g stabilized at a of pH 8.196). Once

the pH was stable, DI was added to reach a final volume of 500ml. A working solution of 1x SB was used during gel electrophoresis.

To make 1.5% agarose gels, 40ml of buffer and 0.6g genetic analysis grade agarose (Thermo Fisher Scientific) were mixed, and heated in a microwave until all agar dissolved. DI water was added to bring the volume up to 40ml as needed. Agarose was allowed to cool slightly before pouring into a gelbox. Gels were covered with ~2mm of buffer (570ml). Five microliters of PCR product was combined with 1ul of 6x loading dye (Affymetrix®) and placed into a well. Next, 5ul of 1kbp+ (Affymetrix®) and 100bp (Thermo Fisher Scientific) DNA ladders were added to their respective wells. All gel running times were dependent on voltage (PowerPac™ Basic) and were run at 5V/cm for 40min, 6.67V/cm for 30min, or at 10V/cm for 23min. Gels were stained in a 0.1% ethidium bromide solution for 5min then de-stained in DI water for 1min before being imaged.

Sequencing and Sequence Processing

All 143 isolates were subjected to *vvhA* sequencing for phylogenetic analysis and for several isolates, 16S rDNA was sequenced to confirm their identification. Ten microliters of PCR product was placed into a SimpleSeq™ (Eurofins Genomics, Huntsville, Alabama) tube and sent to Eurofins Genomics for standard sequencing using either BAC-8f or F-vvh785 primers.

Sequence sample names in the data files were altered to include additional information and present it in a visually appealing and useful manner on a phylogenetic tree. Each sample was renamed as follows: isolate# _C/E (clinical or environmental

isolate)_ A/B/AB (16S type)_origin W/H/O/S (W=water, H=human, O=oyster, and S=sediment). For example isolate number 10 was of environmental origin and was type A so it was renamed: 10 E A W. After additional information was received from Dr. Moore isolates were renamed again to include the additional information as follows: isolate#_C/E_origin_16S type_*vcg* type (C/E)_*pilF* polymorphism (+/-)_mannitol fermentation (+/-). For example isolate 10 was an environmental isolate from water with a 16S type of A, *vcg*E, and was negative for the *pilF* polymorphism and mannitol fermentation so it was renamed: 10_E_W_A_E - -.

Sequences were trimmed using the program DNASTAR® SeqMan™ Pro (DNASTAR Inc., Madison, Wisconsin). The 5' ends were trimmed to remove as many N's as possible and the 3' end was set to 180. If N's were present at the 3' end they were removed. However, if a TGGAAT was present the sequence was ended after the final "T" or within the TGGAAT sequence if it was not complete. The TGGAAT sequence was used as a cutoff point because it was at the 3' end, easily identifiable, and present in virtually all sequences.

The online sequence alignment tool Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used to generate sequence alignments for PHYLIP. ClustalW2 and MUSCLE were used for maximum likelihood and neighbor joining analysis with MEGA.

PHYLOGENETIC ANALYSIS

Isolates were subjected to phylogenetic analysis of a 205bp (useable sequence length ≥140bp) region of the *vvhA* gene that was obtained from PCR when the isolates

were identified as *V. vulnificus*. Two reference *vvhA* sequences from strains fj03-x2

(GenBank accession number KC821520) and VvMBC105 (GenBank accession number

KF255393) were included in all analyses. Both the neighbor joining (Dnadist and

Neighbor) and maximum likelihood (Dnamlk) tools in PHYLIP 3.695

(http://evolution.genetics.washington.edu/phylip/getme.html) were used (Felsenstein

1989). MEGA V6.0 was also utilized for aligning sequences and constructing

phylogenetic trees. Data were bootstrapped and used to construct phylogenetic trees.

Dnamlk utilize the maximum likelihood approach and includes the molecular clock

hypothesis. MEGA V6.0 was used as it can perform analyses quickly and is user friendly.

Neighbor joining was included as it is a fast method to construct generally accurate

phylogenies and is a common technique (Saitou and Nei 1987; Tateno *et al.*1994). Using

the aforementioned tests, profiles of each isolate were made and used to differentiate

isolates based on their genotype.

Analysis with PHYLIP (V 3.695)

Phylogenetic analysis was performed using the maximum likelihood program

Dnamlk which utilizes the molecular clock hypothesis (Felsenstein 2013). Dnadist and

Neighbor were used to construct a distance matrix and neighbor joining tree respectively.

When prompted to input seed or jumble numbers, a number was chosen randomly.

Bootstrapping of data was conducted with 100 replicates (see Appendix for detailed step

by step methods).

Analysis with MEGA 6.0 (Tamura *et al.* 2013)

The program MEGA v6.0 was used to align sequences and construct phylogenetic trees using the maximum likelihood and neighbor joining methods. Parameters for analysis were determined by using the Find Best DNA/Protein models for maximum likelihood. Data were bootstrapped 1000 times for maximum likelihood and 2000 times for neighbor joining (see Appendix for specific instructions).

RESULTS

All analyses were conducted on the 142 previously confirmed *V. vulnificus* isolates from a previous study (Moore and Mott 2013) and one isolate collected during this study for a total of 143 confirmed *V. vulnificus* isolates.

PHENOTYPIC IDENTIFICATION

Media Comparison

One hundred and forty two confirmed *V. vulnificus* isolates were streaked onto each of four selective and differential media typically used to grow *V. vulnificus*. Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) (Difco), CHROMagar™ Vibrio (CAV) (CHROMagar), *Vibrio vulnificus* Agar (VVA) (BAM Media M190: *Vibrio vulnificus* Agar (VVA) 2013), and Colistin Polymyxin B Cellobiose Agar (CPC+) (Warner and Oliver 2007) were utilized in the growth and color comparison. Color on some plates could not be viewed properly (distorted coloration or faint color changes) with the imager and were viewed manually. Faint color changes were determined as best

as possible by comparing to controls. Three distinct color patterns were present. The first and most numerous was the typical yellow (Y), green (G), Y, turquoise (T) profile on VVA, TCBS, CPC+, and CAV respectively (Figure 1 and Table 3). A second profile consisted of Y, Y, Y, T (Table 4) and a third profile was Y, G, Y, W (Table 5) on VVA, TCBS, CPC+, and CAV respectively. Three isolates did not match any of these profiles or each other (Table 6).

Table 3. Typical, and most commonly observed, media profile (colony colors) of *V. vulnificus.* Abbreviations are as follows: Y, yellow; G, green; T, turquoise.

# of isolates	Origin	Medium			
		VVA	TCBS	CPC+	CAV
65	Water	Y	G	Y	T
53	Clinical	Y	G	Y	T
8	Sediment	Y	G	Y	T
4	Oyster	Y	G	Y	T

VVA   TCBS

http://farm9.static.flickr.com/8440/7748443902_97c17e3f4f.jpg   http://cit.vfu.cz/alimentarni-onemocneni/xvp/xvp04s.jpg

CPC+   CAV

Handbook of Culture Media for Food and Water Microbiology
http://books.google.com/books?id=iQIuAgAAQBAJ&pg=PA392&lpg=PA392
&dq=cpc+agar&source=bl&ots=oATZAFswNE&sig=kmeKYUYb7NaZESWC
CXZCppWt1iM&hl=en&sa=X&ei=yvgqU_HnJYfg0gGP6oAQ&ved=0CEcQ6A
EwBQ#v=onepage&q=cpc%20agar&f=false   http://docsdrive.com/images/academicjournals/jm/2013/fig1-2k13-1-12.jpg

Figure 1. Typical appearance of *V. vulnificus* on *Vibrio vulnificus* agar (top left), thiosulfate citrate bile salts sucrose agar (top right), colistin polymyxin B cellobiose agar (bottom left), and CHROMagar™ Vibrio (bottom right).

Table 4. Second most common media color profile of *V. vulnificus* observed. Abbreviations are as follows: Y, yellow; T, turquoise.

Isolate	Origin	Medium			
		VVA	TCBS	CPC+	CAV
95	Blood	Y	Y	Y	T
162	Water	Y	Y	Y	T
217	Blood	Y	Y	Y	T
220	Blood	Y	Y	Y	T
221	Blood	Y	Y	Y	T
252	Blood	Y	Y	Y	T

Table 5. Third most common media color profile of *V. vulnificus* observed. Abbreviations are as follows: Y, yellow; G, green; T, turquoise; W, white.

Isolate	Origin	Medium			
		VVA	TCBS	CPC+	CAV
24	Water	Y	G	Y	W
104	Oyster	Y	G	Y	W
185	Water	Y	G	Y	W

Table 6. Other combinations of colony colors of *V. vulnificus* isolates observed. Abbreviations are as follows: Y, yellow; G, green; T, turquoise; W, white; NG, no growth.

Isolate	Origin	Medium			
		VVA	TCBS	CPC+	CAV
68	Sediment	Y	Y	Y	W
75	Sediment	Y	G	NG	T
101	Oyster	Y	Y	G	T

Next, the ability of each medium to presumptively identify *V. vulnificus* was compared. All isolates grew with the characteristic *V. vulnificus* colors on VVA and TCBS. CPC+ failed to presumptively identify two isolates and CAV was unable to properly identify four isolates (Table 7) i.e. incorrect color for *V. vulnificus* or failure to grow. No association between isolate source (clinical and environmental) and media profile was apparent except for yellow on TCBS. Five out of six isolates that had the Y, Y, Y, T media profile were of clinical origin (Table 8).

Table 7. The ability of each medium to presumptively identify known *V. vulnificus* was assessed and relative effectiveness of each type of media was determined. Abbreviations are as follows: Y, yellow; G, green; T, turquoise; W, white; NG, no growth; ID, identification.

		Medium			# of isolates with profile	% with profile
	VVA	TCBS	CPC+	CAV		
	Y	G	Y	T	130	91.5
	Y	Y	Y	T	6	4.2
	Y	G	Y	W	3	2.1
	Y	Y	Y	W	1	0.7
	Y	G	NG	T	1	0.7
	Y	Y	G	T	1	0.7
% correct ID	100	100	98.6	97.2		
Total # isolates					142	

Table 8. The percentage of clinical and environmental isolates within each medium color profile. Abbreviations are as follows: Y, yellow; G, green; T, turquoise; W, white; NG, no growth.

		Medium			# of isolates	% clinical	% environmental
	VVA	TCBS	CPC+	CAV			
	Y	G	Y	T	130	40.8	59.2
	Y	Y	Y	T	6	83.3	16.7
	Y	G	Y	W	3	0.0	100.0
	Y	Y	Y	W	1	0.0	100.0
	Y	G	NG	T	1	0.0	100.0
	Y	Y	G	T	1	0.0	100.0
Total # isolates					142		

Table 9. Presumptive identification of unconfirmed *V. vulnificus* isolates (identified as *V. vulnificus* by VVA) by each medium. Key: Y, yellow; G, green; T, turquoise; P, purple/mauve; W, white; WY, weakly/pale yellow; V, variable color; ID, identification.

	Medium				# of isolates with profile	% of isolates with profile
	VVA	TCBS	CPC+	CAV		
	Y	G	Y	T	6	7.3
	Y	Y	G	T	2	2.4
	Y	G	G	T	1	1.2
	Y	Y	Y	W	42	51.2
	Y	Y	Y	P	6	7.3
	Y	Y/G	V	V	16	19.5
	B/WY	Y/G	V	V	9	11.0
% correct ID	89.0	100	87.8	11.0		
Total # isolates					82	

Biolog Microbial Identification System (Biolog MIS)

The 142 isolates previously identified as *V. vulnificus* and one new isolate collected in this study were analyzed using the Biolog MIS, Generation III Microplates™. Biolog MIS was able to correctly identify 137/142 (96%) of the confirmed *V. vulnificus* isolates at a confidence level greater than 0.5. Three isolates (Isolate #10, 71, and 163: all of environmental source) returned a "no identification" and two were misidentified as the closely related species *Vibrio mimicus* (Isolate #96: clinical

source) and *Photobacterium damselae* (Isolate #28: environmental source). The newly collected Virginia environmental isolate was identified as *V. vulnificus*.

Antibiotic Resistance Analysis

A comprehensive survey of antibiotic resistance in *V. vulnificus* was conducted and it was found that drug panel selection in previous studies was highly variable (Table 10). However, there were several antibiotics common to many studies, many of which are included in the CLSI M45-A2 recommendations for *V. vulnificus.* In this study the *V. vulnificus* isolates exhibited little resistance to the 12 tested antibiotics (Appendix Tables A1-A4). None of the oyster, sediment, or water isolates were completely resistant to any of the tested antibiotics (Table 11, Appendix Tables A2, A3, and A4 respectively). A total of four isolates from humans, sediment, and water showed intermediate resistance to at least one antibiotic (Appendix Tables A1, A3, and A4 respectively). Interestingly, only two isolates, isolated from human blood, were fully resistant to one antibiotic: cefepime or ampicillin (Table 11, Appendix Table A1).

Table 10. Results of literature survey of antibiotic resistance studies conducted on *V. vulnificus*. A- color legend for antimicrobial classes, B- antimicrobial resistance results. A "1" indicates at least one isolate was resistant to the tested antibiotic while "0" indicates no isolates were resistant. "Instances of resistance" are the total number of studies that had an isolate that was resistant to the tested antibiotic; "instances of use" are the total number of studies that included the antibiotic regardless of the isolate's susceptibility. Total n value represents the number of isolates included in the study.

A

Color or group	Antibiotic class
1	Penicillins
2	Aminoglycoside
3	Tetracycline
4	Quinolone
5	Dihydrofolate reductase inhibitor
6	Sulfonamide
7	MurA inhibitor
8	Carbapenem
9	Cephalosporin 1st gen
10	Cephalosporin 2nd gen
11	Cephalosporin 3rd gen
12	Cephalosporin 4th gen
13	Cephem
14	Amphenicol
15	Fluoroquinolone
16	Polymyxin
17	Macrolide
18	Nitrofuran
19	Other
20	Rifampin
21	Lincosamides

	Antibiotic	Hollis et al. 1976	Oliver et al. 1982	Oliver et al. 1983	Tison and Kelly 1986	Biosca et al. 1996	Radu et al. 1998	Dalsgaard et al. 1999	Li et al.1999	Ottaviani et al. 2001	Hsueh et al. 2004	Wang et al.2006	Han et al. 2007	Zaldenstein et al. 2008	Roig et al. 2009	Baker-Austin et al. 2009	Horseman and Surani 2010	Kitiyodom et al. 2010	Mahmud et al. 2010	Matsumoto et al. 2010	Okoh and Igbinosa 2010	Banerjee et al. 2011	Kim et al. 2011	Diaz and Mott 2012	Elhadi 2012	Instances of Resistance	Instances of Use
1	ampicillin	0			0	0	1	0	1	1	0	1	0	0		1	0	1		0	1	1	1	0	1	10	20
1	penicillin	0	1	0	0	1	1	0		1		1				1				1						7	11
1	carbenicillin	0			0	0	1			1	0	1														3	7
1	piperacillin tazobactam																	0		0			1	0		1	4
1	aztreonam									0								0		0				0		0	4
1	amoxicillin/clavulanic acid (augmentin)																						1	0	1	2	3
1	ampicillin sulbactam														1		0						1			2	3
1	amoxicillin							1											1							2	2
1	piperacillin																			0			1			1	2
1	methicillin												1													1	1
1	ticarcillin																								1	1	1
2	gentamicin	0			0	0	1	0	1		0	0	0			1	0		1	0	1	1	0		1	7	17
2	amikacin				0	0			1		0					1				1	1		1	0	1	6	10
2	streptomycin				1	1	0	0	1		0			0		1		0			1					5	10
2	kanamycin	1			0	0	1		1	1				0					0							4	8
2	neomycin							0		1										0						1	3
2	tobramycin					1														0						1	2
2	apramycin																	1								1	1

2	netilmicin					1															1	1
																							1
3	tetracycline	0	0	1	1	0	1	0	0	0	0	0	1	1	1	1	0	1	0	0	8	9
3	oxytetracycline					1				1	1										3	3
3	doxycycline					0									0						0	2
3	minocycline						0						0								0	2
4	nalidixic acid		1	1		0	0		0		0	1	1			1					5	9
4	oxolinic acid		1		0		0				1					0					2	5
5	trimethoprim sulfamethoxazole (cotrimoxazole)	0		0		0		0		0	1	1	0	0	1	0	1	0	0		4	4 (1)
5	trimethoprim		1		1	0		0			1		1		1						5	7
6	sulfamethoxazole			0	1		1					1		1							4	5
6	sulfadimethoxine		1																		1	1
6	sulphatriad					1															1	1
6	sulfathiazole									1											1	1
6	sulphamethiazole					1															1	1
6	sulfanilamide		1																		1	1
6	sulphadiazine			0																	0	1
6	sulfisoxazole	0																			0	1
7	fosfomycin		1																		1	1
8	imipenem					0	0		0		0	0		0	0		0	0			0	9
8	meropenem					0					1			0	0		1	0			2	6
8	ertapenem																0				0	1
9	cephalothin	0	0		0		1	0	0		0	1		1	1		1				5	11
9	cefazolin										1	0		0			1	1			3	5
9	cephalexin																				0	0
10	cefuroxime					1								1		1					3	3
10	cefoxitin										1					1					2	2
10	cefaclor																	1	1	1		

	cefamandole	0			0	1
1 0	cefotiam		0		0	1
1 0	cefmetazole		0		0	1
1 0	cefotaxime	0 0 0 1	0 1 0	2	7
1 1	ceftazidime	1 1 0	0 1		3	5
1 1	ceftriaxone	0 0 1	0 0		1	5
1 1	latamoxef (moxalactam)	1	0		1	2
1 1	cefoperazone	0	0		0	2
1 1	cefditoren		0		0	1
1 1	cefpodoxime		0		0	1
1 1	ceftiofur	0			0	1
1 2	cefepime		0 1 1		2	3
1 4	chloramphenicol	0 0 0 1 0 1 0 0 0 0 0 1 0 1 0 1		5	6	1
1 4	flumequine	0 1		1	2
1 4	florfenicol				0	0	1
1 5	ciprofloxacin	0 0 0 0 0 0 0 0 1 0 0 1 1 0		3	4
1 5	levofloxacin	0	0 1 0		1	4
1 5	enrofloxacin	0	1 1		2	3
1 5	novobiocin	0 1 1			2	3
1 1	ofloxacin	0 1	1		2	3

515	norfloxacin																			0	0			0	0	3
515	moxifloxacin								0			1												1	2
515	gatifloxacin								0						0									0	2
515	lomefloxacin								0															0	1
515	sparfloxicin								0															0	1
515	pefloxacine (pefloxacin)																							0	0
516	colistin	1	1	1	1			1		0														5	6
516	polymyxin b				1		1	1									1							4	4
517	erythromycin	1		0	0	1	0			1		1	0		1		1	0						6	1
517	azithromycin											1												1	1
518	nitrofurantoin	0		1			0		0		1	1					0		0					3	8
518	furazolidone												0											0	1
519	fusidic acid								1															1	1
519	pteridine		0																					0	1
520	rifampicin/rifampin	0		0		0	1																	1	4
521	clindamycin	1						1	1															3	3
521	lincomycin					1																		1	1
	Total number of antibiotic resistance found	4	2	1	1	13	0	1	0	4	3	1	0	0	10	23	0	7	2	1	16	1	22	2	6

Total n value	38	9	3 3	3 0	8 9	3 6	3 2	1 2	7 1	84	1	15 1	25	13 0	16 1	1	83	33	21	18	19	31	13 7	10

Table 11. Isolates, out of all 143 tested, that displayed any resistance to the tested antibiotics. Abbreviations are as follows: TZP-piperacillin-tazobactam, GM-gentamicin, TE-tetracycline, MEM-meropenem, IPM-imipenem, SXT-trimethoprim-sulfamethoxazole, FEP-cefepime, CRO-ceftriaxone, AM-amoxicillin, C-chloramphenicol, AN-amikacin, CIP-ciprofloxacin.

| | | Antibiotic | | | | | | | | | | | |
Specimen #	Source	TZP	GM	TE	MEM	IPM	SXT	FEP	CRO	AM	C	AN	CIP
137	Blood	S	S	S	S	S	S	S	S	S	S	I	S
237	Blood	S	S	S	S	S	S	S	S	R	S	S	S
248	Blood	S	S	S	S	S	S	R	I	S	S	S	S
170	Sediment	S	S	S	S	S	S	S	S	I	S	S	S
13	Water	S	S	S	S	S	S	S	S	S	S	S	I

DNA BASED ANALYSIS

Multiplex PCR of *vvhA* and 16S

A multiplex PCR protocol and comparison of three gel electrophoresis buffers was conducted on confirmed *V. vulnificus* isolates. The purpose of the multiplex PCR was to amplify both the bacterial 16S gene and the *vvhA* gene, the latter being unique to *V. vulnificus*. This multiplex reaction was designed to accomplish several things: verify that DNA was present in the sample, indicate that PCR reactions were not inhibited, identify *V. vulnificus*, and allow for DNA bands in the gel to be removed for sequencing if desired. Additionally, the PCR reaction conditions were optimized as were gel running conditions.

Tris acetate EDTA (TAE), tris borate EDTA (TBE), and sodium borate (SB) (Brody and Kern 2004) gel electrophoresis running buffers were compared based on their ability to separate high (16S 1500bp) and low (*vvhA* 205bp) molecular weight fragments produced by the proposed multiplex PCR. PCR product was formed for

both 16S and *vvhA* under initial conditions (Figure 2). However, *vvhA* appeared to be

preferentially amplified as the 205bp band appeared brighter.
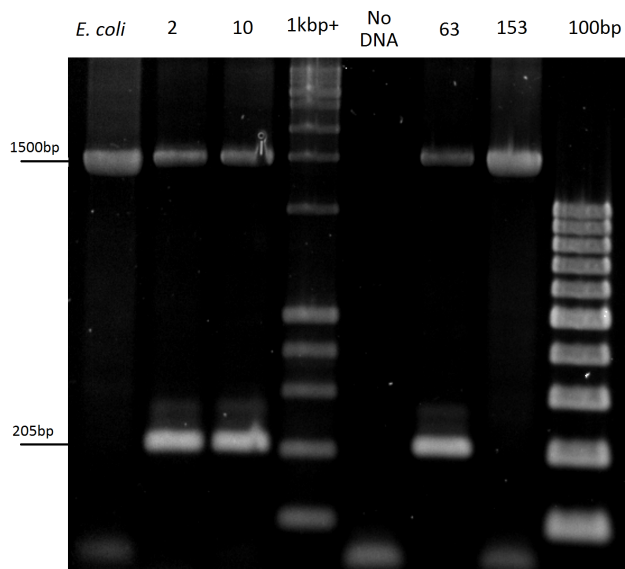
Figure 2. Initial multiplex PCR conditions. Agarose gel electrophoresis was
conducted for 40min at 6.7V/cm on a 1.5% TBE gel. Confirmed *V. vulnificus* isolates
2, 10, and 63 were used as both 16S and *vvhA* positive controls. *E. coli* and an
unknown presumptively identified *V. vulnificus* isolate (153) were used as positive
controls for 16S gene amplification and a negative control for *vvhA* amplification.

A buffer comparison using PCR product from the initial multiplex PCR

conditions was performed and all samples were run at 6.7V/cm for 30min (Figure 3

A, B, and C). Both TAE and TBE were able to separate out the high molecular weight

(16S) product with good resolution. SB appeared to cause smearing of the 16S

product, this effect was repeatable (data not shown). Both TBE and SB, to a greater

extent, were able to separate low molecular weight products with high resolution.

Greatest band separation can be seen in SB at low molecular weights (Figure 3 C).

Reduction of *vvhA* primer concentration resulted in more even amplification

relative to 16S amplification as determined by brightness (Figure 4 A). Additionally,

buffer comparison was performed at a high voltage (10V/cm) for rapid runs. A

similar trend was observed in that 16S resolution was highest in TAE and TBE; SB

and TBE had greater *vvhA* separation, and high molecular weight smearing in SB was

present (Figure 4 A, B, and C).

As MgCl$_2$ concentration increased so did band intensity. However, at 3.5mM

MgCl$_2$ and above nonspecific amplification was present (Figure 5). The pattern for

band resolution and separation remained unchanged at 5V/cm. A faint band in

negative controls lanes was noted and determined to be from sample migrating into

the empty well during gel preparation (data not shown)

Application of Multiplex PCR

Eight of the new presumptive *V. vulnificus* isolates (isolated from Virginia)

were subjected to multiplex PCR to determine its effectiveness. The positive control

(*V. vulnificus* isolate number 2) and one of the new environmental isolates tested were

confirmed to be *V. vulnificus* as shown by the band at 205bp. All isolates had

amplification of the 16S gene (band at 1500bp) (Figure 6). Species confirmation of

isolates by *vvhA* PCR, according to Panicker and Bej (2005), had been conducted

previously and only isolates 2 and 320 were confirmed as *V. vulnificus* (data not

shown).
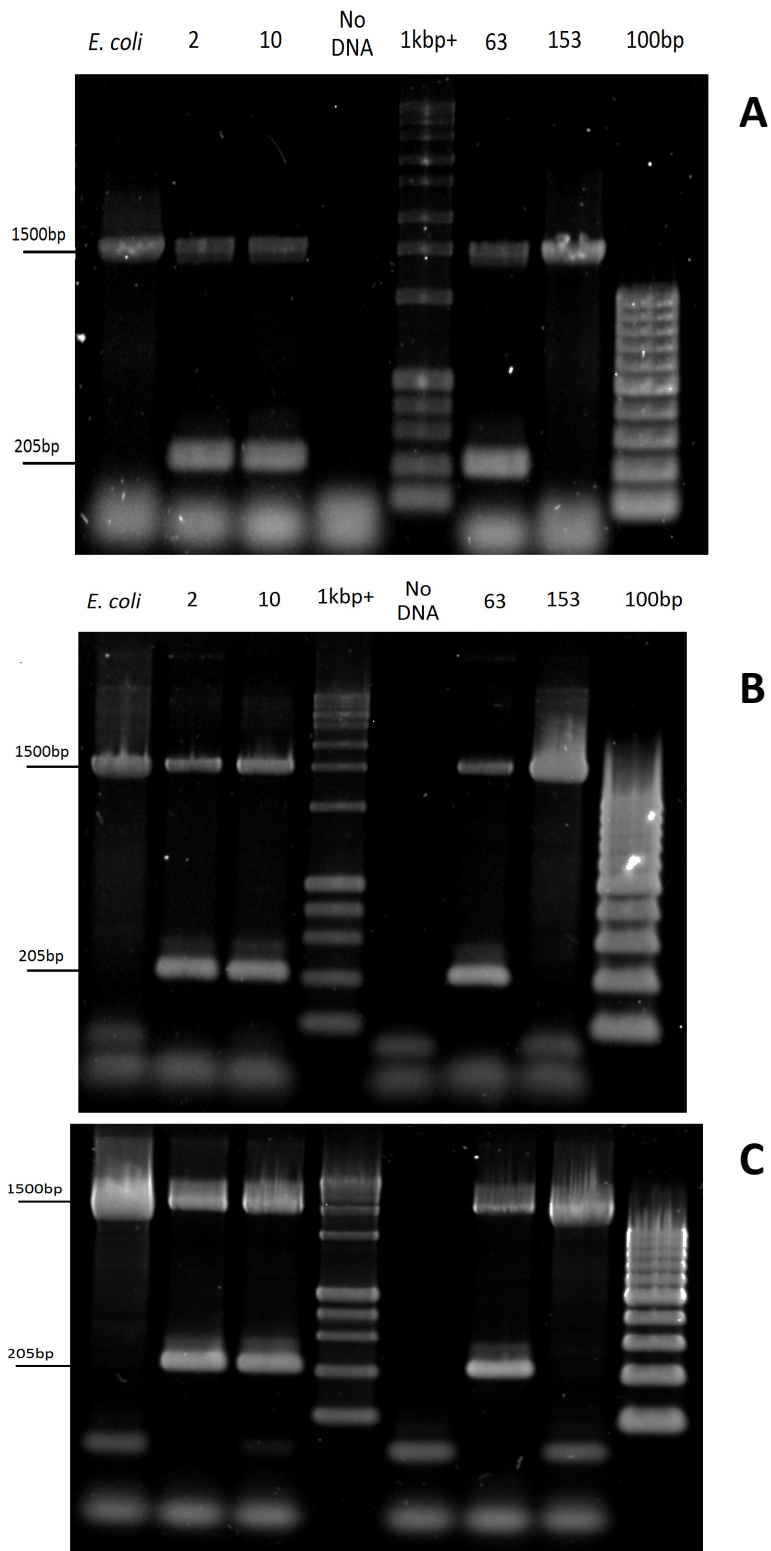
Figure 3. Buffer comparison of TAE (A), TBE (B), and SB (C) at 6.7V/cm for 30min in 1.5% agarose gels. . Confirmed *V. vulnificus* isolates 2, 10, and 63 were used as both 16S and *vvhA* positive controls. *E. coli* and an unknown presumptively identified

V. vulnificus isolate (153) were used as positive controls for 16S gene amplification and a negative control for *vvhA* amplification.
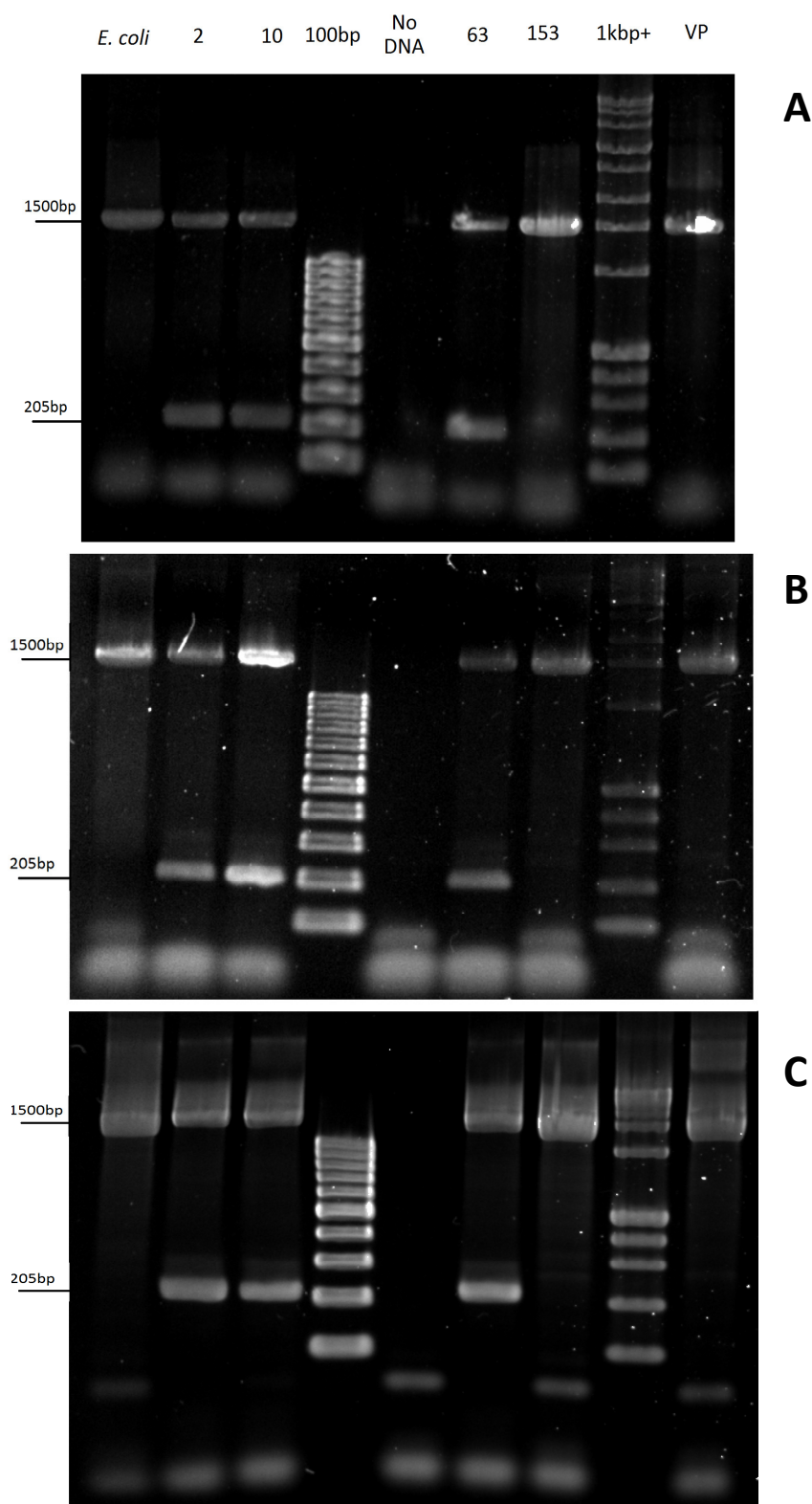
Figure 4. Buffer comparison of TAE (A), TBE (B), and SB (C) at 10V/cm for 23min in 1.5% agarose gels. *vvhA* primer concentration was reduced to 0.2uM from 0.4uM. .

Confirmed *V. vulnificus* isolates 2, 10, and 63 were used as both 16S and *vvhA* positive controls. *E. coli,* an unknown presumptively identified *V. vulnificus* isolate (153), and *V. parahaemolyticus* (VP) were used as positive controls for 16S gene amplification and a negative control for *vvhA* amplification.
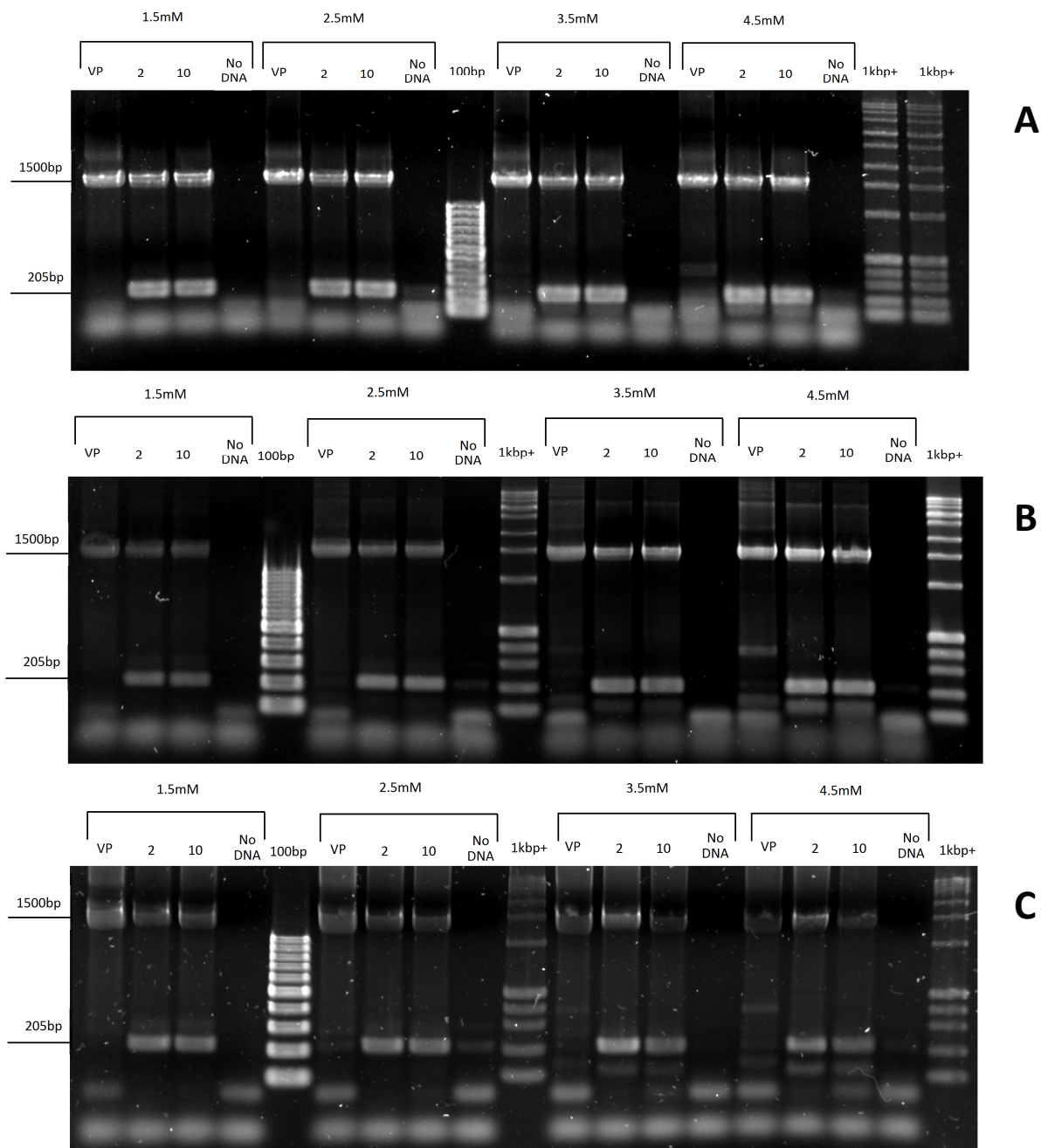
Figure 5. MgCl$_2$ optimization with 1.5mM used as the control and increased to 2.5, 3.5, and 4.5mM. Agarose gel electrophoresis was conducted at 5V/cm for 40min with 1.5% agarose gels in TAE (A), TBE (B), and SB (C). Confirmed *V. vulnificus* isolates

2 and 10were used as both 16S and *vvhA* positive controls. *V. parahaemolyticus* (VP) was used as a positive control for the 16S gene amplification and a negative control for *vvhA* amplification.
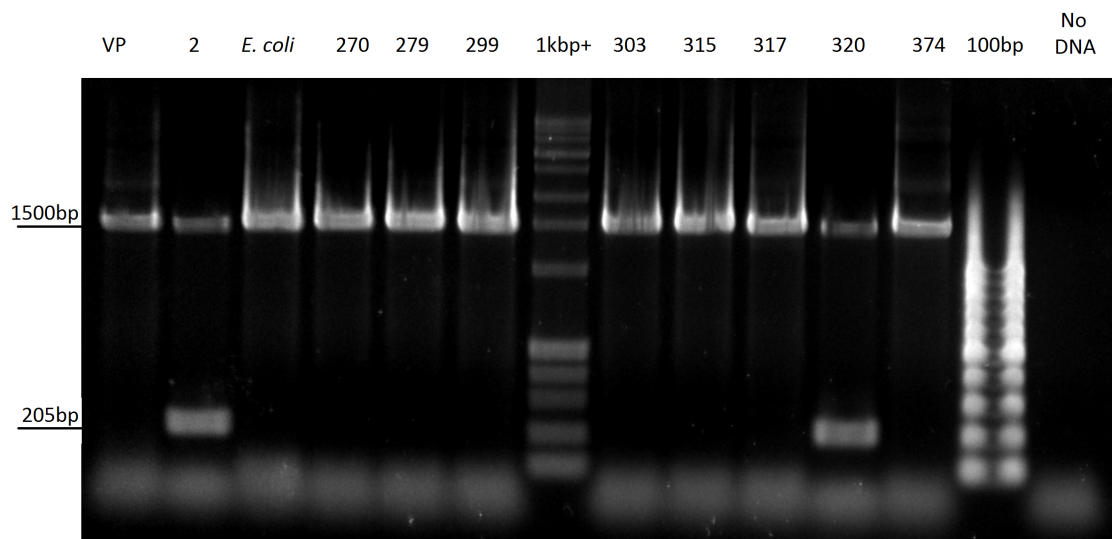
Figure 6. Application of multiplex PCR to crude DNA extract. Agarose gel electrophoresis was conducted at 6.7V/cm for 30min in TBE. *V. parahaemolyticus* and *E. coli* were used as positive controls for 16S gene amplification and negative controls for *vvhA* amplification. Confirmed *V. vulnificus* isolate number 2 was used as a positive control for both 16S and *vvhA* amplification. The remaining isolates 270, 279, 299, 303, 315, 317, 320, and 374 were presumptively identified as *V. vulnificus* by CHROMAgar Vibrio.

Phylogenetic Analysis of *vvhA*

Phylogenetic analysis of the hemolysin A genes (*vvhA*) (~160bp with gaps and ~90bp without gaps) from 143 *V. vulnificus* isolates was conducted using the maximum likelihood and neighbor joining strategies. *vvhA* was used for analyses as it is unique to *V. vulnificus* (Wright *et al.* 1985), used to identify *V. vulnificus* (Panicker and Bej 2005), and PCR product was available. Maximum likelihood analysis was chosen as it produces the most accurate phylogenetic trees. Neighbor joining was also

used as it produces generally reliable trees much faster than maximum likelihood. To
further test the reliability of each tree generated, bootstrapping of the dataset was
employed. Analysis was conducted with Phylip V3.695 (Appendix Figures A1-A7)
and MEGA V6.0 and portions of the *vvhA* gene from two isolates (VvMBC105and
fj03-x2) from the NCBI database were included as references. A bootstrap percent of
$\geq$70% was used to indicate a significant relationship (Hillis and Bull 1993).

Virulence data were provided by Dr. Moore and analysis was performed again
using only MEGA V6.0. The effects of including or deleting all gaps in the
sequences, when using maximum likelihood, or including all gaps or pairwise
deletion, for neighbor joining, were compared. Keeping all gap sites created multiple
subgroups with the maximum likelihood method (Figure 7 and sp9). Fewer branched
groups were present when gaps were deleted (Figure 8 and sp10). However, several
isolates and subgroups overlapped. The same trend was also present when alignments
were constructed using MUSCLE when including all gaps (Figure 9 and sp11) and
deleting all sites with gaps (Figure 10 and sp12). When all gaps were included in the
analysis, all but one of the clinical isolates that associated with the environmental
isolates, were grouped into two clusters: I and II (Figure 9 B and C) However, more
distinct sub groups were present in the MUSCLE alignment compared to ClustalW2
(Figures 7 and 9). Both maximum likelihood and neighbor joining trees produced by
MEGA had two distinct branches and correlated with 16S type A and *vcg* type E
(environmental) or 16S type B and *vcg* type C (clinical) no matter how the
phylogenetic trees were constructed (typically a frequency of 99%). Virtually all
other sub branches had non-significant bootstrap frequencies (data not shown).
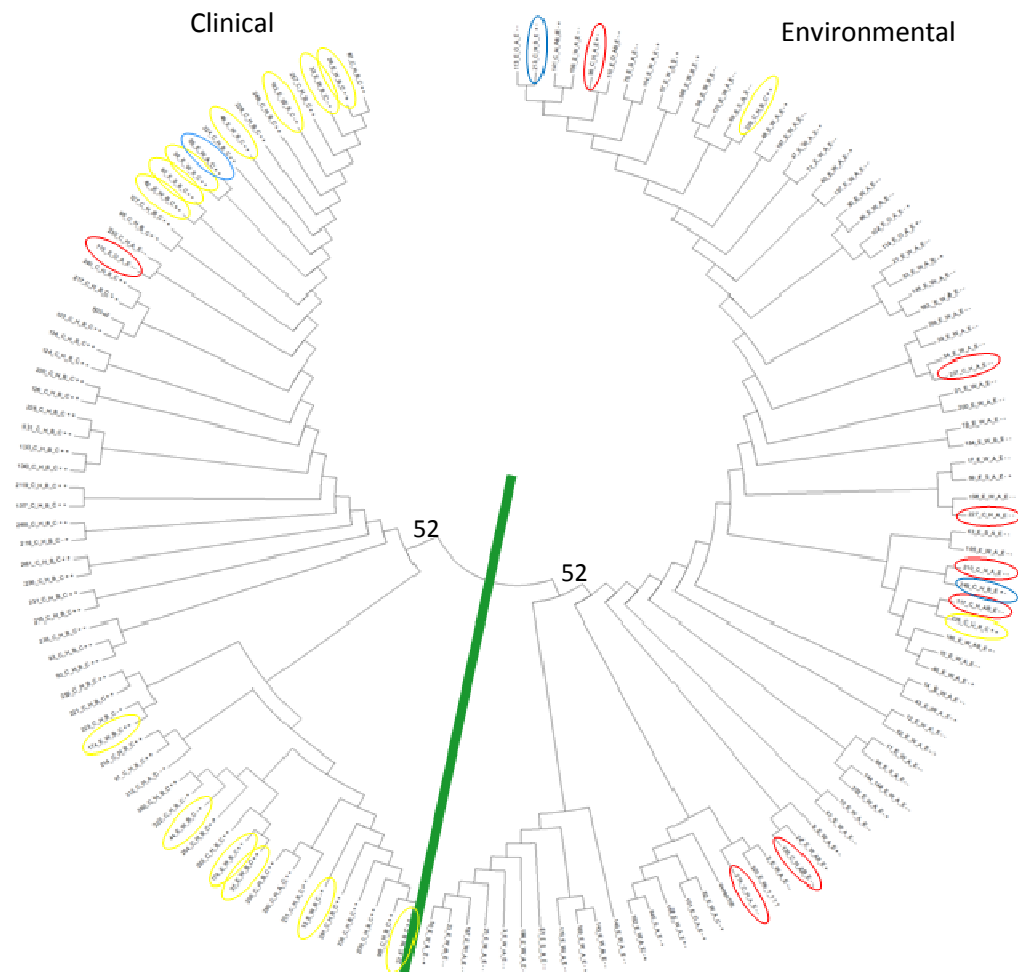
Figure 7. Phylogenetic tree constructed using MEGA V6.0 maximum likelihood without deleting gaps and 1000 bootstrap replicates. ClustalW2 was used for sequence alignment. Bootstrap frequency (%) for main branches shown. Circled isolates represent environmental isolates on clinical branch or clinical isolates on the environmental branch. Red: 16S type A/AB, *vcg* type E, *pilF*-, mannitol fermentation-; yellow: 16S type B, *vcg* type E, *pilF*+, mannitol fermentation+; blue: 16S type does not match *vcg* type. Correction to isolate 141, it is listed as 141_C_H and should be 141_E_W.
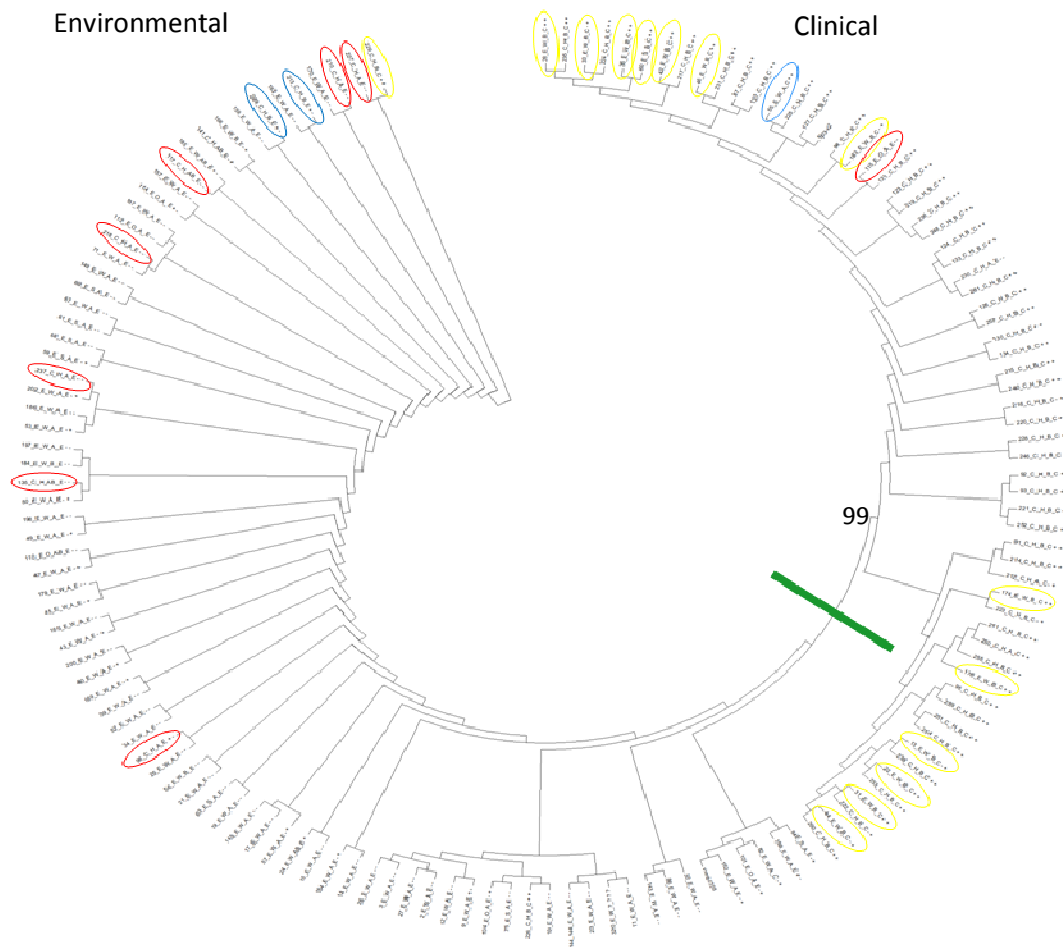
Figure 8. Phylogenetic tree constructed using MEGA V6.0 maximum likelihood, 1000 bootstrap replicates, and all gap sites were deleted. ClustalW2 was used for sequence alignment. Bootstrap frequency (%) for main branch shown. Circled isolates represent environmental isolates on clinical branch or clinical isolates on the environmental branch. Red: 16S type A/AB, *vcg* type E, *pilF-*, mannitol fermentation-; yellow: 16S type B, *vcg* type E, *pilF+*, mannitol fermentation+; blue: 16S type does not match *vcg* type. Correction to isolate 141, it is listed as 141_C_H and should be 141_E_W.

A

Clinical   Environmental

48   48

I   II

B

2

53

C

0

169_E_W_A_E-+
179_E_W_A_E-.
135_C_H_AB_E-.
143_E_W_A_E-.
210_C_H_A_E-.
187_E_W_A_E-.
198_E_W_A_E-.
227_C_H_A_E-.
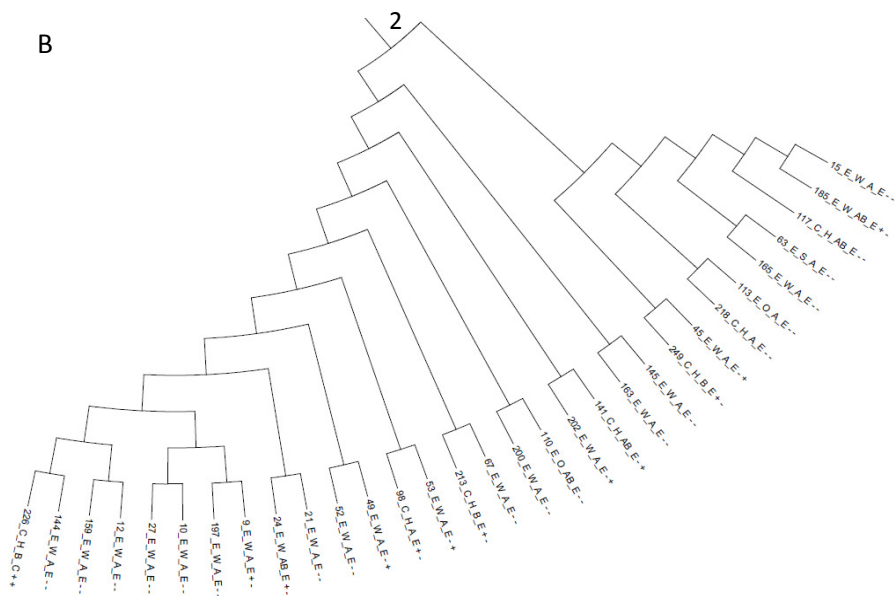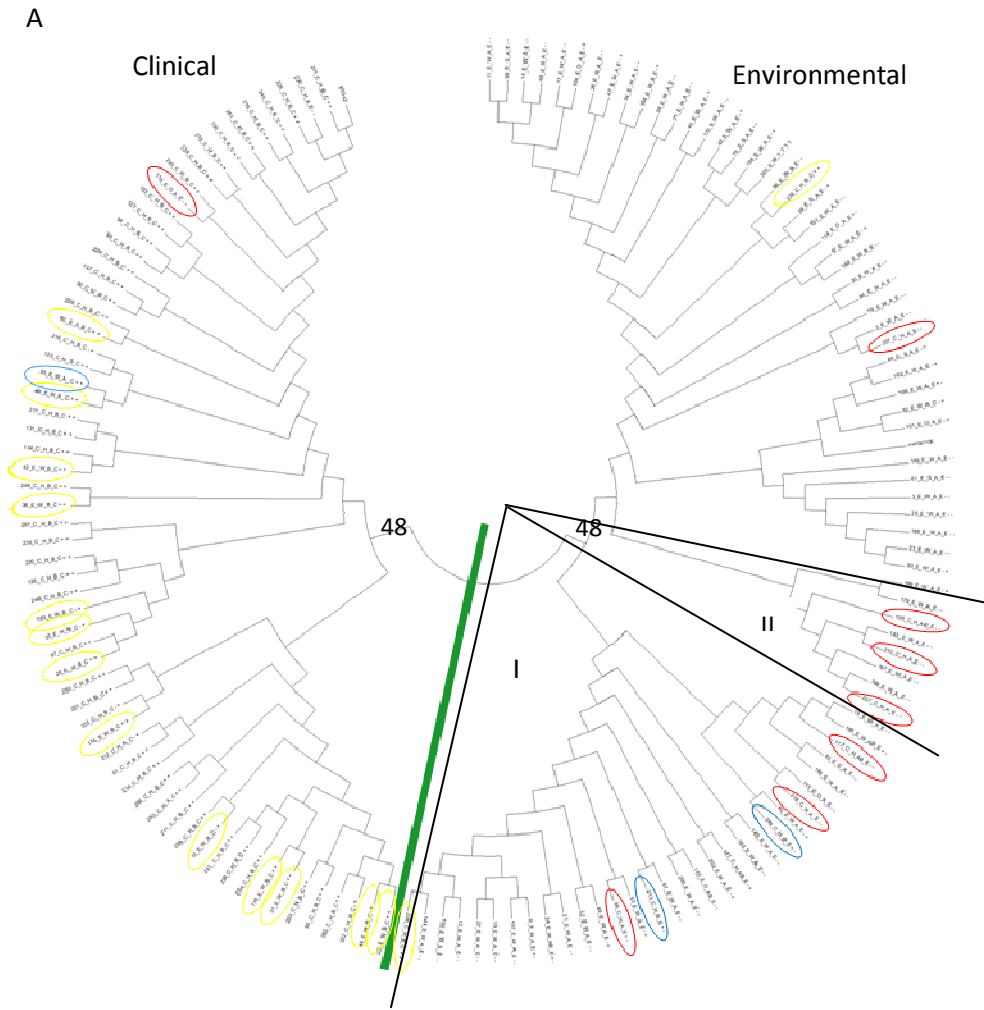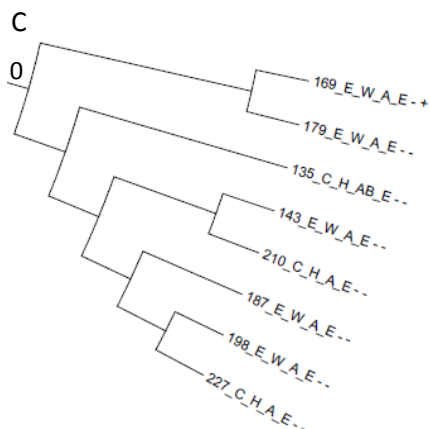
Figure 9. A: Phylogenetic tree constructed using MEGA V6.0 maximum likelihood without deleting gaps and 1000 bootstrap replicates. MUSCLE was used for sequence alignment, B: subgroup I, and C: subgroup II. Bootstrap frequency (%) for main branch shown in A and for sub branches B and C. Circled isolates represent environmental isolates on clinical branch or clinical isolates on the environmental branch. Red: 16S type A/AB, *vcg* type E, *pilF*-, mannitol fermentation-; yellow: 16S type B, *vcg* type E, *pilF*+, mannitol fermentation+; blue: 16S type does not match *vcg* type. Names are as follows: isolate number, source type clinical (C) or environmental (E), isolate origin water (W), human (H), sediment (S), or oyster (O) 16S type A (environmental) or B (clinical) or AB, *vcg* type E or C, *pilF* polymorphism +/-, and mannitol fermentation +/-. Groups I and II contain most of the clinical isolates present on the environmental branch. Correction to isolate 141, it is listed as 141_C_H and should be 141_E_W.
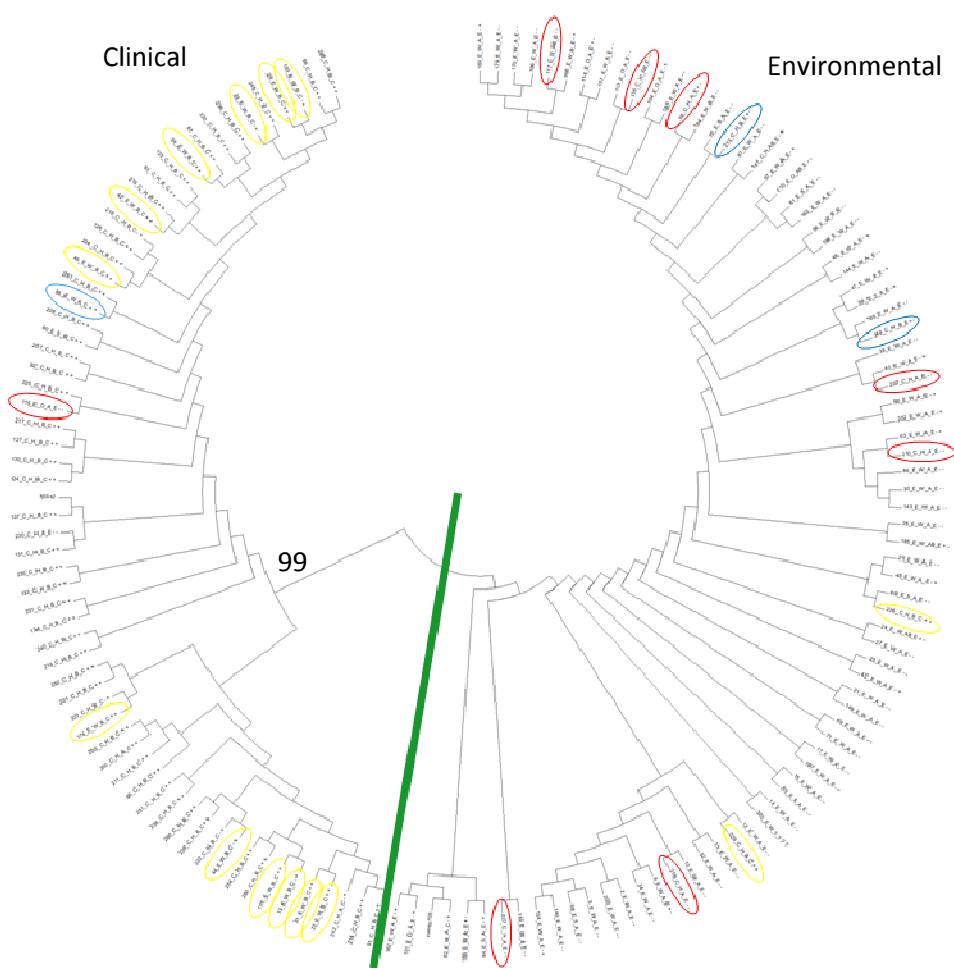
Figure 10. Phylogenetic tree constructed using MEGA V6.0 maximum likelihood, 1000 bootstrap replicates, and all gap sites deleted. MUSCLE was used for sequence alignment. Bootstrap frequency (%) for main branch shown. Circled isolates represent environmental isolates on clinical branch or clinical isolates on the environmental branch. Red: 16S type A/AB, *vcg* type E, *pilF*-, mannitol fermentation-; yellow: 16S type B, *vcg* type E, *pilF*+, mannitol fermentation+; blue: 16S type does not match *vcg* type. Correction to isolate 141, it is listed as 141_C_H and should be 141_E_W.

Neighbor joining trees constructed using pairwise deletion of gap sites (Figure 11 and sp13) appeared markedly different from those with all gap sites deleted (Figure 12 and sp14). Very few distinct sub groups were present in the neighbor joining tree where all gap sites were deleted and most isolates were considered equally related to each other (Figure 12). This was not the case when pairwise deletion was employed. Aside from the major separation of clinical and

environmental isolates, as has been demonstrated in previous phylogenetic trees,

numerous sub groups were created. All of the clinical isolates, present on the

environmental side of the tree formed two clusters in the neighbor joining tree (Figure

11 B. and C). When MUSCLE was used to align sequences the effect of gap deletion

was very pronounced and varied. Again, complete deletion of gap sites resulted in

few sub groups compared to pairwise deletion (Figure 13 and sp15 and Figure 14 and

sp16 respectively). However, subgroups did not appear to correlate well between the

delete all gap sites tree (Figure 13) and the pairwise deletion tree (Figure 14).

A

B

16

198_E_W_A_E_--
187_E_W_A_E_--
227_C_H_A_E_--
210_C_H_A_E_--
10_E_W_A_E_--
143_E_W_A_E_--
213_C_H_B_E_+-
113_E_O_A_E_--
237_C_H_A_E_--
320_E_W_?_???
162_E_W_A_E_-+

C

0

135_C_H_AB_E_--
24_E_W_AB_E_+
249_C_H_B_E_+-
165_E_W_A_E_--
218_C_H_A_E_--
229_C_H_B_C_++
98_C_H_A_E_+-
159_E_W_A_E_--
141_C_H_AB_E_-+
145_E_W_A_E_--
226_C_H_B_C_++
117_C_H_AB_E_--
163_E_W_A_E_--
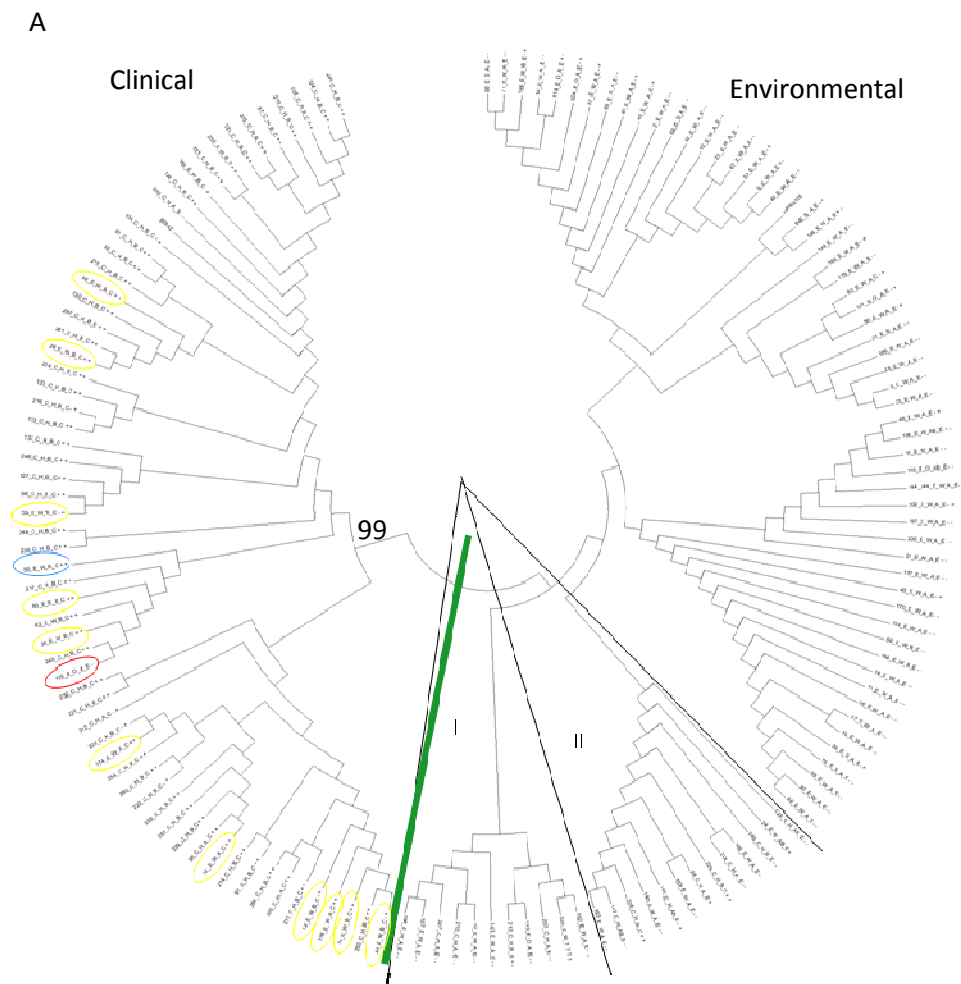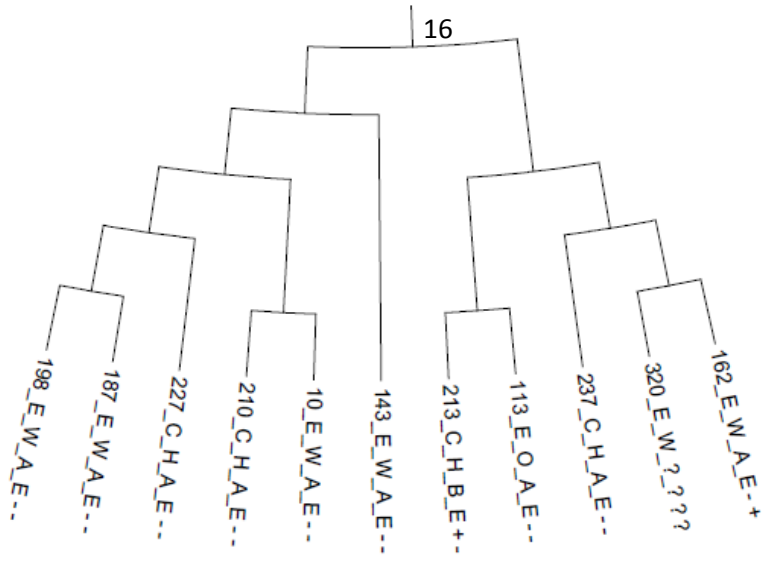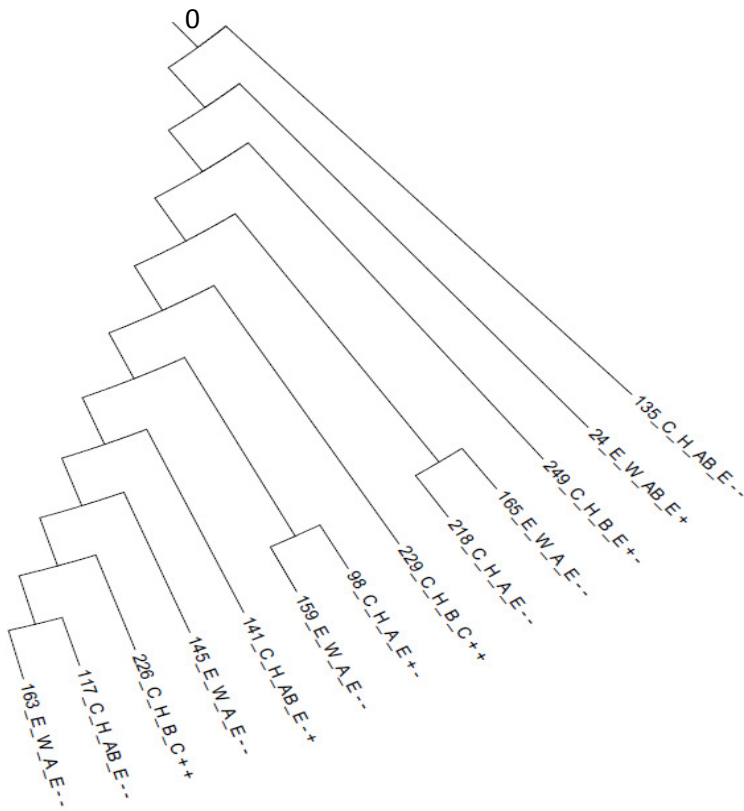
Figure 11. A: Phylogenetic tree constructed using MEGA V6.0 neighbor joining, 2000 bootstrap replicates, and pairwise deletion of gap sites. ClustalW2 was used for sequence alignment, B: subgroup I, C: subgroup II. Bootstrap frequency (%) for main branch shown for the whole tree in A and in sub branches B and C. Circled isolates represent environmental isolates on clinical branch or clinical isolates on the environmental branch. Red: 16S type A/AB, *vcg* type E, *pilF*-, mannitol fermentation-; yellow: 16S type B, *vcg* type E, *pilF*+, mannitol fermentation+; blue: 16S type does not match *vcg* type. Names are as follows: isolate number, source type clinical (C) or environmental (E), isolate origin water (W), human (H), sediment (S), or oyster (O) 16S type A (environmental) or B (clinical) or AB, *vcg* type E or C, *pilF* polymorphism +/-, and mannitol fermentation +/-. Groups I and II contain clinical isolates that were associated with the majority of environmental isolates. Correction to isolate 141, it is listed as 141_C_H and should be 141_E_W.
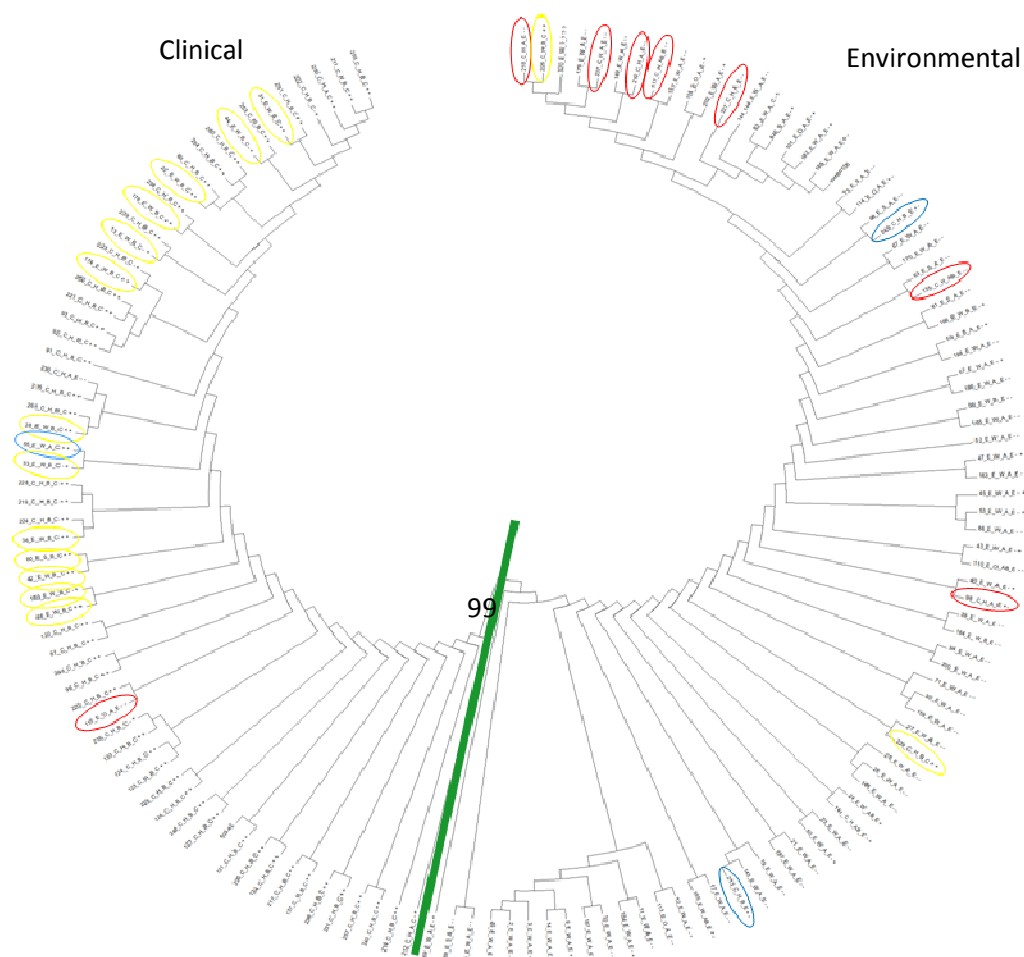
Figure 12. Phylogenetic tree constructed using MEGA V6.0 neighbor joining, 2000 bootstrap replicates, and deletion of all gap sites. ClustalW2 was used for sequence alignment. Bootstrap frequency (%) for main branch shown. Circled isolates represent environmental isolates on clinical branch or clinical isolates on the environmental

branch. Red: 16S type A/AB, *vcg* type E, *pilF-*, mannitol fermentation-; yellow: 16S type B, *vcg* type E, *pilF+*, mannitol fermentation+; blue: 16S type does not match *vcg* type. Correction to isolate 141, it is listed as 141_C_H and should be 141_E_W.
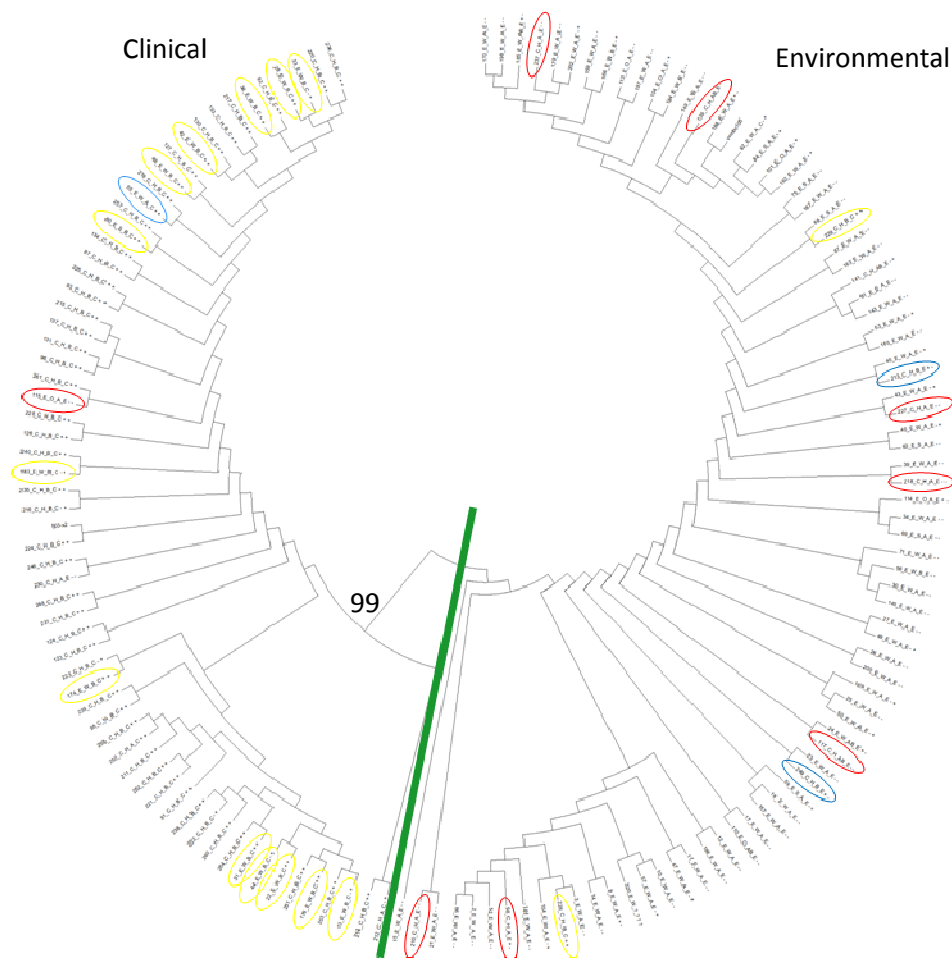
Figure 13. Phylogenetic tree constructed using MEGA V6.0 neighbor joining, 2000 bootstrap replicates, and deletion of all gap sites. MUSCLE was used for sequence alignment. Bootstrap frequency (%) for main branch shown. Circled isolates represent environmental isolates on clinical branch or clinical isolates on the environmental branch. Red: 16S type A/AB, *vcg* type E, *pilF-*, mannitol fermentation-; yellow: 16S type B, *vcg* type E, *pilF+*, mannitol fermentation+; blue: 16S type does not match *vcg* type. Correction to isolate 141, it is listed as 141_C_H and should be 141_E_W.
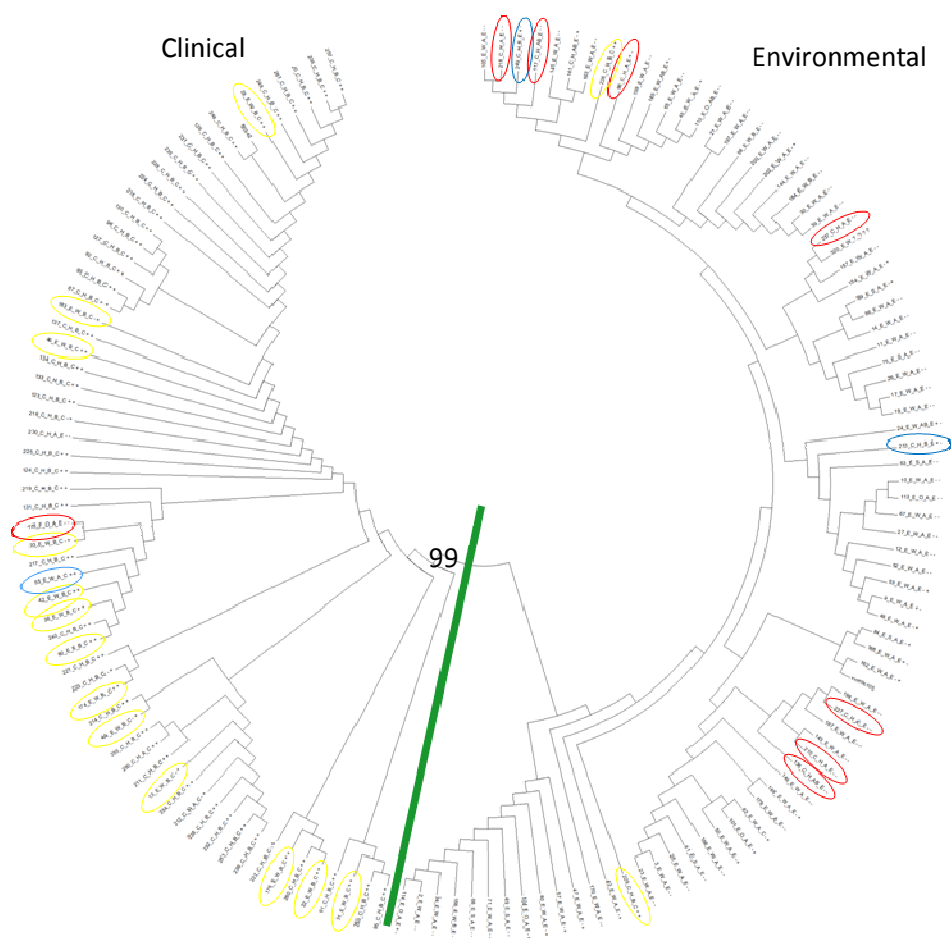
Figure 14. Phylogenetic tree constructed using MEGA V6.0 neighbor joining, 2000 bootstrap replicates, and pairwise deletion of gap sites. MUSCLE was used for sequence alignment. Bootstrap frequency (%) for main branch shown. Circled isolates represent environmental isolates on clinical branch or clinical isolates on the environmental branch. Red: 16S type A/AB, *vcg* type E, *pilF*-, mannitol fermentation-; yellow: 16S type B, *vcg* type E, *pilF*+, mannitol fermentation+; blue: 16S type does not match *vcg* type. Correction to isolate 141, it is listed as 141_C_H and should be 141_E_W.

Gap treatment substantially altered grouping of isolates within the clinical and environmental branches. Deletion of sites with gaps resulted in reduced branching of trees in general and different groupings of isolates. However, some subgroups overlapped between gap treatments. Location of isolate origin did not appear to correlate with the constructed phylogeny. Although, *pilF* and mannitol fermentation

also associated with the constructed phylogeny, 16S, and *vcg* types. The tested

alignment strategies had some effect on tree topology. Regardless of the differences

with alignment or tree construction strategy the same set of 11 clinical isolates

appeared on the environmental side of all tested phylogenetic trees (Figures 7-14)

(Table 12). Also, the same set of 13 environmental isolates appeared on the clinical

origin branch (Table 13). The five isolates not correctly identified by Biolog MIS did

not appear to be grouped separately from other isolates. Including gaps generally

resulted in increased sub branching but low bootstrap values (typically <70%) were

obtained for each branch

Table 12. Clinically acquired isolates that grouped with the majority of environmental
isolates regardless of alignment algorithm or phylogenetic tree construction strategy.
Names are as follows: isolate number, source type clinical (C) or environmental (E),
isolate origin water (W), human (H), sediment (S), or oyster (O) 16S type A
(environmental) or B (clinical) or AB, *vcg* type E or C, *pilF* polymorphism +/-, and
mannitol fermentation +/-.

Isolate	Infection Type	location
249_C_H_B_E+-	Septicemia	Florida
135_C_H_AB_E --	Septicemia	Florida/Louisiana
98_C_H_A_E+-	Cutaneous	Texas
229_C_H_B_C++	Cutaneous	Texas
117_C_H_AB_E--	Unknown	Texas
140_C_H_A_E--	Septicemia	Texas
143_C_H_B_E+-	Septicemia	Texas
148_C_H_A_E--	Septicemia	Texas
226_C_H_B_C++	Septicemia	Texas
227_C_H_A_E--	Septicemia	Texas
237_C_H_A_E--	Septicemia	Texas

Table 13. Environmentally acquired isolates present on the clinical branch of all phylogenetic trees with isolate origin. Names are as follows: isolate number, source type clinical (C) or environmental (E), isolate origin water (W), human (H), sediment (S), or oyster (O) 16S type A (environmental) or B (clinical) or AB, *vcg* type E or C, *pilF* polymorphism +/-, and mannitol fermentation +/-.

Isolate	location
13_E_W_B_C-+	Texas
22_E_W_B_C++	Texas
28_E_W_B_C++	Texas
31_E_W_B_C++	Texas
33_E_W_B_C-+	Texas
36_E_W_B_C++	Texas
42_E_W_B_C++	Texas
44_E_W_B_C-+	Texas
46_E_W_B_C++	Texas
55_E_W_A_C++	Texas
60_E_S_B_C++	Texas

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DISCUSSION

PHENOTYPIC ANALYSIS

Media Comparison

Differential and selective media offer rapid low cost screening of samples for *Vibrio* isolates. In this study, the ability of several media to presumptively identify *V. vulnificus* was compared. Currently the FDA recommends using VVA to isolate *V.*

vulnificus followed by additional verification (FDA BAM 2013). This medium selects

through high pH and a single carbon source: cellobiose. While all confirmed *V.*

vulnificus isolates grew as yellow colonies on VVA in this study, i.e. it was effective

at supporting *V. vulnificus* growth, when environmental isolations were performed it

was shown not to be effective as a selective medium as numerous false positives were

present. CPC+ was developed to better reduce background flora and enhance

V. vulnificus recovery by adjusting the concentrations of antibiotics (colistin and

polymyxin B) and salts (Warner and Oliver 2007). When Jones *et al.* (2013)

compared the ability of VVA and CPC+ to isolate *V. vulnificus* for enumeration as

well as for preferential isolation of virulent genotypes no difference was observed.

While less background flora grows on CPC+, numerous false positives can occur. To

determine if CAV, which can differentiate several species of *Vibrio*, is more effective

than CPC+ at isolating *V. vulnificus* and reducing false positives, Williams *et al.*

(2013) used each to isolate *V. vulnificus* from the environment. Both media had

numerous false positives when *V. vulnificus* numbers were low, similar to results in

this study. Williams *et al.* (2013) demonstrated that CAV was able to differentiate *V.*

alginolyticus, which appeared as white on CAV, from *V. vulnificus* (turquoise). Some

strains of *V. alginolyticus* have the ability to ferment cellobiose which results in a

false positive on CPC+ (Williams *et al.* 2013). In this study numerous isolates, shown

not to be *V. vulnificus* by PCR, were presumptively identified as *V. vulnificus* on both

VVA and CPC+, presumably due to their ability to ferment cellobiose. Very few,

however, produced turquoise colonies on CAV (Table 10). Thus while these media

are effective for growth of *V. vulnificus* they are less effective at selecting *V.*

vulnificus. To reduce the need for multiple steps and costs, Williams *et al.* (2013) developed a triple plating method in which environmental samples were plated on CAV and presumptive *V. vulnificus* colonies (turquoise) were then plated onto CPC+ and TCBS. If colonies appeared as yellow and green on CPC+ and TCBS respectively, they had a 93% probability of being *V. vulnificus* (Williams *et al.* 2013). However, when the triple plating method was tested by Froelich *et al.* (2014) only 68% of isolates were correctly identified. The difference was attributed to a sub population of sucrose fermenting *V. vulnificus*. In this study ~17% of the previously confirmed isolates were able to ferment sucrose on TCBS, the same proportion as the study by Froelich *et al.* (2014). However, all of the isolates grew on TCBS in contrast to 23% of isolates that failed to grow on TCBS in Froelich *et al.* (2014).

Due to the ability of VVA to presumptively identify all of the known isolates (confirmed by PCR), it was used as the initial isolation media for environmental water samples. Yellow colonies were picked and subjected to media comparison as well as PCR verification. The majority of all 82 isolates collected during this study appeared as yellow colonies on VVA, TCBS, and CPC+ while white on CAV; none of which were identified as *V. vulnificus* by PCR. It is unusual such a high number of non *V. vulnificus* isolates were able to grow in the presence of colistin and polymyxin B in CPC+ agar. It is probable that by using VVA as the initial isolation medium that bacteria with similar traits to *V. vulnificus* were selected for, resulting in a high number of false positives. CAV was the most selective medium with very few isolates being presumptively identified, in contrast to the other media, on which most of the colonies were presumptively identified (88-100%) as *V. vulnificus*. Potentially,

colistin and polymyxin B could be added (or increased if present) to CAV to increase its selectivity even further but testing would be needed. Due to PCR confirming only a single isolate, which had the characteristic media profile, it can be concluded that CAV is a superior initial isolation medium as the fewest isolates grew with the *V. vulnificus* color, i.e. it produced the fewest false positives. High numbers of false positives from isolation increase the amount of downstream work, decrease efficiency, and increase cost. Therefore, use of CAV as the initial isolation medium followed by plating onto the other media, as in Williams *et al.* (2013), may provide a better path for screening samples. Use of VVA may be most useful as a routine growth medium for known VV isolates.

Biolog Microbial Identification System

Identification of species can be performed using a variety of methods such as amplification of unique genes (e.g. *vvhA*) or sequencing of 16S fragments. Identification of isolates by PCR is considered the gold standard as a target gene that is unique to a species can be amplified. However, phenotypic approaches have been developed to identify isolates without the need for molecular analysis, and may also generate additional information. Biolog MIS uses unique substrate utilization profiles to determine the identity of an isolate. Similarly to Biolog MIS, API 20E and 20NE tests use substrate utilization to resolve species identity. A potential problem with identifying *V. vulnificus* using these methods is the phenotypic diversity and plasticity of the species (Harwood *et al.* 2004). Multiple studies have utilized each of the three previous systems to identify *V. vulnificus* with varying degrees of success. API 20NE

was unable to identify any *V. vulnificus* isolates in two comparisons (Sanjuán *et al.* 2009; Fouz *et al.* 2006). Biolog MIS GN (100%) (Fouz *et al.* 2006) and Generation II Microplates (84%) (Sanjuán *et al.* 2009) proved to be as good as or better than API 20E (60-100% correct identification) when compared.

Three *V. vulnificus* biotypes exist and differentiation of biotypes can be problematic due to metabolic differences. Sanjuán *et al.* (2009) found no correlation between phenotype and isolate origin, biotype, or serovar. In this study Biolog Generation III Microplates™ were used and 96% of isolates were correctly identified, suggesting this method can be an effective, accurate phenotypic test for *V. vulnificus*. Misidentification or no identification of the five isolates, in this study, may have been due to variation within the species. *P. damselae* appeared as the identity, or most probable identity, of two confirmed *V. vulnificus* isolates in this study. Vandenberghe *et al.* (2003) differentiated *Vibrio* into different phenotypic groups/species and found that *V. vulnificus* was related to *Vibrio harveyi* and *Vibrio aestuarianus* but not *Photobacterium damselae*. However, upon examination of the phylogenetic trees constructed in this study, these five isolates did not appear unique. While expensive (approximately $10 per isolate), Biolog MIS is a relatively simple, standardized method which also provides more information than just species identification. Metabolic profiles of isolates can be compared and used for further analysis.

Antibiotic Resistance Analysis

Antibiotic susceptibility testing of *V. vulnificus* is important for monitoring of resistance to therapeutic agents such as doxycycline and ceftriaxone and may provide

information on exposure to antibiotics in various environments. Generally few

isolates have been resistant to agents recommended for treatment (Table 3). However,

antibiotic resistance appears to be widespread but incredibly variable (Table 3). In

this study 143 isolates were tested for resistance to 12 antibiotics and only two, out of

58 isolates isolated from clinical samples, displayed complete resistance to a single

antibiotic (cefepime or amoxicillin). Three isolates, one of clinical and two of

environmental origin, exhibited intermediate resistance to a single antibiotic

(amoxicillin, amikacin, or ciprofloxacin), none of which are used for treatment of

infections in humans (Appendix Table A1). Location of isolation as well as local

agriculture/aquaculture has been implicated in some antibiotic resistance variability.

Kitiyodom *et al.* (2010) observed frequent resistances to tetracycline,

fluoroquinolone, and sulfonamides in isolates from farmed shrimp, all of which are

used heavily as prophylactics on local shrimp farms.

Antibiotic resistance profiling of *V. vulnificus* from oysters has been

conducted for safety concerns and to determine if there is a difference in antibiotic

resistance between isolates found in oysters or in water. Han *et al.* (2007) tested

oysters from Louisiana and found that all *V. vulnificus* isolates (n=151) were

susceptible to ampicillin while ~20% of *V. parahaemolyticus* isolates were

susceptible. In contrast Okoh and Igbinosa (2010) found that all (18 isolates) tested *V.*

vulnificus in South Africa were resistant to ampicillin and sulfamethoxazole, as have

others (Table 12). Ottaviani (2001) isolated several antibiotic resistant and multidrug

resistant *V. vulnificus* strains that produced ß-lactamase and several isolates were

resistant to the tested ß-lactam based antibiotics. Kim *et al.* (2011) determined that

resistance to several classes of antibiotics was more common in environmental (n=14)

V. vulnificus compared to oysters (n=17). However, a larger sample size is needed to

be more definitive. Shaw *et al.* (2014) reported that few *V. vulnificus* (21/120) were

resistant to any of the antibiotics tested. Interestingly, they noted that intermediate

resistance to many of the tested antibiotics was common in contrast to the results of

this study.

Treatment of patients with antibiotic resistant *V. vulnificus* infections can be

complicated. *V. vulnificus* infections progress rapidly and an effective treatment plan

must be implemented quickly before the patient is overwhelmed and dies (Oliver

2013). Ciprofloxacin and cefotaxime have been reported to work synergistically to

kill *V. vulnificus in vitro* more effectively than doxycycline and cefotaxime (the

currently recommended treatment) (Kim *et al.* 2005). More recently, Jang *et al.*

(2014) determined that the synergy worked *in vivo* to decrease mortality of mice.

Ciprofloxacin also significantly (P<0.05) reduced transcription of the exotoxin

repeats-in-toxin (RtxA1) analog PrtxA1. It was believed that suppression of RtxA1,

which is involved in organ damage during sepsis, decreased the mortality rate (Jang *et

al.* 2014). However, with antibiotic resistance a real concern and on the rise, it is

likely that strains resistant to therapeutic drugs will emerge more frequently. An

alternate strategy is to utilize bacteriophages to treat infections. Jun *et al.* (2014)

demonstrated that phage therapy was able to prevent mouse mortality when

challenged with a lethal dose of a multi-drug resistant pandemic strain of *V.

parahaemolyticus*.

Due to the low numbers of resistant organisms, no correlation between source (clinical or environmental) or isolate origin (human, oyster, sediment, or water) and antibiotic resistance could be made in this study. However, increasing the number of antibiotics may identify resistance to other antimicrobials and allow for better comparisons to be made. The 12 antibiotics used in this study were of medical importance for treatment, which is useful for public health. This study found that over a range of geographic locations and sources *V. vulnificus* isolates exhibited little resistance to common antibiotics, implying antibiotic treatment may still be an effective protocol for *V. vulnificus* infections, if the infection is identified quickly. However, including antibiotics that are common in agriculture (farm runoff) and aquaculture may provide additional perspective. Continued monitoring of resistance in *V. vulnificus* will be important in identifying increases in resistance and appropriate treatments.

MULTIPLEX PCR OF *vvhA* AND 16S OPTIMIZATION

Multiplex PCR allows for amplification of multiple target genes at the same time. Various multiplex PCR protocols have been developed to identify *V. vulnificus* and determine its genotype. Several multiplex protocols have been developed to differentiate potentially pathogenic *Vibrio* spp. Bauer and Rørvik (2007) differentiated *V. parahaemolyticus, V. cholerae,* and *V. vulnificus* based on their *toxR* gene polymorphisms. However, some *V. alginolyticus* isolates yielded a false positive for *V. parahaemolyticus.* They developed a multiplex protocol specific for *V. vulnificus* that could identify *V. vulnificus* and determine its genotype; though it is

limited to biotype 1 only (Warner and Oliver 2008). In this study a novel multiplex

PCR was created to amplify the 16S gene and a portion of the *vvhA* gene which can

be used to identify *V. vulnificus*. The purpose of the multiplex PCR reaction was to

validate the reaction and ensure no inhibitors were in the DNA sample by

amplification of the 16S gene and test it in a real world scenario. This method has the

advantage over standard PCR (Panicker and Bej 2005) in that it not only identifies the

species as *V. vulnificus,* but it has a built in positive control, and if desired, bands can

be removed from the gel for sequencing.

Several types of buffers have been created to improve band resolution as

well as reduce costs. The three buffers (tris acetate EDTA, tris borate EDTA, and

sodium borate) compared in this study appeared to perform differently with respect

to voltage and fragment size. TAE provided the highest band resolution with the 16S

fragment, SB proved to be the best at separating low molecular weight fragments, and

TBE was overall the best as it provided good separation and resolution of high and

low molecular weight fragments. A similar trend was observed by Brody and Kern

and it was shown that SB was able to maintain resolution of high molecular weight

products at very high (350V) voltages (Brody and Kern 2004).

Voltage did not appear to have a large effect on band resolution with the

tested buffers and PCR products. TBE appeared retain the highest overall separation

at 6.7V/cm. However, the same pattern of separation based on buffer type was still

present at all voltages tested. The ability to run at high voltage without sacrificing

resolution is important to save time. Based on these results, TAE appears to be best

suited to high molecular weight, SB low molecular weight, and TBE high and low

molecular weight fragments. Other buffers have been proposed to further enhance band resolution at very low (<100bp) molecular weights such as 1mM lithium boric acid which was able to achieve 1bp resolution (Brody *et al.* 2004). Each type of buffer has unique characteristics and can be chosen based on cost, fragment size, voltage, and duration of electrophoresis to optimize band separation.

PHYLOGENETIC ANALYSIS

In this study a short fragment (~90-160bp) of the *vvhA* gene, obtained from species identification PCR, from 143 isolates was sequenced. *vvhA* was used for analysis as it is used to identify *V. vulnificus,* unique to *V. vulnificus,* and PCR product was available from identification assays. Two programs PHYLIP V3.695 and MEGA V6.0 were used to help determine the phylogeny of the isolates. PHYLIP has been used for small datasets but as the number of sequences increases the time required to complete the processing quickly becomes overwhelming. MEGA is able to save time by using a modified version of maximum likelihood that reduces the computational burden (Tamura *et al.* 2011). A faster method, neighbor joining, was included to compare with the maximum likelihood method. In addition to comparing strategies for constructing phylogenies, two different sequence alignment strategies were compared: ClustalW2 and MUSCLE. The effects of gap treatment were also explored.

Several genes have been found to correlate with clinical and environmental strains of *V. vulnificus* and have been used to determine the pathogenic potential of isolates. As mentioned previously 16S type (A/B) (Nilsson *et al.* 2003) (real time

PCR version by Vickery *et al.* 2007) and *vcg* type (E/C) have been well correlated

with virulence and putative virulence factors including *pilF* polymorphism (Baker-

Austin *et al.* 2009). Rosche *et al.* (2005) correlated *vcg* type (E/C) with differences in

vvhA between clinical and environmental isolates as well the number of copies of a

heptanucleotide repeat. Additional correlations have been drawn with box PCR

(Staley *et al.* 2011), RAPD PCR, housekeeping genes, and *vvhA* (Rosche *et al.* 2005;

Rosche *et al.* 2010) among others. Rosche *et al.* (2010) reported that the differences

between clinical and environmental isolates were present in the housekeeping genes

recA and *glnA* but the differences were more pronounced in the virulence gene *vvhA*.

Such differences could indicate a deep divide between strains. This difference was

observed with the fragment of the *vvhA* gene used in this study. Both maximum

likelihood and neighbor joining trees produced by MEGA had two distinct branches

and correlated with 16S type A and *vcg* type E (environmental) or 16S type B and *vcg*

type C (clinical) no matter the alignment algorithm (typically a frequency of 99%).

The environmental isolates that were found on the clinical branch may

represent pathogenic strains. Despite the observed differences with gap treatment,

alignment algorithm, and tree construction method, low bootstrap values were

obtained for almost all sub branches (data not shown). This could be due to the

conserved nature of the *vvhA* gene and that a short sequence length was used for

analysis. Further analysis of the entire *vvhA* or 16S gene may lead to more

associations between the previously mentioned virulence indicators and origin.

Additionally, with a longer sequence the effects of tree construction strategy could

become more apparent.

SUMMARY

In this study a group of 143 clinical and environmental isolates were characterized using different phenotypic and genotypic approaches. Media profiles (colony color on selective or differential media) of the confirmed isolates, mostly matched those characteristic of *V. vulnificus*. Most of the tested media had very high false positive rates with the new presumptive *V. vulnificus* isolates from Virginia but CAV was determined to have the fewest false positives. Biolog Microbial Identification System, correctly identified 96% of the confirmed *V. vulnificus* isolates and was determined to be an accurate method for identification of *V. vulnificus* species. Approximately 9% of the clinical isolates and ~1% of the environmental were able to ferment sucrose (yellow appearance on TCBS). However, no other associations were found between colony color on the tested media and isolate origin. Almost all the *V. vulnificus* isolates were susceptible to all the antibiotics used (only five isolates displayed any type of resistance) and thus no relationship with origin could be established. A multiplex PCR protocol was developed, optimized, and tested to identify an isolate as *V. vulnificus* and include a positive control by amplification of *vvhA* (Panicker and Bej 2005) and the 16S (Fierer and Jackson 2006) gene respectively. Buffer optimization was simultaneously conducted with the multiplex PCR and TBE was determined to be the best buffer of those tested, for gel electrophoresis with the produced amplicons. Finally, phylogenetic analysis of the *vvhA* gene resulted in phylogenetic trees with two distinct branches related to clinical or environmental isolate origin. Virulence factors associated with clinical or environmental branches but sample origin (water, sediment, or oyster) did not. This

study was conducted to further characterize a group of well studied *V. vulnificus* isolates and to find methods to differentiate clinical from environmental isolates. Continued analysis of these and additional isolates may further our knowledge of the species and reveal more characteristics indicative of pathogenicity.

APPENDIX I

Antibiotic Resistance Analysis

Table A1. Antibiotic resistance profiles of clinical isolates (obtained from human samples). Isolates that displayed an intermediate resistance are yellow and full resistances are red.

Specimen #	Source	TZP	GM	TE	MEM	IPM	SXT	FEP	CRO	AM	C	AN	CIP
									Antibiotic				
87	Blood	S	S	S	S	S	S	S	S	S	S	S	S
91	Blood	S	S	S	S	S	S	S	S	S	S	S	S
92	Blood	S	S	S	S	S	S	S	S	S	S	S	S
93	Blood	S	S	S	S	S	S	S	S	S	S	S	S
95	Blood	S	S	S	S	S	S	S	S	S	S	S	S
96	Blood	S	S	S	S	S	S	S	S	S	S	S	S
117	Blood	S	S	S	S	S	S	S	S	S	S	S	S
123	Blood	S	S	S	S	S	S	S	S	S	S	S	S
124	Blood	S	S	S	S	S	S	S	S	S	S	S	S
126	Blood	S	S	S	S	S	S	S	S	S	S	S	S
127	Blood	S	S	S	S	S	S	S	S	S	S	S	S
130	Blood	S	S	S	S	S	S	S	S	S	S	S	S
131	Blood	S	S	S	S	S	S	S	S	S	S	S	S
133	Blood	S	S	S	S	S	S	S	S	S	S	S	S
134	Blood	S	S	S	S	S	S	S	S	S	S	S	S
135	Blood	S	S	S	S	S	S	S	S	S	S	S	S
137	Blood	S	S	S	S	S	S	S	S	S	S	I	S
210	Blood	S	S	S	S	S	S	S	S	S	S	S	S
212	Blood	S	S	S	S	S	S	S	S	S	S	S	S
213	Blood	S	S	S	S	S	S	S	S	S	S	S	S
214	Blood	S	S	S	S	S	S	S	S	S	S	S	S
215	Blood	S	S	S	S	S	S	S	S	S	S	S	S

216	Blood	S	S	S	S	S	S	S	S	S	S	S	S
217	Blood	S	S	S	S	S	S	S	S	S	S	S	S
218	Blood	S	S	S	S	S	S	S	S	S	S	S	S
219	Blood	S	S	S	S	S	S	S	S	S	S	S	S
220	Blood	S	S	S	S	S	S	S	S	S	S	S	S
221	Blood	S	S	S	S	S	S	S	S	S	S	S	S
222	Blood	S	S	S	S	S	S	S	S	S	S	S	S
224	Blood	S	S	S	S	S	S	S	S	S	S	S	S
225	Blood	S	S	S	S	S	S	S	S	S	S	S	S
226	Blood	S	S	S	S	S	S	S	S	S	S	S	S
227	Blood	S	S	S	S	S	S	S	S	S	S	S	S
230	Blood	S	S	S	S	S	S	S	S	S	S	S	S
231	Blood	S	S	S	S	S	S	S	S	S	S	S	S
235	Blood	S	S	S	S	S	S	S	S	S	S	S	S
236	Blood	S	S	S	S	S	S	S	S	S	S	S	S
237	Blood	S	S	S	S	S	S	S	S	R	S	S	S
238	Blood	S	S	S	S	S	S	S	S	S	S	S	S
240	Blood	S	S	S	S	S	S	S	S	S	S	S	S
248	Blood	S	S	S	S	S	S	R	I	S	S	S	S
249	Blood	S	S	S	S	S	S	S	S	S	S	S	S
250	Blood	S	S	S	S	S	S	S	S	S	S	S	S
251	Blood	S	S	S	S	S	S	S	S	S	S	S	S
252	Blood	S	S	S	S	S	S	S	S	S	S	S	S
255	Blood	S	S	S	S	S	S	S	S	S	S	S	S
257	Blood	S	S	S	S	S	S	S	S	S	S	S	S
260	Blood	S	S	S	S	S	S	S	S	S	S	S	S
261	Blood	S	S	S	S	S	S	S	S	S	S	S	S
246	Stool	S	S	S	S	S	S	S	S	S	S	S	S
98	Wound	S	S	S	S	S	S	S	S	S	S	S	S
211	Wound	S	S	S	S	S	S	S	S	S	S	S	S

223	Wound	S	S	S	S	S	S	S	S	S	S	S	S
228	Wound	S	S	S	S	S	S	S	S	S	S	S	S
229	Wound	S	S	S	S	S	S	S	S	S	S	S	S
239	Wound	S	S	S	S	S	S	S	S	S	S	S	S
253	Wound	S	S	S	S	S	S	S	S	S	S	S	S
254	Wound	S	S	S	S	S	S	S	S	S	S	S	S

Table A2. Antibiotic resistance profiles of isolates obtained from oysters.

| Specimen # | Source | Antibiotic | | | | | | | | | | | |
		TZP	GM	TE	MEM	IPM	SXT	FEP	CRO	AM	C	AN	CIP
101	Oyster	S	S	S	S	S	S	S	S	S	S	S	S
104	Oyster	S	S	S	S	S	S	S	S	S	S	S	S
110	Oyster	S	S	S	S	S	S	S	S	S	S	S	S
113	Oyster	S	S	S	S	S	S	S	S	S	S	S	S
114	Oyster	S	S	S	S	S	S	S	S	S	S	S	S
115	Oyster	S	S	S	S	S	S	S	S	S	S	S	S

Table A3. Antibiotic resistance profiles of isolates obtained from sediment. Isolates that displayed an intermediate resistance are in yellow.

| Specimen # | Source | Antibiotic | | | | | | | | | | | |
		TZP	GM	TE	MEM	IPM	SXT	FEP	CRO	AM	C	AN	CIP
61	Sediment	S	S	S	S	S	S	S	S	S	S	S	S
63	Sediment	S	S	S	S	S	S	S	S	S	S	S	S
84	Sediment	S	S	S	S	S	S	S	S	S	S	S	S
169	Sediment	S	S	S	S	S	S	S	S	S	S	S	S
170	Sediment	S	S	S	S	S	S	S	S	I	S	S	S

Table A4. Antibiotic resistance profiles of isolates obtained from water. Isolates that displayed an intermediate resistance are in yellow.

Specimen #	Source	Antibiotic											
		TZP	GM	TE	MEM	IPM	SXT	FEP	CRO	AM	C	AN	CIP
2	Water	S	S	S	S	S	S	S	S	S	S	S	S
3	Water	S	S	S	S	S	S	S	S	S	S	S	S
9	Water	S	S	S	S	S	S	S	S	S	S	S	S
11	Water	S	S	S	S	S	S	S	S	S	S	S	S
12	Water	S	S	S	S	S	S	S	S	S	S	S	S
13	Water	S	S	S	S	S	S	S	S	S	S	S	I
14	Water	S	S	S	S	S	S	S	S	S	S	S	S
15	Water	S	S	S	S	S	S	S	S	S	S	S	S
17	Water	S	S	S	S	S	S	S	S	S	S	S	S
19	Water	S	S	S	S	S	S	S	S	S	S	S	S
21	Water	S	S	S	S	S	S	S	S	S	S	S	S
22	Water	S	S	S	S	S	S	S	S	S	S	S	S
23	Water	S	S	S	S	S	S	S	S	S	S	S	S
24	Water	S	S	S	S	S	S	S	S	S	S	S	S
25	Water	S	S	S	S	S	S	S	S	S	S	S	S
26	Water	S	S	S	S	S	S	S	S	S	S	S	S
27	Water	S	S	S	S	S	S	S	S	S	S	S	S
28	Water	S	S	S	S	S	S	S	S	S	S	S	S
30	Water	S	S	S	S	S	S	S	S	S	S	S	S
31	Water	S	S	S	S	S	S	S	S	S	S	S	S
33	Water	S	S	S	S	S	S	S	S	S	S	S	S
34	Water	S	S	S	S	S	S	S	S	S	S	S	S
36	Water	S	S	S	S	S	S	S	S	S	S	S	S
39	Water	S	S	S	S	S	S	S	S	S	S	S	S
40	Water	S	S	S	S	S	S	S	S	S	S	S	S
42	Water	TZP	GM	TE	S	S	SXT	S	CRO	S	C	AN	CIP
43	Water	S	S	S	S	S	S	S	S	S	S	S	S
44	Water	S	S	S	S	S	S	S	S	S	S	S	S
45	Water	S	S	S	S	S	S	S	S	S	S	S	S
46	Water	S	S	S	S	S	S	S	S	S	S	S	S
47	Water	S	S	S	S	S	S	S	S	S	S	S	S
49	Water	S	S	S	S	S	S	S	S	S	S	S	S
50	Water	S	S	S	S	S	S	S	S	S	S	S	I
52	Water	S	S	S	S	S	S	S	S	S	S	S	S
53	Water	S	S	S	S	S	S	S	S	S	S	S	S

55	Water	S	S	S	S	S	S	S	S	S	S	S	S
56	Water	S	S	S	S	S	S	S	S	S	S	S	S
57	Water	S	S	S	S	S	S	S	S	S	S	S	S
59	Water	S	S	S	S	S	S	S	S	S	S	S	S
60	Water	S	S	S	S	S	S	S	S	S	S	S	S
63	Water	S	S	S	S	S	S	S	S	S	S	S	S
67	Water	S	S	S	S	S	S	S	S	S	S	S	S
68	Water	S	S	S	S	S	S	S	S	S	S	S	S
69	Water	S	S	S	S	S	S	S	S	S	S	S	S
71	Water	S	S	S	S	S	S	S	S	S	S	S	S
75	Water	S	S	S	S	S	S	S	S	S	S	S	S
82	Water	S	S	S	S	S	S	S	S	S	S	S	S
86	Water	S	S	S	S	S	S	S	S	S	S	S	S
141	Water	S	S	S	S	S	S	S	S	S	S	S	S
143	Water	S	S	S	S	S	S	S	S	S	S	S	S
144	Water	S	S	S	S	S	S	S	S	S	S	S	S
145	Water	S	S	S	S	S	S	S	S	S	S	S	S
149	Water	S	S	S	S	S	S	S	S	S	S	S	S
154	Water	S	S	S	S	S	S	S	S	S	S	S	S
157	Water	S	S	S	S	S	S	S	S	S	S	S	S
159	Water	S	S	S	S	S	S	S	S	S	S	S	S
162	Water	S	S	S	S	S	S	S	S	S	S	S	S
163	Water	S	S	S	S	S	S	S	S	S	S	S	S
165	Water	S	S	S	S	S	S	S	S	S	S	S	S
166	Water	S	S	S	S	S	S	S	S	S	S	S	S
174	Water	S	S	S	S	S	S	S	S	S	S	S	S
176	Water	S	S	S	S	S	S	S	S	S	S	S	S
179	Water	S	S	S	S	S	S	S	S	S	S	S	S
183	Water	S	S	S	S	S	S	S	S	S	S	S	S
184	Water	S	S	S	S	S	S	S	S	S	S	S	S
185	Water	S	S	S	S	S	S	S	S	S	S	S	S
186	Water	S	S	S	S	S	S	S	S	S	S	S	S
187	Water	S	S	S	S	S	S	S	S	S	S	S	S
188	Water	S	S	S	S	S	S	S	S	S	S	S	S
197	Water	S	S	S	S	S	S	S	S	S	S	S	S
198	Water	S	S	S	S	S	S	S	S	S	S	S	S
200	Water	S	S	S	S	S	S	S	S	S	S	S	S
202	Water	S	S	S	S	S	S	S	S	S	S	S	S
320	Water	S	S	S	S	S	S	S	S	S	S	S	S

APPENDIX II

PHYLOGENETIC ANALYSIS

Supplemental Methods

PHYLIP v3.695

Maximum Likelihood: Dnamlk (Felsenstein 2013)

When using Dnamlk the following options were selected: for Global

rearrangements yes was selected, Randomize input order was selected using 99 as a

seed number and 7 as jumble number (when using bootstrapped data 3 was used as a

seed and 2 for the jumble number, and Analyze multiple datasets was used with 100

data sets.

Drawgram

(http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=drawgram)

Drawgram was used to view phylogenetic trees generated by Dnaml and

Dnamlk.

Neighbor Joining Step One: Dnadist (Felsenstein 2013)

The aligned sequence file was loaded into Dnadist. Default values were used

except that multiple data sets was changed to yes and 100 data sets was entered

Neighbor Joining Step Two: Neighbor (Felsenstein 2013)

To construct a neighbor joining tree, Neighbor was used with the file

generated by Dnadist and sequence input was randomized with '9 and 9' as seed

numbers and multiple data sets was changed to 'yes' for bootstrapped sequences with a value of 100.

Bootstrapping with Seqboot and Consense (Felsenstein 2013)

To undergo resampling the alignment file was loaded into Seqboot. Ninetynine was used as the seed number and the default 100 replicates were used. Analysis was then conducted with Dnaml/Dnamlk/Dnadist and processed as before. Phylogenetic tree files were then loaded into Consense and the Majority rule was selected and then the phylogenetic tree was viewed with Drawgram.

MEGA v6.0

Sequence alignment and processing

Trimmed sequences were imported and aligned using either ClustalW2 or MUSCLE. After alignment, gaps were present at the 5' and 3' ends so sequences were further trimmed to remove most of the gaps.

Selecting a Proper Model

Sequence alignments were imported and Models was selected then Find Best DNA/Protein models (ML) was selected then protein coding DNA and analysis was run. An output of recommended running parameters was then displayed. To compare the effect of using all sites/gaps versus deleting sites with gaps (complete deletion) the appropriate selection was made under Gaps/Missing Data Treatment.

Maximum Likelihood

Using the parameters outlined in the step above maximum likelihood analysis

was conducted. Phylogeny> Construct/Test Maximum Likelihood Tree was selected

and parameters were adjusted. One thousand bootstrap replications were used,

complete deletion of gap sites; other options remained at default unless changed as

recommended by Selecting the Proper Model or all sites were included.

Neighbor Joining

Under the Phylogeny tab Construct/Test Neighbor-Joining Tree was selected

and all default values were used (Maximum Composite Likelihood, Complete

Deletion of gaps etc.) except sequences had 2000 bootstrap replicates. To compare

the effect of gap deletion both pairwise and complete deletion of gaps were used

DISCUSSION

PHYLIP was initially used for maximum likelihood analysis with a bootstrap

size of 100 replicates. The program Dnamlk was run for 62hr on an AMD FX™-6300

3.5GHz processor using seed numbers three and two (Figure A1, Figure SP2), an

additional run using different seed numbers was attempted but did not complete after

62hr. However, type B, or clinical isolates, tended to form subgroups away from type

A, environmental, isolates (Figure A1 top half). Very little branching was present in

the consensus tree compared to the individually constructed trees (Supplemental file

P1).

To compare methods, neighbor joining trees were constructed using PHYLIP. Each tree used different seed numbers for comparison. Three distinct groups were present, environmental/type A (I), clinical/type B (III), and both (II) (Figure A2, Figure SP3). Interestingly, the distinct clinical and environmental branches present in individual maximum likelihood trees (supplemental file P1) are more similar in topology to Figure A2 than the maximum likelihood consensus tree (Figure A1). To verify that the produced tree was accurate a second tree using different seed numbers was constructed (Figure A3, Figure SP4). No major change in topology between trees was noted and the same three branches were present (environmental/type A (I), both (II), and clinical/type B (III)).

Due to the large amount of time required to run the maximum likelihood analysis in PHYLIP, failure of some programs to finish, and distinct differences in tree topology, MEGA V6.0 was utilized. Maximum likelihood analysis was conducted again and two different alignment algorithms were used: ClustalW2 and MUSCLE. ClustalW2 alignment produced a tree with two distinct branches designated: clinical (left) and environmental (right) (Figure A4 and SP5). Six sub groups were present but did not appear to associate with any of the virulence factors or origin (Figure A4). Minor changes to grouping of a few isolates, between alignment strategies, was present (Figure A5 and SP6).

Again, neighbor joining trees were constructed to compare the two alignment strategies as well as tree constructing methods. Little difference between alignment strategies was found. More sub groups were present on the environmental (right side) on both neighbor joining trees (Figures A6 and A7; supplemental Figures SP7 and

SP8) compared to those produced by the maximum likelihood method (Figures A4
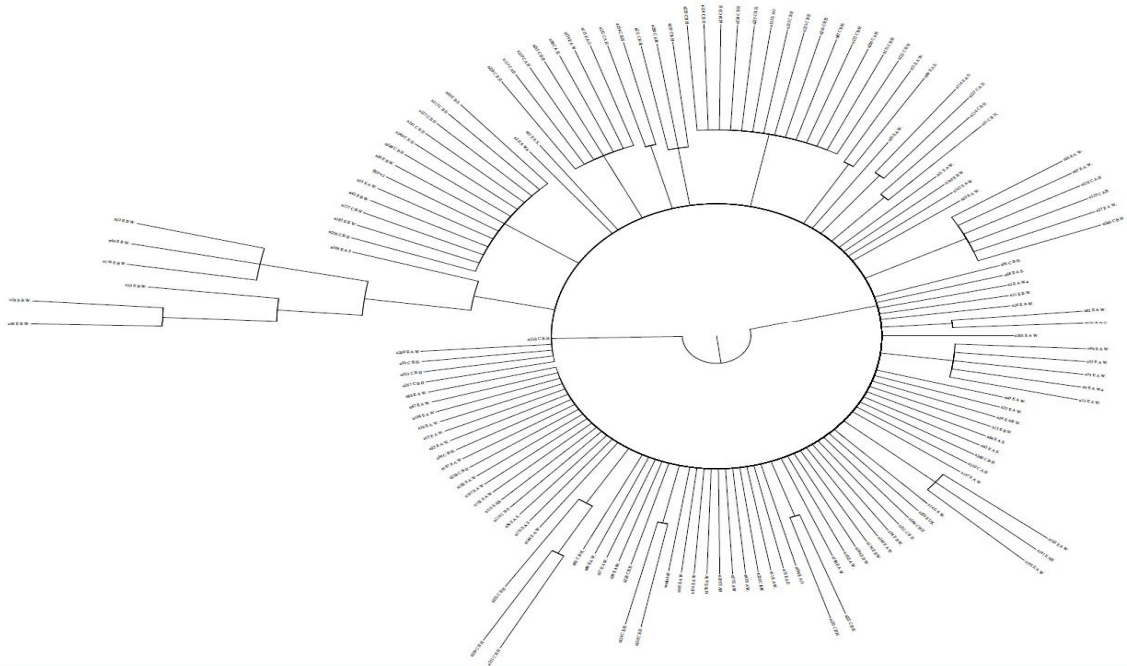
and A5).

Figure A1. Consensus phylogenetic tree of 145 *vvhA* genes constructed using Dnamlk with 100 bootstrapped replicates. Names are as follows: isolate number, source type clinical (C) or environmental (E), 16S type A (environmental) or B (clinical) or AB, and isolate origin water (W), human (H), sediment (S), or oyster (O).
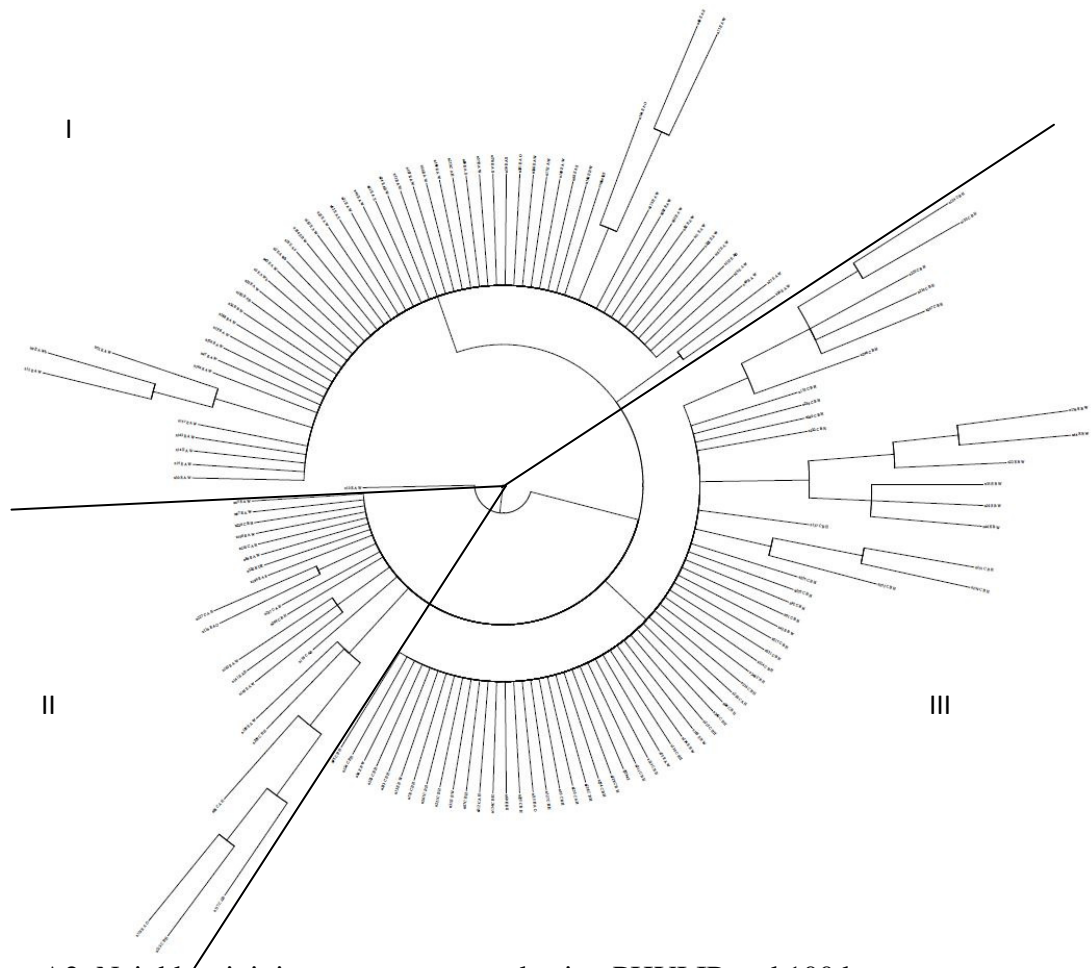
Figure A2. Neighbor joining tree constructed using PHYLIP and 100 bootstrap replicates. Groups are separated by lines: environmental/type A (I), clinical/type B (III), and both (II). Names are as follows: isolate number, source type clinical (C) or environmental (E), 16S type A (environmental) or B (clinical) or AB, and isolate origin water (W), human (H), sediment (S), or oyster (O).
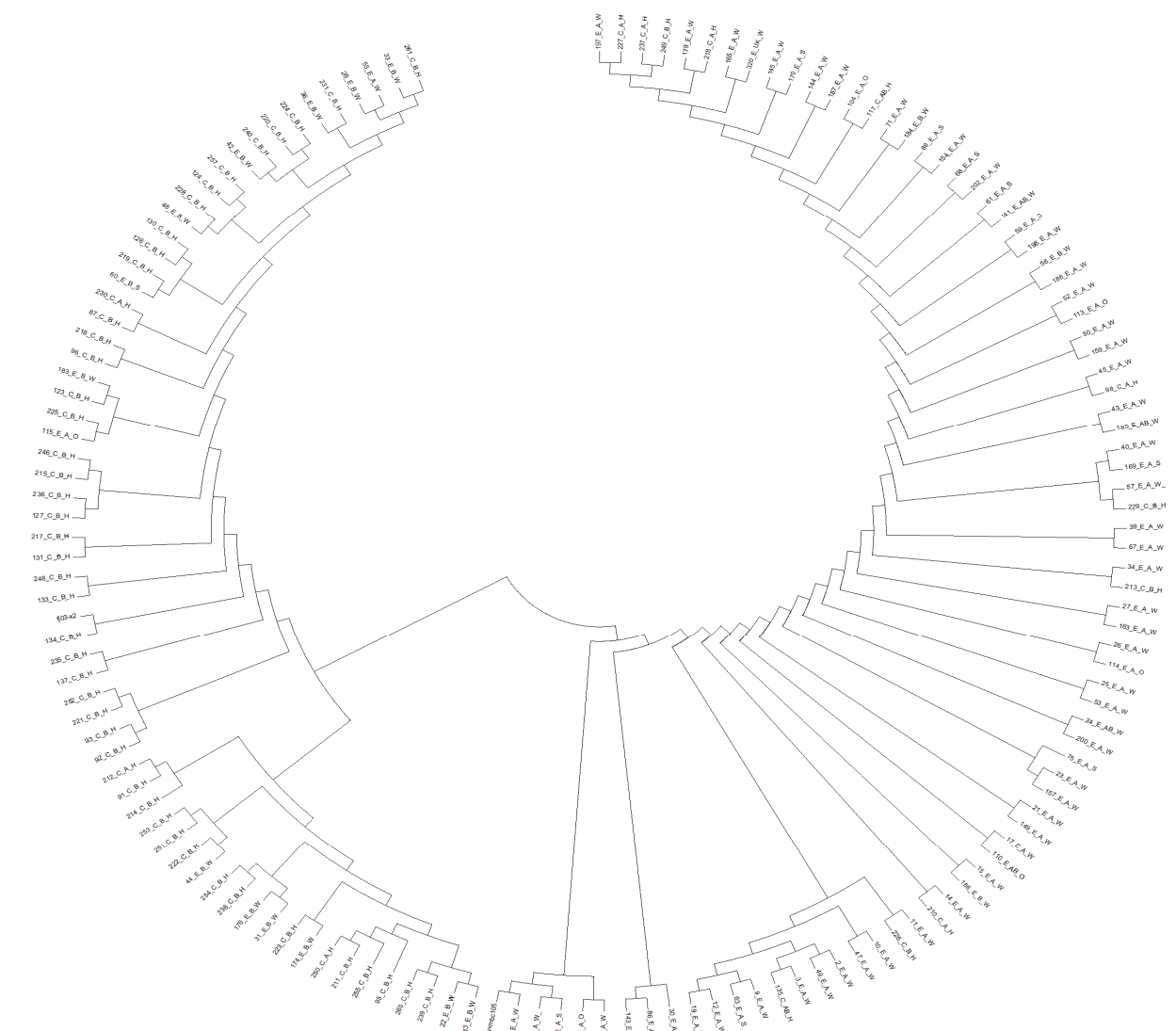
Figure A3. Alternate neighbor joining tree constructed using PHYLIP and 100 bootstrap replicates. Groups are separated by lines: environmental/type A (I), clinical/type B (III), and both (II). Names are as follows: isolate number, source type clinical (C) or environmental (E), 16S type A (environmental) or B (clinical) or AB, and isolate origin water (W), human (H), sediment (S), or oyster (O).

Figure A4. Phylogenetic tree constructed using MEGA V6.0 maximum likelihood method with 1000 bootstrap replicates. ClustalW2 was used for sequence alignment. Sub-groups (I-VI) are separated by lines. Names are as follows: isolate number, source type clinical (C) or environmental (E), 16S type A (environmental) or B (clinical) or AB, and isolate origin water (W), human (H), sediment (S), or oyster (O).
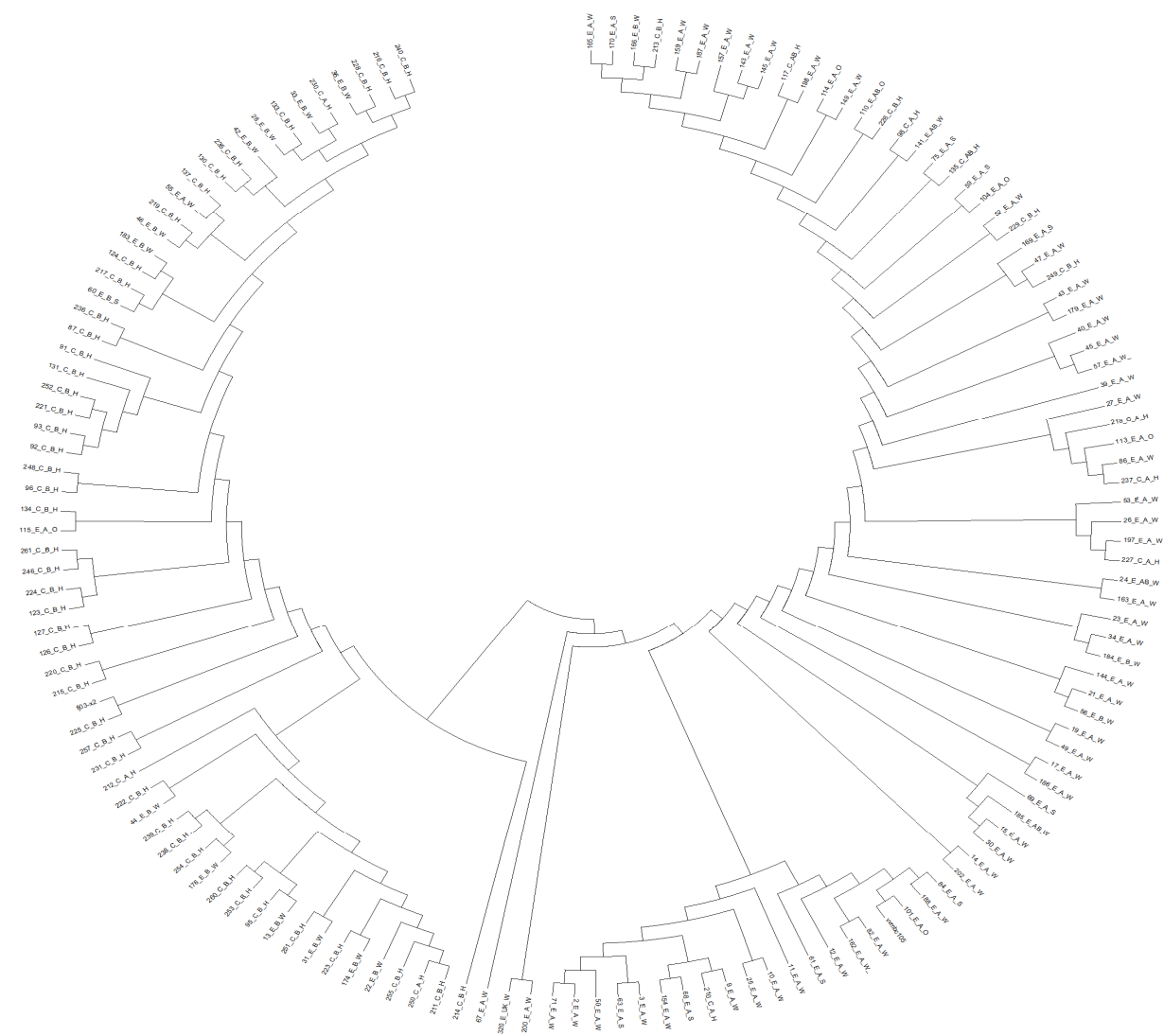
Figure A5. Phylogenetic tree constructed using MEGA V6.0 maximum likelihood method with 1000 bootstrap replicates. MUSCLE was used for sequence alignment. Names are as follows: isolate number, source type clinical (C) or environmental (E), 16S type A (environmental) or B (clinical) or AB, and isolate origin water (W), human (H), sediment (S), or oyster (O).
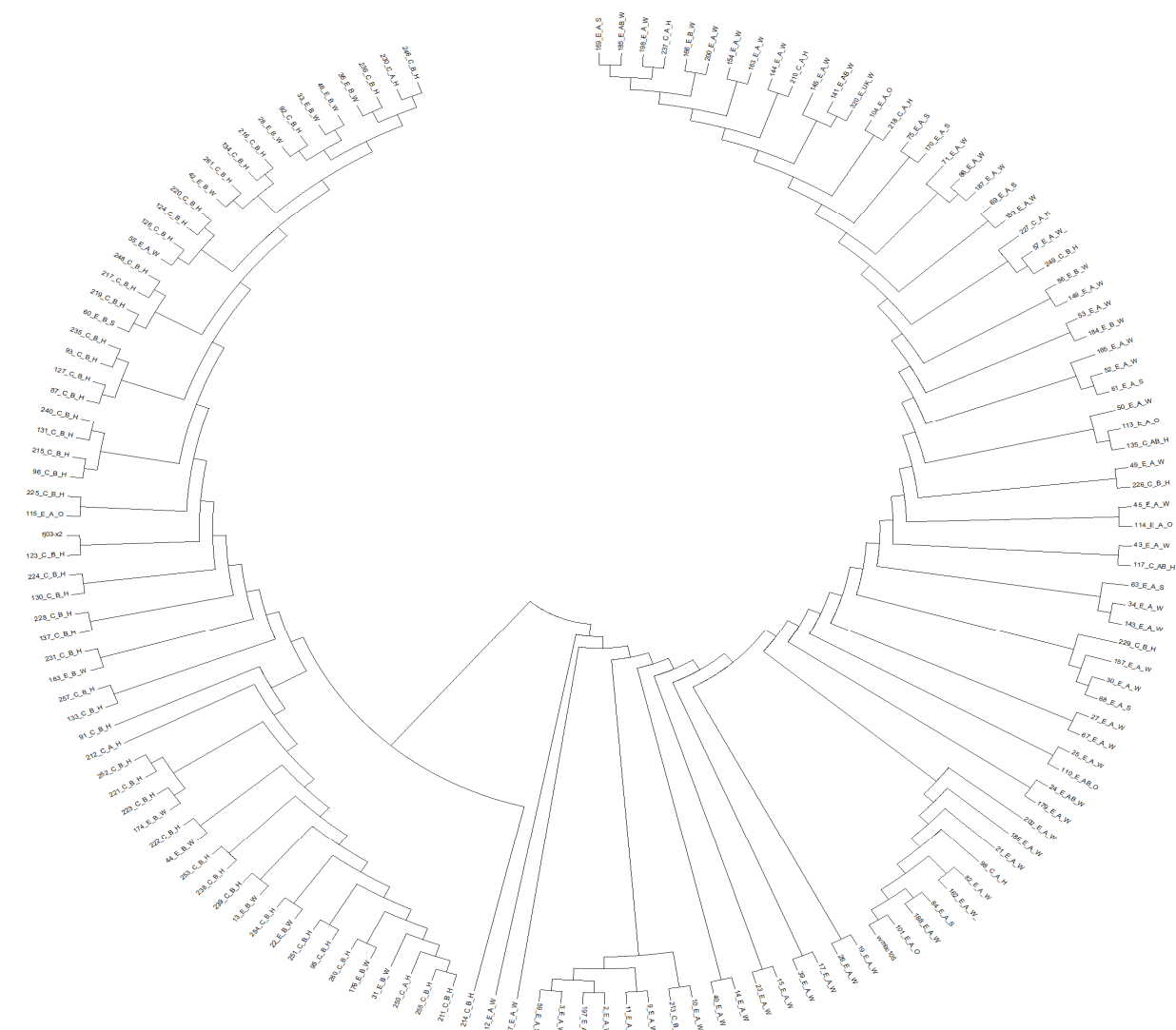
Figure A6. Phylogenetic tree constructed using MEGA V6.0 neighbor joining with 2000 bootstrap replicates. ClustalW2 was used for sequence alignment. Names are as follows: isolate number, source type clinical (C) or environmental (E), 16S type A (environmental) or B (clinical) or AB, and isolate origin water (W), human (H), sediment (S), or oyster (O).

Figure A7. Phylogenetic tree constructed using MEGA V6.0 neighbor joining with 2000 bootstrap replicates. MUSCLE was used for sequence alignment. Names are as follows: isolate number, source type clinical (C) or environmental (E), 16S type A (environmental) or B (clinical) or AB, and isolate origin water (W), human (H), sediment (S), or oyster (O).

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