# DETECTION OF *VIBRIO VULNIFICUS* PATHOGENIC ISOLATES THROUGH USE OF PRIMERS FOR THE SIGMA-38 GENE AND HEMOLYSIN/CYTOLYSIN GENE

A Thesis

by

VINOSHNA SAMA

BS, Sreenidhi Institute of Science and Technology, 2018

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This thesis meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

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December 2021

# ABSTRACT

*Vibrio vulnificus (V. vulnificus)* is a halophilic, asporogenous Gram-negative mesophilic bacterium belonging to the Gammaproteobacteria. It lives in marine and estuarine waters and grows at high levels in the Gulf of Mexico between April and October. Activation of virulence genes in *V. vulnificus* is regulated by various environmental factors, such as temperature, salinity, and pH. Transcription of these genes is regulated by a specific sigma factor, RpoS, encoded by the *rpoS* gene which is present in most members of Proteobacteria and is highly conserved. RpoS is seen under stress, starvation and stationary phase, and controls expression of the virulence factor gene, *vvhA*, which produces a cytolysin that leads to hemolysis of erythrocytes and leucocytes, causing release of iron. Previous primers designed to amplify *rpoS* no longer worked to amplify a critical and highly conserved essential gene, and the problem arose on how to detect this locus in *V. vulnificus.* This study allowed increased accuracy for correctly detecting pathogenic *V. vulnificus* isolates by l) redesigning *rpoS* primers to perform conventional end point polymerase chain reaction (PCR) of 28 *V. vulnificus* isolates; 2) redesigning *vvhA* primers to perform conventional end point polymerase chain reaction (PCR) of 28 *V. vulnificus* isolates; and 3) developing a multiplex PCR assay to simultaneously detect both *rpoS* and *vvhA* genes.

# ACKNOWLEDGMENTS

Thanks to God for his showers of blessings and for giving me the best, wonderful, and benevolent advisor. In a world where millions of girls are out of school. I am extremely fortunate to get a master's degree. I do not take this privilege for granted.

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# CHAPTER I

# INTRODUCTION

## Background and Relevance:

*V. vulnificus* is a Gram-negative halophilic bacterium inhabiting marine and estuarine aquatic environments (Froelich *et al.* 2013). This bacterium may be free-living or exist as a biofilm on the skin of finfish and on the shells of mollusks and crustaceans. *V. vulnificus* may also be a human pathogen (Jones and Oliver, 2009). Upon ingestion through undercooked seafood or entry into wounds it may cause three distinct syndromes: 1) primary septicemia; 2) wound infection, leading to necrotizing fasciitis; and 3) gastroenteritis. Primary septicemia is caused by consumption of raw shellfish (Oliver *et al.* 2007). When the organism enters the body, the infection will start developing into different stages with the symptoms such as swollen blisters, pain, and tenderness (Kuo *et al.* 2007). Necrotizing fasciitis is an inflammatory infection that occurs in fascia, but rapidly develops into the secondary stage of necrosis of the subcutaneous tissues. The initial stage of infection begins with an area of erythema at the site of infection. The skin will start forming a purpuric lesion that will then form fluid-filled blisters or ecchymoses and coalesce to form large hemorrhagic bullae leading to tissue death. An alternative to stop the contamination and limit the risk of mortality is to debride or even amputate the infected parts to prevent necrosis from spreading (Hollis *et al.* 1976; Klontz *et al.* 1988; Kuo *et al.* 2007). *V. vulnificus* belongs to the class Gammaproteobacteria. This genus of bacteria interacts with the different organisms in estuarine and marine habitats. Other *vibrio* species usually pathogenic for humans include *Vibrio cholerae* and *Vibrio parahaemolyticus* (Thompson *et al.* 2004). *V. vulnificus* is highly lethal and responsible for the majority of seafood-related deaths in the United States, which includes ingestion of shrimp, fish, and oysters (Feldhusen *et al.* 2000; Oliver *et al.* 2007).

# CHAPTER II

## BIOTYPES

*V. vulnificus* strains may be categorized in several ways. The species is classified by Biotype 1, Biotype 2, and Biotype 3. Most human infections are caused by Biotype 1 while Biotype 2 members are primarily eel pathogens. Biotype 3 is a hybrid of types 1 and 2, and this third biotype will cause human wound infections. *V. vulnificus* isolates may also be classified by genotypes, formerly classified as ecotypes. One such gene locus is the virulence-correlated gene *vcg*, whose function is yet defined, and consists of two possible alleles. These genotypes strongly indicate potential virulence (Rosche *et al.* 2005; Rosche *et al.* 2010). Another method for discrimination between *V. vulnificus* strains is to use the 16S rRNA gene and spacer regions (Aznar *et al.* 1994). Dissimilarities in the 16S rRNA gene will differentiate the clinical type (B) from the environmental type (A), i.e., A or B (Aznar *et al.* 1994; Vickery *et al.* 2007; Nilsson *et al.* 2003).

Virulence Factors of *V. vulnificus*:

Other factors related to the pathogenesis of *V. vulnificus* include capsular polysaccharide (CPS), cytotoxins, hydrolytic enzymes, and cytolysin (Amako *et al.* 1984, Gray and Kreger 1987; Hor and Chen 2013) as well as the production of lipase, protease, and collagenase (Moreno *et al.* 1998). Variation of *Vibrio* species to environmental changes, as well as to changes in their individual hosts, is basic to their survival as a planktonic organism. One key factor for natural endurance and transmission is the capacity to form biofilms (Yildiz and Visnick 2009), which might be a reaction to supplemental limitation of nutrients. Biofilms will initially form at nutrient rich surfaces but then detach when there is a decrease in the amount of nutrients available. In various members of the genus *Vibrio*, motility plays an important role in virulence (Milton *et al.* 1996; Yang *et al.* 2014). For example, adhesion, which is the first step in colonization, is lacking with the absence

of the flagellin genes in the non-motile *V. vulnificus* (Kim *et al.* 2014). CPS is an important virulence factor, as its role is to protect the bacteria from phagocytosis and allow it to survive in the host (Amako *et al.* 1984; Grau *et al.* 2008). It was discovered that there is variation in virulence between the encapsulated and the unencapsulated *Vibrio* (Wright and Morris *et al.* 1991; Wright *et al.* 1999). CPS promotes the adherence of the bacterium and is also responsible for the inhibition of non-specific host defense mechanisms such as complement and complement mediated opsonophagocytosis (Amako *et al.* 1984; Zuppardo and Siebeling 1998).This project focused on two major virulence factors: the alternative sigma factor RpoS and hemolysin/cytolysin VvhA.

Sigma Factor RpoS:

During transcription, the RNA polymerase holozyme must bind to a promoter region and the normal factor is the RpoD protein or sigma-70. Under different environmental stresses (e.g., pH changes, oxidative stress, hyposalinity, hyposmolarity, nutrient depletion), another sigma factor, RpoS or sigma-38, allows binding to genes expressed under those conditions. The role of RpoS in *V. vulnificus* was initially shown to be required for cells growing to stationary phase under many types of environmental stresses, including exposure to hydrogen peroxide, hyperosmolarity, and acidic conditions (Hulsmann *et al.* 2003), later confirmed with cells growing exponentially under oxidative stress (Park *et al.* 2004) and with cells growing under low salinity of 0.1-0.4% (Tan *et al.* 2010). *V. vulnificus* tolerates different levels of osmotic stress and can survive in hyperosmotic conditions (Hülsmann *et al.* 2003; Lee *et al.* 2006, ) and in this situation *rpoS* is expressed instead of RpoD (Hengge-Aronis *et al.* 2002). The role of RpoS during stress holds true for *V. vulnificus* and several other bacteria such as *Pseudomonas aeruginosa* (Suh *et al.* 1999) and *Escherichia coli* (Hengge-Aronis *et al.* 2002, Gawande *et al.* 2005). Indeed, there is strong correlation between the expression of virulence factors that require RpoS and general cellular stress responses (Dulebohn

*et al.* 2017), nutrient starvation (Aldsworth *et al.* 1999), oxidative stress (Gawande *et al.* 2005), ultraviolent radiation and osmotic stress (Berney *et al.* 2006), heat, and acid or alkaline treatment (Bhagwat *et al.* 2006). In the growth phase of the bacteria, *rpoS* will be expressed in the stationary phase (Jishage *et al.* 1995). RpoS also has the ability to control the expressions of other virulence genes such as elastase (vvpE), *hap*, *vvhA*. (Fang *et al.* 1992; Kowarz *et al.* 1994; Beltrametti *et al.* 1999; Hengge-Aronis *et al.* 2000; Hulsmann *et al.* 2003).

Hemolysin/Cytolysin VvhA:

One virulence gene that requires RpoS as a transcriptional factor is *vvhA*, which encodes a cytolysin-hemolysin cytotoxin of *V. vulnificus* (Kim *et al.* 2008). One of the most significant elements for bacterial survival and virulence is the ability of bacteria to acquire iron from its surroundings (Andrews 1999). The key role of this toxin is to cause lysis of erythrocytes and leukocytes, releasing iron that is then taken up by the bacterial cell via siderophores (Wright *et al.* 1991; Yokochi *et al.* 2013). Iron is the fundamental metal typically involved in all functions of the bacterium, and iron uptake occurs through siderophores. The role of iron is to form complex bindings with bacterial proteins as iron is a co-factor for numerous enzymes. To invade the host cell and initiate an infection, bacteria must rely on their capacity to survive in the host cell by utilizing iron bound to high-affinity iron binding proteins such as hemoglobin, methemoglobin, and hematin (Helms *et al.* 1984).

Genome Variability of *V. vulnificus* DNA:

*V. vulnificus* contains genomic islands (GI), which are large chromosomal regions obtained from other bacteria by horizontal gene transfer (HGT), including transformation and transduction. Acquisition of such large chromosomal regions unique to the strains of *V. vulnificus* suggests that this genus may obtain novel sequences to encode factors that enable the organism to survive

environmental changes (Baker-Austin and Oliver 2018). These GI did not enable previous investigators to differentiate pathogenic from non-pathogenic strains (Quirke *et al.* 2006). With greater numbers of isolates analyzed (Mullis *et al.* 2019); however, such comparisons might now be more feasible.

Initially, *V. vulnificus* was suspected to contain fewer genetic elements such as transposons and phages in addition to regions of divergent G+C content, which is indicative of HGT (Heidelberg *et al.* 2000; Makino *et al.* 2003). Further studies (Karaolis *et al.* 1994; Karaolis *et al.* 1995; Waldor *et al.* 1996; Boyd *et al.* 2000; Rowe Magnus *et al.* 2001; Rowe Magnus *et al.* 2002; Rowe Magnus *et al.* 2003) have confirmed HGT contributes to a few significant attributes of *Vibrios,* such as pathogenicity and the ability to function in the natural environment.

# CHAPTER III

## PRELIMINARY FINDINGS

As part of the genomic analysis, a preliminary comparative analysis was performed involving a gene comparison study on several of the *V. vulnificus* isolates, *Vibrio parahaemolyticus*, and *Vibrio alginolyticus* (described in Appendix E) identified in previous works using BLAST and other graphical user interface software (Altschul *et al.* 1990; Ramirez *et al.* 2009; Mullis *et al.* 2019). Using the rpoS gene from ATCC 27562 as a reference, a comparative analysis was performed to determine the percentage similarity between these isolates, and revealed they are 96 – 99% similar. To determine if the primers in (Table 1) annealed to this respective gene, a simple analysis of primers from Planas-Costas (2014) was performed by aligning the existing primers to the gene sequence. According to theory (Sambrook 2012), the selected sequences of the primers must be complementary to the complementary region of the target DNA, i.e., the forward primer binds to the template strand sequence, which is complementary to the target DNA, while the reverse primer binds to the coding strand, producing a strand identical to the template strand, which is complementary to the coding strand (the target DNA). So, a simple analysis was performed in which the forward primer must align to the template DNA sequence while the reverse primer aligns to the target DNA in a reverse and complementary manner. This analysis gave negative results for the existing primers, concluding that the primers are not aligning to the respective gene, as previously described.

Table – 1: Primers for end point PCR

| | Primers (Planas Costas - 2014) | |
|---|---|---|
| *vvhA* | 1418F 5'-AATTTGGGGAATGTGATGAACTGC-3' | 705 pmol/uL |
| *vvhA* | 2147R 5'-CGTTGGAAACCCACATTACA-3' | 895 pmol/uL |
| *rpoS* | 573F 5'-CGTGGTTTATTCGGGTAACG-3' | 804.5 pmol/uL |
| *rpoS* | 2004R 5'-GCTTCTAACGCTTGGCGTGG-3' | 836.5 pmol/uL |

Research Gap:

According to the initial description of isolates (Ramirez *et al.* 2009, these strains were considered as *V. vulnificus*. However, few of these isolates annealed to the respective gene using the *rpoS* primers previously designed. What could be the possible reasons? First, confusion over forward and reverse primer annealing. Second, technical aspects of performing the lysis procedure. This possibility was excluded by other investigators who were able to obtain the 1,431 bp amplicon, but not consistently; eventually a 301 bp amplicon was seen. Third, changes in the genome or plasticity of the DNA, as described by several investigators (Quirke *et al.* 2006; Baker-Austin and Oliver 2018). An in-silico study of the primers demonstrated that the *rpoS* primers should anneal to the 42 genomes sequenced by the Virginia Department of Health; however, this in-silico analysis revealed that the primers which are currently in use no longer annealed to the respective gene (Ramirez *et al.* 2009; Mullis *et al.* 2019). Fourth, the elongation step of PCR might be a problem for *Taq* polymerase, as the product length of the PCR primers is 1,431 bp. While previous investigators (Galvan 2009; Planas-Costas 2014) were able to obtain amplicons greater than 1400 bp, these results were not reproducible. Ideally, both forward and reverse primer length will be

approximately 18-20 nucleotides and during PCR the cycles will be repeated 25-35 times (Sambrook and Green, 2012).

Objectives:

Previous studies by Galvan (2009) and Planas-Costas (2014) have provided valuable information on *rpoS* through PCR analysis. The primers in these studies should anneal the gene forming an amplicon at 1,431 bp. When the work was repeated, the PCR amplicon obtained was at 301 bp. Therefore, this study will investigate why there is a change in the amplicon obtained with no change in the genome or plasticity of DNA. The following objectives will be accomplished.

1. Design new primers for *rpoS* gene of *V. vulnificus* using recent data from the Virginia Dept. of Health, the goal is to design primers with the product length ranging from 300-400 bp to perform end-point PCR that will anneal to 28 of the 42 isolates being examined as the remaining isolates were identified as other species of *Vibrio*.
2. Confirm the presence of *vvhA* gene in the new and old isolates of *V. vulnificus* by using newly designed primers.
3. Evaluate primer binding efficiency towards *vvhA* and *rpoS* genes using an in-silico approach.
4. Design the Multiplex PCR primers for *vvhA* and *rpoS* genes of *V. vulnificus*.

# CHAPTER IV

# MATERIALS AND METHODS

## Study Site

The isolates currently used in this study (Table 2) were part of a study done previously (Ramirez *et al.* 2009). These isolates were collected from different sites in the Corpus Christi region (Fig.11).

*Vibrio* isolates used in this study:

Table 2:Isolates from the Coastal Bend Region (Ramirez, 2008; Ramirez *et al.* 2009)

| Isolate | Location Source | Date of isolation | Actual Organism |
|---|---|---|---|
| ARA 0040407-39 | Aransas Bay | 07 April 2007 | *V. vulnificus* |
| ARA 0040407-40 | Aransas Bay | 07 April 2007 | *V. vulnificus* |
| ATCC 27562 | "Wildtype" American Type Culture Collection, Manassas, VA | N/A | *V. vulnificus* |
| BI 0607-1 | Bird Island | 07 June 2007 | *V. ostreicida* |
| BS 0607-5 | Bayside | 07 June 2007 | *V. vulnificus* |
| CB 1006-27 | Copano Bay | 06 October 2007 | *V. vulnificus* |
| NB 0507-7 | Nueces Bay | 07 May 2007 | *V. vulnificus* |
| BS 0507 - 11 | Bayside | 17 May 2007 | *V. vulnificus* |
| BS 0507 - 16 | Bayside | 17 May 2007 | *V. vulnificus* |
| BS 0607 - 31 | Bayside | 13 June 2007 | *V. vulnificus* |
| CB 0507 - 20 | Copano Bay | 21 May 2007 | *V. vulnificus* |
| CB 0507 - 16 | Copano Bay | 21 May 2007 | *V. vulnificus* |
| CB 0507 - 30 | Copano Bay | 21 May 2007 | *V. vulnificus* |

| | | | |
|---|---|---|---|
| CB 0607 - 2 | Copano Bay | 13 June 2007 | *V. vulnificus* |
| CB 0607 - 9 | Copano Bay | 13 June 2007 | *V. vulnificus* |
| CB 0707 - 35 | Copano Bay | 11 July 2007 | *V. vulnificus* |
| CB 0707 – 42 | Copano Bay | 11 July 2007 | *V. vulnificus* |
| CB 0707 – 82 | Copano Bay | 11 July 2007 | *V. vulnificus* |
| CB 0707 – 91 | Copano Bay | 11 July 2007 | *V. vulnificus* |
| CB 1006 – 12 | Copano Bay | 11 October 2006 | *V. vulnificus* |
| CB 1006 – 49 | Copano Bay | 11 October 2006 | *V. vulnificus* |
| CP 0607 – 10 | Cole Park | 11 June 2007 | *V. alginolyticus* |
| CP 1006 - 27 | Cole Park | 4 October 2006 | *V. vulnificus* |
| CP 1006 - 37 | Cole Park | 4 October 2006 | *V. vulnificus* |
| NB 0507 - 13 | Nueces Bay | 9 May 2007 | *V. vulnificus* |
| NB 0507 - 8 | Nueces Bay | 9 May 2007 | *V. vulnificus* |
| NUE 0400707 - 7 | TGLO site on North Beach | 17 July 2007 | *V. vulnificus* |
| RB 0407 - 13 | Redfish Bay | 11 April 2007 | *V. vulnificus* |
| RB 0906 - 57 | Redfish Bay | 13 September 2006 | *V. vulnificus* |

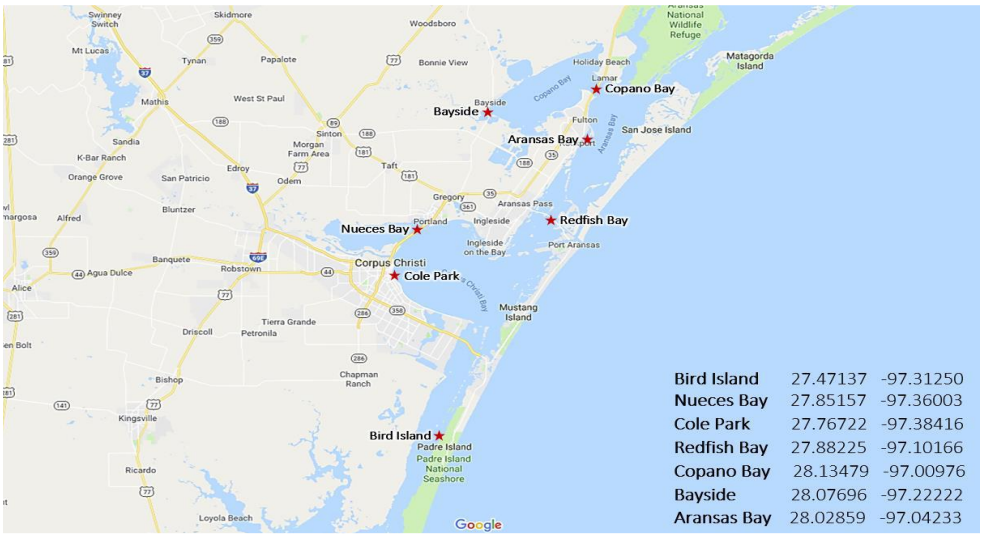


Figure 1: Map of study sites from where isolates were previously sampled (taken from Ramirez 2008; Ramirez *et al.* 2009; Planas-Costas 2014) .

Methodology

Sample Collection:

Sample collection was originally performed from August 2006 – July 2007 as previously described (Ramirez 2008; Ramirez *et al.* 2009). Samples were stored as 1 mL polypropylene tubes with 0.5 mL saturated overnight cultures with 50% (wt/vol) glycerol. These samples were removed from cryogenic storage and were revived onto LBL2N agar slants, then into LBL2N broth and streaked onto fresh TBCS plates as described below.

Regrowth of *vibrio* isolates from cryopreservation:

From the cryogenically preserved bacteria, 10 µL were streaked on to the slants containing Luria-Bertani Lennox agar containing 2% NaCl (LBLA 2N) loosely capped and incubated aerobically 37 °C for 16-24 hours. From the slants a loopful of bacteria wase inoculated in duplicates into Luria-Bertani Lennox with 2% NaCl (LBL2N) liquid media and capped loosely. These tubes were aerobically incubated at 37 °C for 16-24 hours.

Preparation of Bacterial crude isolates for PCR analysis:

The crude lysates were prepared as described (Parvathi *et al.* 2005). Briefly, 500 µL of saturated overnight culture of *V. vulnificus* were centrifuged in a 2 mL microfuge tube at 12,000 xg for 10 min. Cultures were washed with 1X TE buffer and the pellet resuspended in 100 µL of 1X TE buffer followed by a boiling step at 100°C for 10 min. Microfuge tubes were flash cooled at 4°C before centrifugation at 12,000 xg for 10 min. Supernatant was discarded and 50 µL of Triton X-100 solution added to the pellet and the boiling step repeated before a spin at 12,000 xg for 5 min. The supernatant of 37 µL was collected, and two microliters added to 18 µL PCR reaction tubes. PCR assay was performed according to the revised procedure by Dr. Gregory Buck, Githzette Planas-Costas & Joshua Carbaugh (unpublished). The PCR conditions were provided in Table 5.

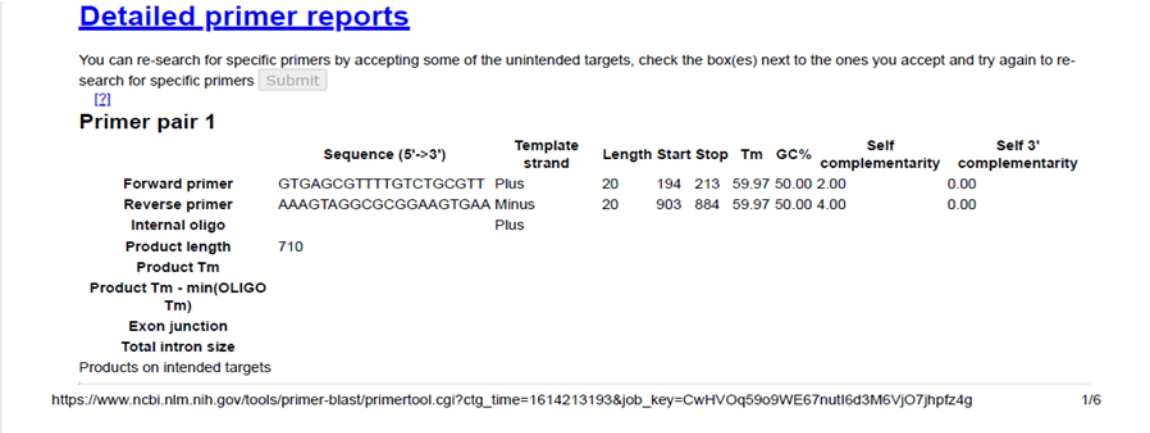
PRELIMINARY STUDIES: INFORMATICS AND IN-SILICO PCR

The purpose of this preliminary study was the detection of the *rpoS* and *vvhA* genes by using conventional end point PCR. The previous primers used by (Galvan 2009; Planas-Costas 2014) were found not to align to the respective gene after multiple trials. An in-silico PCR was also performed to confirm this observation. The primers were redesigned for optimal alignment to the respective gene.

Primer design:

Primers play a vital role in the PCR technique. *Taq* polymerase and other important components are also crucial. These components will only initiate the replication process, primers are the vital elements that give the desired product. To design primers for *rpoS* (Sambrook and Russell, 2001, p.8.14), these procedures will be followed. The tool used to design primers was NCBI-Primer-Blast. The target gene sequence was uploaded in the FASTA format in the PCR template section. All the PCR parameters were set by default. In the Primer Pair Specificity Checking Parameters

database, the field was changed to “required option,” and the required organism *V. vulnificus* was given in the field option called “organism”. The output was a graphical view of primer pairs and detailed primers report (Fig. 2). The detailed report consisted of the sequences of forward and reverse primers, length of the primers (bp), product length (bp), start, stop, G-C Content (%), melting temperature (°C) and self-complementarity. Primers were selected based on these factors.



**Detailed primer reports**

You can re-search for specific primers by accepting some of the unintended targets, check the box(es) next to the ones you accept and try again to re-search for specific primers Submit
[2]

**Primer pair 1**

| | Sequence (5'->3') | Template strand | Length | Start | Stop | Tm | GC% | Self complementarity | Self 3' complementarity |
|---|---|---|---|---|---|---|---|---|---|
| Forward primer | GTGAGCGTTTTGTCTGCGTT | Plus | 20 | 194 | 213 | 59.97 | 50.00 | 2.00 | 0.00 |
| Reverse primer | AAAGTAGGCGCGGAAGTGAA | Minus | 20 | 903 | 884 | 59.97 | 50.00 | 4.00 | 0.00 |
| Internal oligo | | Plus | | | | | | | |
| Product length | 710 | | | | | | | | |
| Product Tm | | | | | | | | | |
| Product Tm - min(OLIGO Tm) | | | | | | | | | |
| Exon junction | | | | | | | | | |
| Total intron size | | | | | | | | | |

Products on intended targets

https://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi?ctg_time=1614213193&job_key=CwHVOq59o9WE67nutI6d3M6VjO7jhpfz4g 1/6

Figure 2: Screenshot of the detailed primer report produced by NCBI’s Primer-Blast.

In silico PCR:

In-silico PCR is the technique used to perform virtual or electronic PCR of DNA sequences or gene sequences in programming tools such as Fast PCR (Kalendar *et al.* 2011; Kalendar *et al.* 2017), is the tool which was utilized here and is one among many tools such as UCSC In-silico PCR (Kent WJ *et al.* 2002), In-silico PCR amplification (Bikandi *et al.* 2004). This tool tested both single primers and multiple primers concerning the whole genome or gene in a single attempt and also accepted primers of various lengths. Multiple hits for both forward and reverse primers were in-silico PCR whole DNA of the species or respective gene and the primers designed for the gene were required to submit in the tool in the required format.

Gene comparison analysis:

The main goal was to design primers for end point and for multiplex PCR. A gene comparative analysis was performed for *rpoS* on the list of the genomes as listed in (Mullis *et al.* 2019). The desired gene segments were retrieved from all the genomes and a BLAST search performed to determine the match percentage between the genes by using NCBI Blast.

Informatics:

System Information: All the informatics analysis were performed on a High-Performance Linux operating computer with domain name hpcm01.tamucc.edu with 64-bit architecture having 96 CPUs with 2 threads per core and Intel (R) Xenon (R) processor.

Finding the primer binding efficiency:

Initially, four strains were selected from 42 genome sequences. Whole genome sequences were downloaded from the NCBI database accessed from [https://www.ncbi.nlm.nih.gov/sra?linkname=bioproject_sra_all&from_uid=47526] and extracted to .fna or fasta format and these four genomes were further used as the reference sequences in this experiment. The *vvhA* coding sequence was retrieved from NCBI nucleotide database accessed from the following [https://www.ncbi.nlm.nih.gov/nuccore/155311; https://www.ncbi.nlm.nih.gov/gene/61923430] with a GenBank accession number M34670.1 in a Fasta format; similarly, *rpoS* gene sequences were retrieved from NCBI accessed from [https://www.ncbi.nlm.nih.gov/genome/?term=txid1219061[Organism:noexp] with a Gene ID 61923430 to a fasta file. At the start, *vvhA* gene sequence was aligned to the whole genome sequence of the *V. vulnificus* ATCC 27562 strain [https://www.ncbi.nlm.nih.gov/sra?linkname=bioproject_sra_all&from_uid=475262] to get the actual *vvhA* and *rpoS* gene sequence that was eventually used in the further analysis. After getting

both sequences using the custom range option in the NCBI with the help of the blast output coordinates, a few nucleotide blast searches were performed against the four selected strains and the exact aligned sequence was retrieved using the specific coordinates in all the four blast results for both *vvhA* and *rpoS* genes.

Table 3: *V. vulnificus* strains used to check primer binding efficiency.

| Sequence No. | Strain ID | WGS ID | Submitter ID |
|---|---|---|---|
| 1 | RCGD01000000.1 | VA-WGS-18022 | ARA0040407-39 |
| 2 | RCGC01000000.1 | VA-WGS-18023 | BI0607-1 |
| 3 | RCFU01000000.1 | VA-WGS-18031 | BS0607-5 |
| 4 | AMQV01000000.1 | ATCC 27562 | Not applicable |

Analyzing the % identity or mutations in *vvhA* and *rpoS* genome locations:

Mutations and the % identity at *vvhA* and *rpoS* genome locations were analyzed in all the 42 genomes (Mullis *et al.* 2019) using the simple blast alignment. Initially, all the 42 genome assemblies were downloaded from the NCBI on the Linux machine. The same *vvhA* and *rpoS* gene sequences were used as query sequences in the blast search that were previously used in the (Table 3) above. Here, the blast search was automated using a simple bash scripting which resulted in all the blast results at once and the blast output result was used to study the % identity and mutation rate in all the 42 genomes.

```
#/usr/bin/bash
for i in ncbi-genomes-2021-03-15/*.fna
do
        out=${i%%_genomic.fna}
        makeblastdb -in $i -out databases/$out -dbtype nucl -parse_seqids
        blastn -query vvha.fasta -db databases/$out -out $out.out -outfmt 6 >> all_genome_blast_vvha.txt

done
```

Figure 3 : Bash script for blast search using *vvhA* vs 42 genomes.

```
#/usr/bin/bash
for i in ncbi-genomes-2021-03-15/*.fna
do
        out=${i%%_genomic.fna}
        makeblastdb -in $i -out databases/$out -dbtype nucl -parse_seqids
        blastn -query rpos.fasta -db databases/$out -out $out.out -outfmt 6 >> all_genome_blast_rpos.txt

done
```

Figure 4 : Bash script for blast search using *rpoS* vs 42 genomes.

Implementing Blast command-line:

Building a custom database with (local) sequences

Command-1: makeblastdb -in reference.fasta -out database -dbtype nucl -parse_seqids

- makeblastdb - produces BLAST databases from FASTA file.
- reference.fasta - this file contains a set of fasta sequences that are used as reference sequences.
- database - database name
- nucl – referring to nucleotide database, database type.

Performing blastn search

Command-2: blastn -query query.fasta -db database -out results.out -outfmt 6

- query.fasta – query sequences against reference.fasta sequences.
- result.out – blast output file that contains results.

- outfmt – blast output format

Table 4: Output format for blast

| outfmt | Description |
|---|---|
| 0 | pairwise |
| 1 | query-anchored showing identities |
| 2 | query-anchored no identities |
| 3 | flat query-anchored, show identities |
| 4 | flat query-anchored, no identities |
| 5 | XML Blast output |
| 6 | tabular |
| 7 | tabular with comment lines |
| 8 | text ASN.1 |
| 9 | binary ASN.1 |
| 10 | comma-separated values |
| 11 | BLAST archive format (ASN.1) |

Primer Analysis using primers:

To perform the primer analysis the required gene sequence was obtained from the NCBI. This is the sequence described earlier (Yamamoto *et al.* 1990). In the previous studies conducted, the primers were designed with reference to this sequence (Planas-Costas 2014). The primers were designed by using NCBI PRIMER BLAST tool.

>AMQV01000194.1:164337-165753 *Vibrio vulnificus* NBRC 15645 = ATCC 27562 B736contig194, whole genome shotgun sequence

CTAGAGTTTGACTTGTTGTAATGTGGGTTTCCAACGCGCCTGAGTTGCTTCATTTTCAGGGGTTACTTGA
ACATTACGGCCACCGACAATGTTTAGAAGGTAGCGAGTATTGTTGCCATCAACATAGCGGCTGATGAGCT
TATCGCCTTCCCAATACCATTTCTGTGCTAAGTTCGCACCACACTGTTCGACTGTGAGCGTTTTGTCTGC
GTTTACGGTCAAACAACGATCGGATGCCACTCGGCTACGGTAACGTTCTTCTTTATCTAGGCCCCAAACT
TGGTTCCAACTGCCGTGACAGCTCCAGCCGTTAACCGAACCACCCGCAACCGTTTTGTCACCGTTCTCAC
CATAAACATCTAGGCAGAGATCGTTGTTGCTCAGTGATTGTAGTGTAACGTGTGCTTCCGCTTCAAACAG
TGGATGATTCCAGTCGATGCGAATACGTTGTTTCACGGTACTGCTGTTGGTTGACGAGCCCGCAGAACCA
TAAACTGAGAAGAGTGCTGAAGGGATTACCGTACCAAAGCGTGCACGATAGTTGAGTTTCACGCCCATCT
CGAAATCCGTTACGCCGGTTTCAGACACAGGCGCTTCGTACAAAACGTCATAGTTCGGTTTGAAGTTGGA
ATAAGAGATTGGGTTGAACTTCGTCTTATCAAATACCCAGCCACTGCCCCAGTGAGCGGCGGTGAAATAG
CATCCAAGCTCTTGGCGGCGCAGTTCATCACACTCACCAAACTCACGCTCGAATGAAATATCAAAATCAC
TCAGTGATGAGCGGTTGTTGATGCGATAGTCTTTTGTATCAAACACCAAGGTTTTCGAGTAGTTGTAAGT
AAATGAGCCACTGACTTCGCCACCTACTTTCGGGCCGTCTTTGTTCACTTCCGCGCCTACTTTACCGTTA
ATACCGATGGAGTAACCGTAAGTATCACGGTGTTGGTAGTTTTTGTTCTCATTCTGCGGTAGGTCACGCG
CTTTTTTCGGTGTGTAACCCGAAACCGGTTTCACCCAAAGGTCATAACTGCTGGCGAATGGGCCAATGTA
AGTGCGGCGGTTTGCCCAACTCTGGAACCAACTGTGATCTTGCTGTAGCTCGTTAACCAAATGGATACCC
GTGCCAGGCTTGTCGGCATCGACGGTAAAGCGGACAATTTTGGCGTCAGGAGTAAAACCATCTGACGTTG
CCGATTGCACCGAGCGCATCTGTGCCACATTGACGCGAACATCGATGGAAGCGCCCGCATTACACCAGTC
TCGTGTGGCGTTGAAATCACCACTTGTGTGTAGCACGCACTTAATTTTTGAGCTGGTGATATAAATAGGT
TTCTCAACAATCGGCACATATTCTTGTGCGCCAACCTGTACCGCGGTAGCTAAAAGAGAAAGGGTAAACA
GAGTTATTTTTTTCATC

Figure 5: Sequence of *V. vulnificus* ATCC 27562 DNA from 194th contig, containing *rpoS*.

*vvhA* retrieval:

To design the primers, the *vvhA* sequence from ATCC27562 was used as reference gene and retrieved by using BLAST tool. The whole genome sequence of ATCC27562 was sequenced previously (Li *et al.* 2012). To retrieve the target gene sequence *vvhA* from ATCC27562, its accession number must be given in the NCBI database. Using blast command line on the Linux platform the required sequence can be retrieved (Fig. 5).

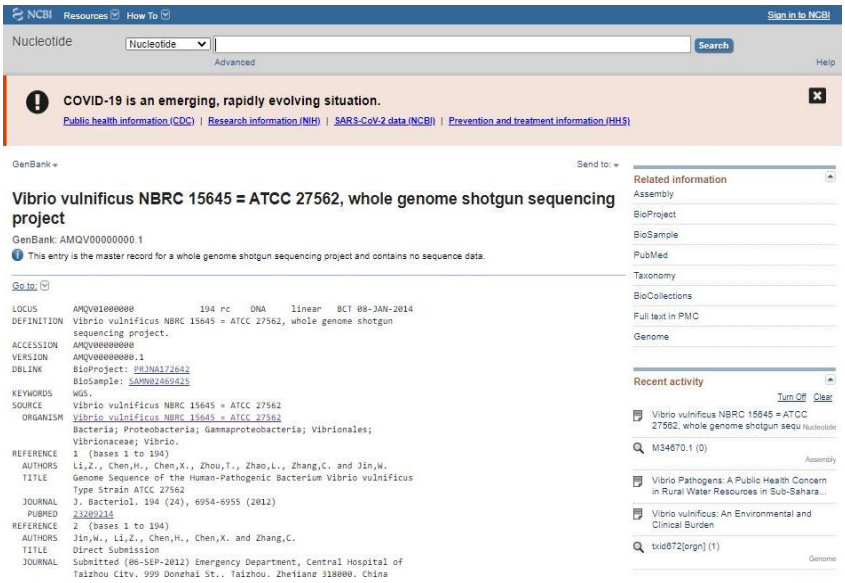



Figure 6: *V. vulnificus* ATCC 27562 whole genome shotgun sequencing project retrieval from NCBI

This whole genome was downloaded by using BLAST commands on the Linux platform (Fig. 5). From the output it is analyzed that *vvhA* lies in 194th contig out of 194 in ATCC27562 (Fig. 3). Now, by directing to whole genome shotgun sequencing project in NCBI, the 194th contig was downloaded (Fig. 6). With the output, the exact range of sequence of *vvhA* can be determined. In the NCBI after directing to the ATCC 27562 whole genome shotgun sequencing project, the option is then entered with the range of sequence to display (Fig. 7) and the target gene sequence of *vvhA* from ATCC27562 can be downloaded from the NCBI.

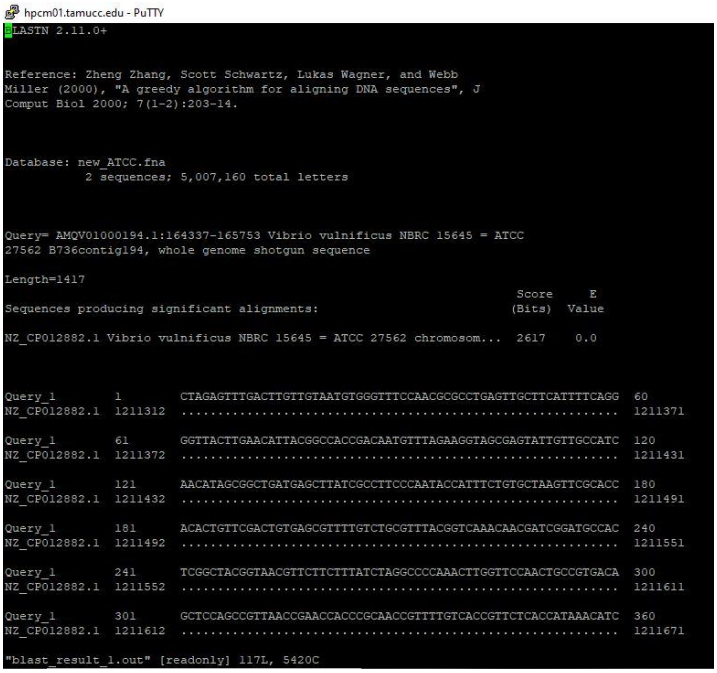



Figure 7: *vvhA* gene range of sequence identification in the whole genome on Linux

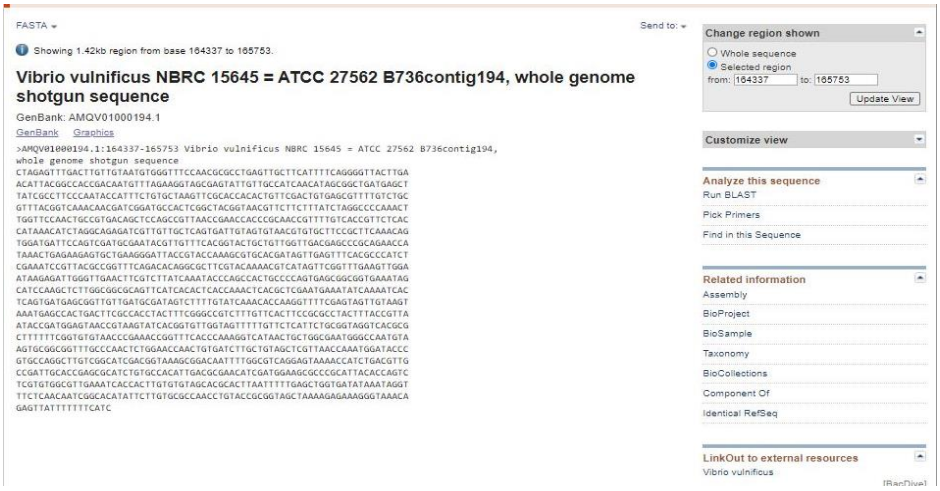



Figure 8: *vvhA* gene retrieval from NCBI (after giving the exact range on the right-hand corner.)

# CHAPTER V

## PRIMER DESIGNING

To design primers with respect to the target gene, NCBI PRIMER BLAST tool was used. In this tool gene sequence was pasted in PCR template (Fig. 8). In the primer parameter section and exon-intron section all the option will be default. But under the primer pair specificity check parameters (Fig. 8) database must be set to nr(non-redundant) and "organism" as required.

Example: *Vibrio vulnificus* and hit get primers.

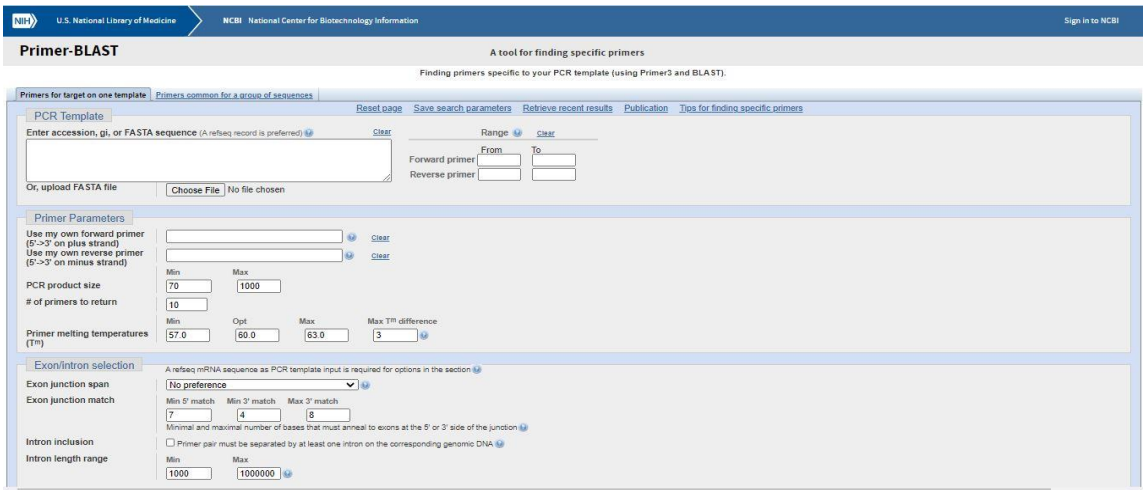



Figure 9 : NCBI primer Blast tool

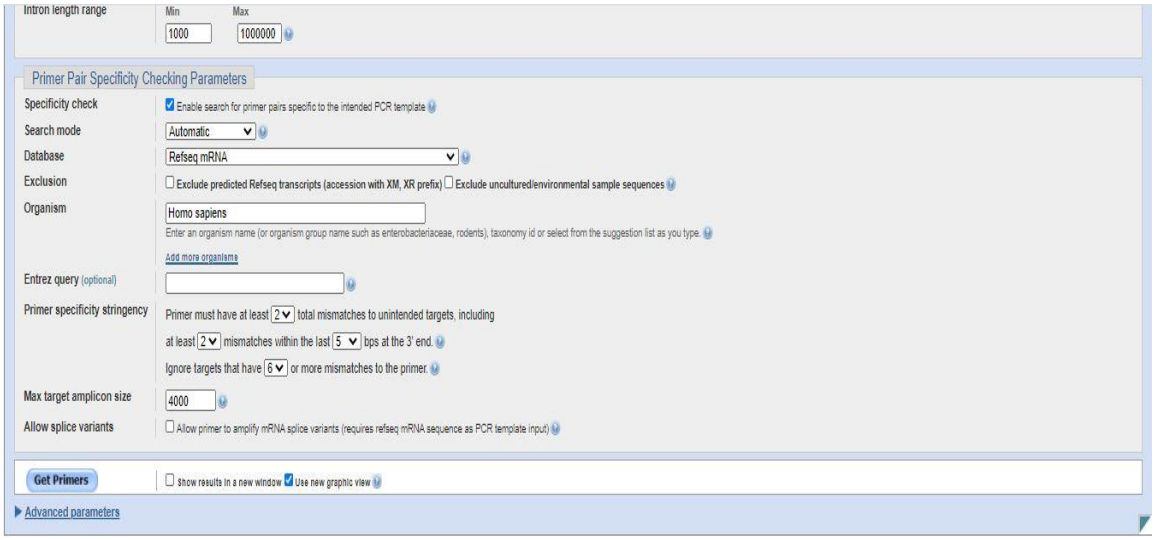



Figure 10 : NCBI Primer Blast tool

Output:

Ten pairs of primers sets were exhibited with the graphical view of primers, followed by a detailed report of primers.

Primer Dilution:

Primers were ordered from Eurofins Operon (Louisville, KY, USA) as 50 nanomolar scale lyophilized salt-free stocks. Just before use, primers were diluted with deionized water from the stocks to make a working solution of 10 µM.

Table 5: Preparation for master mix:

| Components | Final Volume |
|---|---|
| 5x Green Go Taq Reaction Buffer | 4.0 µL |
| dNTPs (deoxyribonucleotidetriphosphates) | 0.3 µL |
| *rpoS* 573F Forward Primer | 0.2 µL |
| *rpoS* 2004R Reverse Primer | 0.2 µL |
| dI Water | 15.1 µL |
| Go Taq DNA polymerase | 0.2 µL |

Single-plex Polymerase Chain Reaction (PCR) Assay:

Polymerase chain reactions (PCR) consisted of either single-plex or multiplex. The former was performed as described previously (Galvan 2009; Sambrook and Green 2012; Planas-Costas 2014; Nilsson and Turner 2016) using the re-designed primers and following the protocol described (Parvathi *et al.* 2005) as defined in Table 5 & 6 will be the conditions for PCR. Polymerase chain reaction was performed by adding 2.0 µL of lysates to 18.0 µL of PCR mix in clean PCR tubes,

which were placed into the Bio-Rad Laboratories PCR Thermal Cycler T100™ to obtain amplified DNA and the amplicons are expected as mentioned in Table 8.

Multiplex Polymerase Chain Reaction (PCR) Assay with both *vvhA* and *rpoS*:

Multiplex PCR assays were developed for *vvhA* and *rpoS* as described (Neogi *et al.* 2010).Polymerase chain reaction was performed by adding 2 µL of lysate and 18 µL of master mix and placed in the Bio-Rad Laboratories PCR Thermal Cycler T100™. As suggested by a graduate student in the Turner lab, single-plex PCR with the new primers was successfully attempted then multiplex experiments were performed at varying melting temperatures as described in (Hailey Wallgren, pers. comm.).

Table 6: Polymerase Chain Reaction Conditions for *rpoS*

| PCR Conditions | | Time |
|---|---|---|
| Denaturation | 94 °C | 10 Minutes |
| | 94 °C | 40 Seconds |
| Annealing | 54 °C | 45 Seconds |
| Extension | 72 °C | 45 Seconds |
| Final Extension | 72 °C | 10 minutes |
| Soak | 4 °C | Infinite |
| 36 Cycles | | |

Table 7: Polymerase Chain Reaction Conditions for *vvhA*

| PCR Conditions | | Time |
|---|---|---|
| Denaturation | 94 °C | 10 Minutes |
| | 94 °C | 40 Seconds |
| Annealing | 54 °C | 45 Seconds |
| Extension | 72 °C | 45 Seconds |
| Final Extension | 72 °C | 10 minutes |
| Soak | 4 °C | Infinite |
| 34 Cycles | | |

Table 8: Primers for end-point PCR.

| Genes | Primers | Amplicon size (bp) |
|---|---|---|
| *vvhA* | 887F 5'- CTTCCGCGCCTACTTTACCG-3' | 416 |
| *vvhA* | 1152R 5'-CGCTTTACCGTCGATGCCGAC-3' | 350 |
| *rpoS* | 5783F 5'-CGTGGTTTATTCGGGTAACG-3' | 337 |
| *rpoS* | 6176R 5'-GCTTCTAACGCTTGGCGTGG-3' | 333 |
| | Primers (Planas Costas - 2014) | |
| *vvhA* | 1418F 5'-AATTTGGGGAATGTGATGAACTGC-3' | 705 |
| *vvhA* | 2147R 5'-CGTTGGAAACCCACATTACA-3' | 895 |

| | | |
|---|---|---|
| *rpoS* | 573F 5’-CGTGGTTTATTCGGGTAACG-3’ | 804.5 |
| *rpoS* | 2004R 5’-GCTTCTAACGCTTGGCGTGG-3’ | 836.5 |

Table 9: Amplicon expected for Endpoint PCR.

| Genes | Amplicon Size (bp) |
|---|---|
| *vvhA* 887 F<br>*vvhA* 1152 R | 265 |
| *rpoS* 5783 F<br>*rpoS* 6176 R | 393 |

Gel Electrophoresis Conditions:

The final product of PCR was electrophoresed on 2% agarose gel containing 0.5 µg/mL ethidium bromide in 1X TAE buffer by using a molecular weight marker ranging from 100 – 1400 bp.

# CHAPTER VI

# RESULTS

## IN-SILICO ANALYSIS

### Finding the primer binding efficiency

Blast results were obtained in a tabular format for all four strains. The blast output consists of parameters such as start and end coordinates in query and the reference genome, e-value, mismatches, percent identity, and length of the aligned region. It was observed that the % identity was exactly same across the four strains with the *vvhA* as the query.

```
sstart - Start position in strain
send - End position in strain
qstart - Start positon in rpos gene
qend - End positon in rpos gene

Strain Name  vvha_ATCC  % iden  Length_of_alignment  Gaps sstart send qstart qend e_value NULL


AMQV01000194.1  1       95.763  1416    60      0       164337  165752  1416    1       0.0     2283
RCFU01000005.1  1       95.763  1416    60      0       724     2139    1       1416    0.0     2283
RCGC01000006.1  1       95.763  1416    60      0       635     2050    1       1416    0.0     2283
RCGD01000003.1  1       95.763  1416    60      0       371075  372490  1416    1       0.0     2283
```

Figure 11: Blast search using *vvhA* and four strains.

With the *rpoS* gene as the query sequence, it was observed that the percent identity score slightly varied from 97 – 100 % and the alignment length was found to be 501. It was also evident that the ATCC strain exactly mapped with the *rpoS* gene sequences with no mismatches or gaps.

```
sstart - Start position in strain
send - End position in strain
qstart - Start positon in rpos gene
qend - End positon in rpos gene

Strain Name  rpos_ATCC  % iden  Length_of_alignment  Gaps sstart send qstart qend e_value NULL

RCGD01000008.1  1       97.405  501     13      0       75874   76374   1       501     0.0     854
RCGC01000004.1  1       99.002  501     5       0       408849  409349  501     1       0.0     898
RCFU01000010.1  1       98.004  501     10      0       70455   70955   501     1       0.0     870
AMQV01000110.1  1       100.000 501     0       0       5605    6105    1       501     0.0     926
```

Figure 12: Blast search using *rpoS* and four strains.

Finally, the start and end coordinates in the reference that were observed in the blast output used and the genome sequence present in that specific region was retrieved using the custom range option in NCBI. This range was applied to a specific contig that was observed in the blast output. Later, primer sequences were used to check the binding capacity to all four strains and reported in Table1.

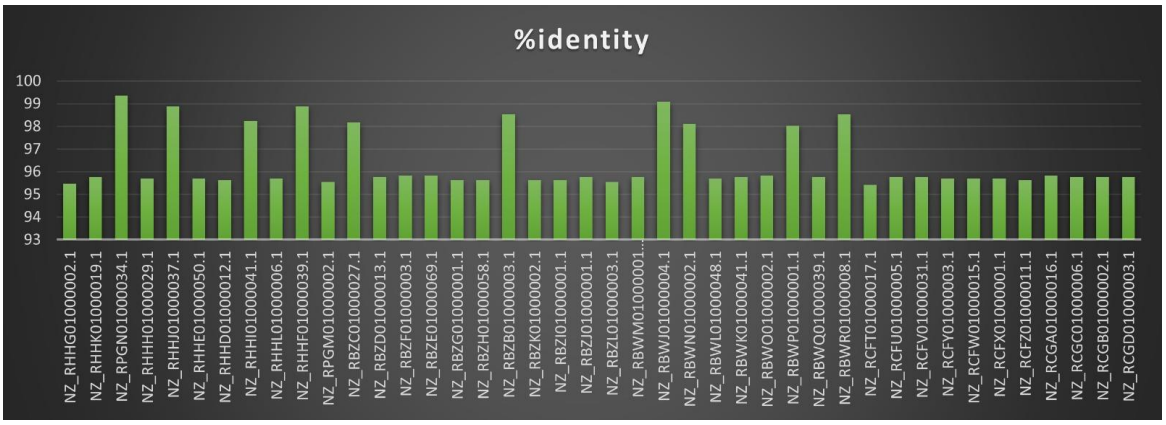


Figure 13: Bar chart showing %identity of *vvhA* in all 42 genomes.

Thirty two out of forty-two genomes are showing that the percent identity ranged between 95 – 96% and in three genomes, was observed at 99%.

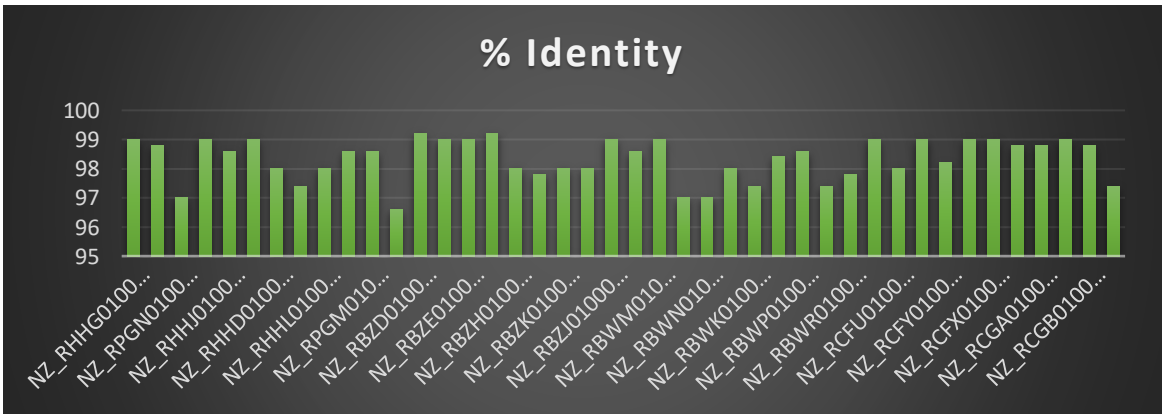


Figure 14: Bar chart showing %identity of *rpoS* for all the 42 genomes.

In-silico PCR:

The whole genome sequence or respective gene sequence was submitted as the query. In the second step, designed primers were submitted. The results report shows the positions of the primer alignment with respect to melting temperature. Fig. 15, shows the position and percentage of alignment with the forward primer and the position and percentage of alignment with the reverse primer. It also displays amplicon size which will be obtained when performing PCR.

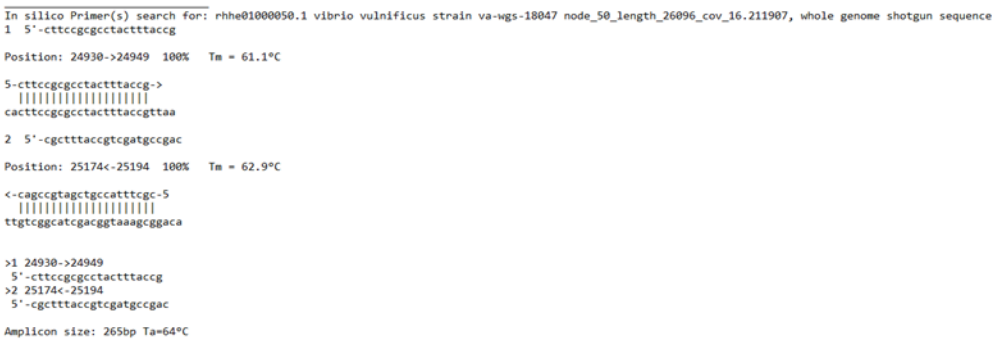



Figure 15: The above figure demonstrates the *vvhA* gene primers alignment with the *vvhA* gene of *V. vulnificus* strain CB 0607-9.

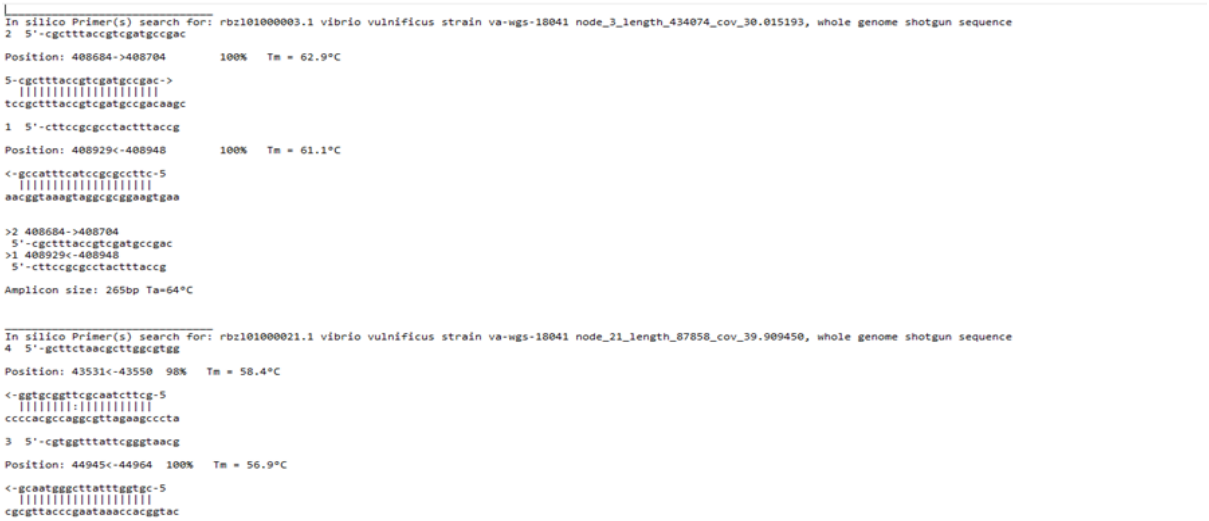



Figure 16: The above figure demonstrates the in-silico multiplex PCR of *vvhA* and *rpoS* gene primers alignment with the *vvhA* and *rpoS* genes of *V. vulnificus* in strain CB 0607-9.

Table 10: vvhA percentage (%) similarity to the strains listed below.

| Reference | Strains | Sequence availability | Match % *vvhA*_ATCC 27562 |
|---|---|---|---|
| ATCC 27562_*vvhA* | *Vibrio vulnificus* 06-2450 | NO | - |
| ATCC 27562_*vvhA* | *Vibrio vulnificus* 96-11-17M | NO | - |
| ATCC 27562_*vvhA* | *Vibrio vulnificus* A1402 | NO | - |
| ATCC 27562_*vvhA* | *Vibrio vulnificus* B2 | YES | 100% |
| ATCC 27562_*vvhA* | *Vibrio vulnificus* BAA87 | NO | - |
| ATCC 27562_*vvhA* | *Vibrio vulnificus* CladeA-yb158 | YES | 96.40% |
| ATCC 27562_*vvhA* | *Vibrio vulnificus* CMCP6 | YES | 96.47% |
| ATCC 27562_*vvhA* | *Vibrio vulnificus* CN7 | NO | - |
| ATCC 27562_*vvhA* | *Vibrio vulnificus* E64MW | NO | - |
| ATCC 27562_*vvhA* | *Vibrio vulnificus* Env1 | YES | 100% |
| ATCC 27562_*vvhA* | *Vibrio vulnificus* JY1305 | YES | 99.57% |
| ATCC 27562_*vvhA* | *Vibrio vulnificus* JY1701 | NO | - |
| ATCC 27562_*vvhA* | *Vibrio vulnificus* MO6-24/O | YES | 95.97% |
| ATCC 27562_*vvhA* | *Vibrio vulnificus* NBRC 15645 = ATCC 27562 | YES | 100% |
| ATCC 27562_*vvhA* | *Vibrio vulnificus* VV | NO | - |
| ATCC 27562_*vvhA* | *Vibrio vulnificus* VVyb1(BT3) | YES | 95.62% |

| | | | |
|---|---|---|---|
| ATCC 27562_*vvhA* | *Vibrio vulnificus* YJ016 | YES | 96.69% |

Common Primers:

In the NCBI database under the taxonomy category the strain names were listed in Table 9. The whole genomes were not available for all the strains listed in the NCBI. The availability of the genome sequence was listed in the above table.

Single-plex PCR:

*rpoS* Gene Identification:

A 2% agarose gel with 0.5 µg/mL Ethidium bromide was run at 7.5 V/cm and visualized. Lane 1 contained 50 ng/µL Marker MW Ladder; last two lanes contained the positive control (*V. vulnificus* ATCC 27562 DNA at 60 ng/µL), and the negative control as H2O. Polymerase chain reactions (PCR) seen from Figure 17 - 20 shows results with the primers of *rpoS*, which yielded an amplicon of 393 bp for all experimental samples, and bands are sharp and clear, with no traces of DNA in the background.

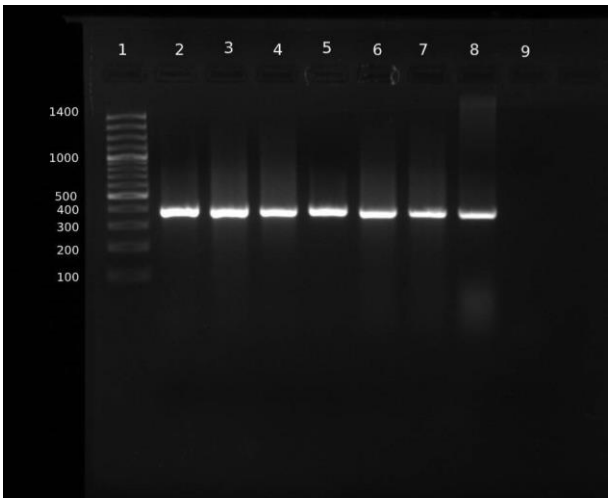



Figure 17 : Agarose gel electrophoresis of *V. vulnificus* samples from PCR for *rpoS* gene identification. Bands were visualized using 0.5 ug/mL of ethidium bromide. Lane 1 - 50 ng/uL Marker MW Ladder; Lanes 2,3 – Lanes ARA0040407-39; Lanes 4,5 - ATCC 27562; Lanes 6,7 – CB 0607-2; Lane 8 - Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 9 - negative control.

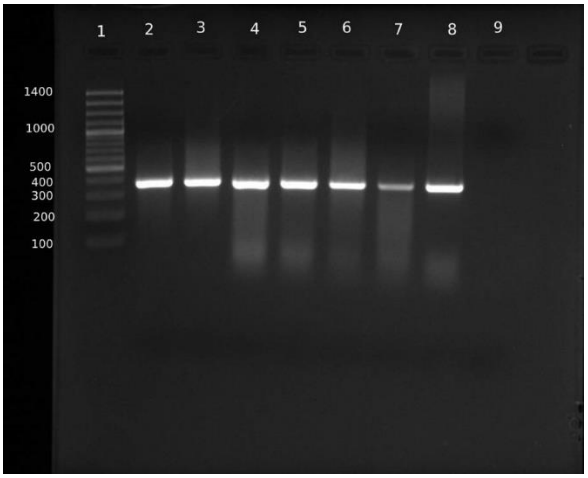



Figure 18 : Agarose gel electrophoresis of *V. vulnificus* samples from PCR for *rpoS* gene identification. Bands were visualized using 0.5 ug/mL of ethidium bromide. Lane 1 - 50 ng/uL Marker MW Ladder; Lanes 2,3 - ATCC 27562; Lanes 4,5 – BS 0607-5; Lanes 6,7 – NB 0507-7; Lane 8 - Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 9 - negative control.

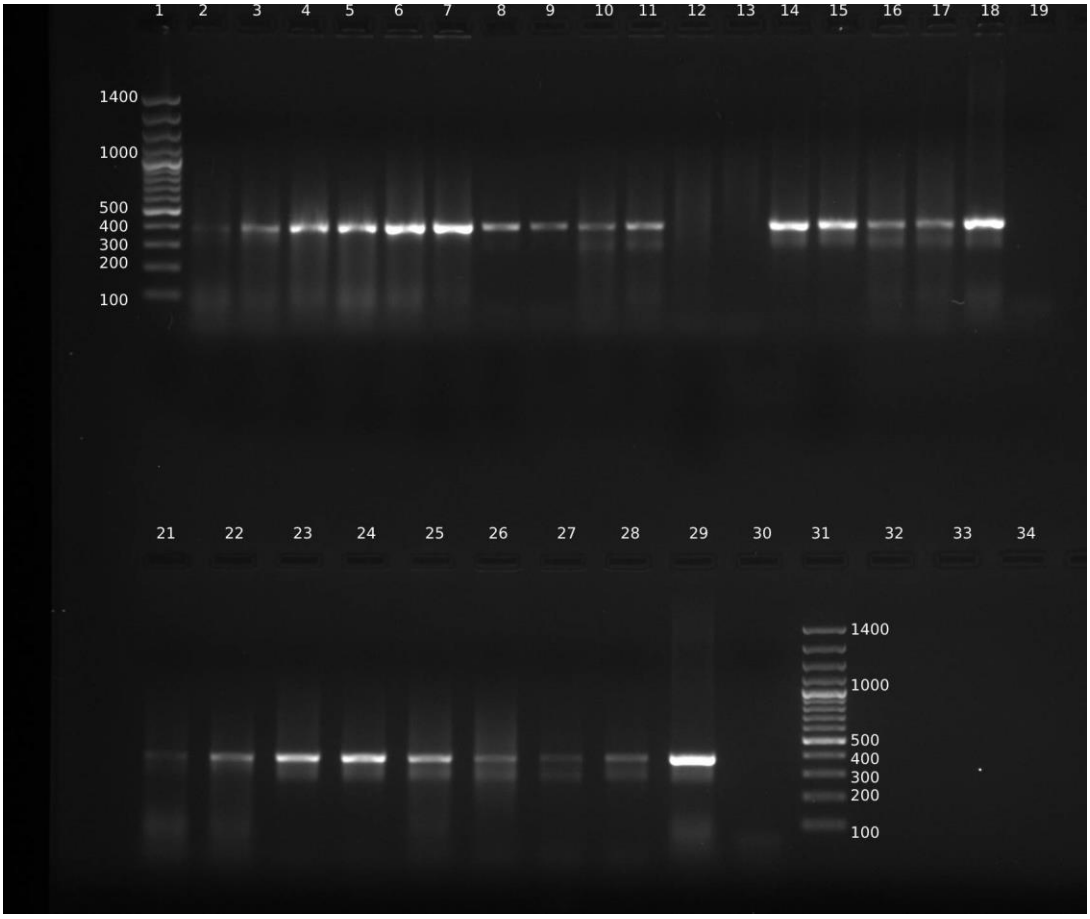



Figure 19 : Agarose gel electrophoresis of *V. vulnificus* samples from PCR for *rpoS* gene identification. Bands were visualized using 0.5 ug/mL of ethidium bromide. Lane 1 - 50 ng/uL Marker MW Ladder; Lanes 2,3 - ATCC 27562; Lanes 4,5 – CB 0507-16; Lanes 6,7 – CB 0507-30; Lanes 8,9 – CB 0707-23; Lane 10,11 – CB 0707-42; Lane 12,13 – CB 0707-82; Lane 14,15 – CB 0707-91; Lane 16,17- CB 1006-12; Lane 18 - Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 19 - negative control; Lane 21,22 - ATCC 27562; Lane 23,24 – CB 1006-49; Lane 25,26 - CP 0607-10; Lane 27,28 - CP 1006-27; Lane 29 - Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 30 - negative control; Lane 31 - 50 ng/uL Marker MW Ladder.

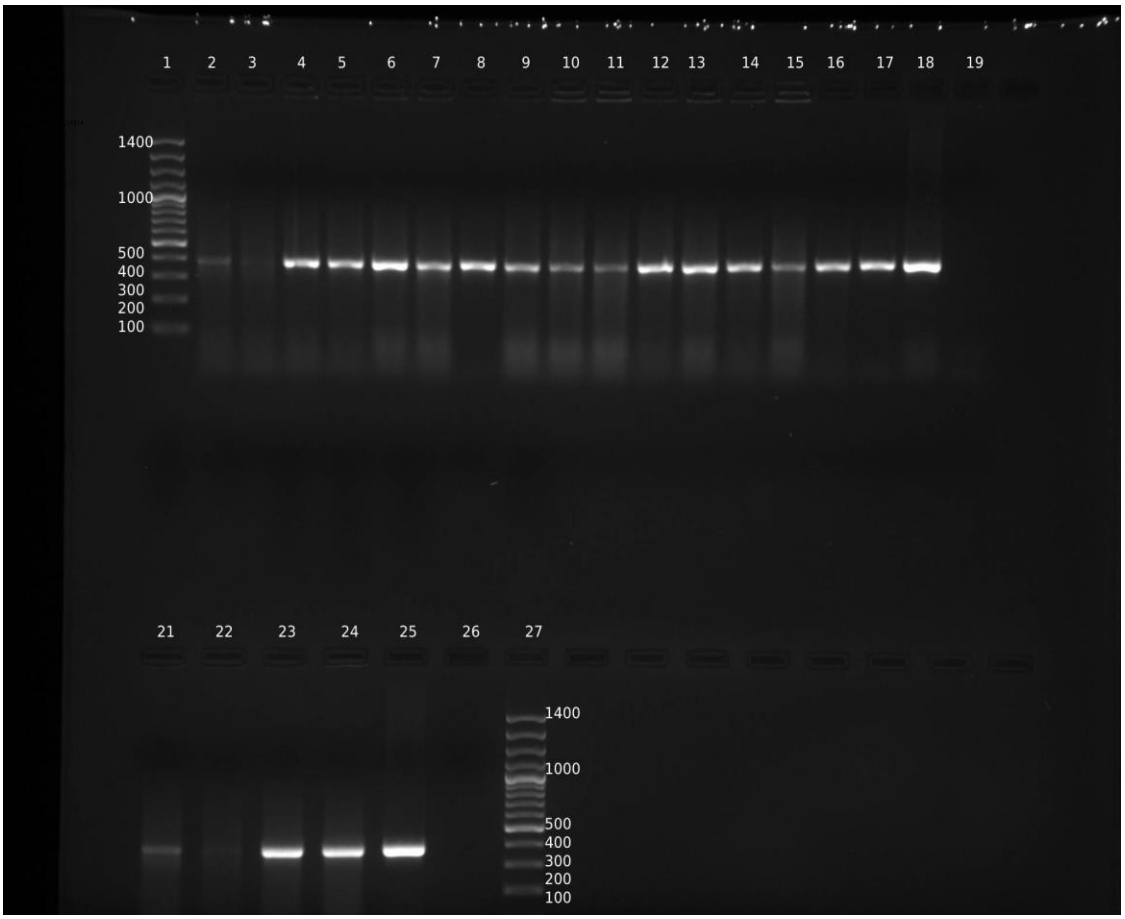



Figure 20 : Agarose gel electrophoresis of *V. vulnificus* samples from PCR for *rpoS* gene identification. Bands were visualized using 2.5 of ethidium bromide. Lane 1 - 50 ng/uL Marker MW Ladder; Lanes 2,3 - ATCC 27562; Lanes 4,5 – ARA0040407-40; Lanes 6,7 – CB 1006-27; Lanes 8,9 – CP1006-37; Lane 10,11 – NB 0507-8; Lane 12,13 – NB 0507-13; Lane 14,15 – NUE 0400707-7; Lane 16,17- RB 0407-13; Lane 18 - Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 19 - negative control; Lane 21,22 - ATCC 27562; Lane 23,24 – RB 0906-57; Lane 25 - Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 26 - negative control; Lane 27 - 50 ng/uL Marker MW Ladder.

*vvhA* Identification:

A 2% agarose gel with 0.5 µg/mL ethidium bromide was run at 7.5 V/cm and visualized. Lane 1 contains 50 ng/µL Marker MW Ladder; last two lanes after the sample are the positive and negative control. Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/µL and the negative control is H2O. Polymerase chain reaction (PCR) from Figure 21 - 24 shows results with the primers of *vvhA*, which yielded clear, distinct amplicons of 265 bp for all lanes containing experimental samples.

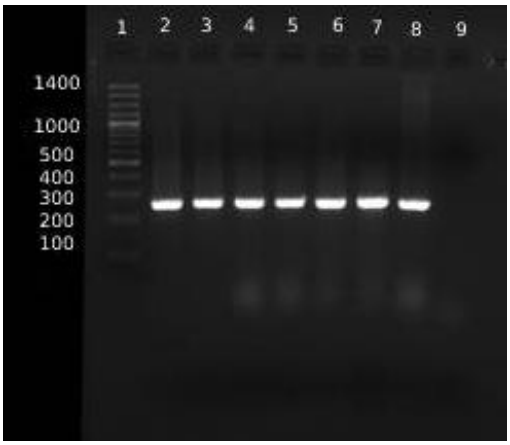



Figure 21 : Agarose gel electrophoresis of *V. vulnificus* samples from PCR for *vvhA* gene identification. Bands were visualized using 0.5 ug/mL of ethidium bromide. Lane 1 - 50 ng/uL Marker MW Ladder; Lanes 2,3 - ATCC 27562; Lanes 4,5 –ARA 0040407-40; Lanes 6,7 –CB 0607-02; Lane 8 - Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 9 - negative control.

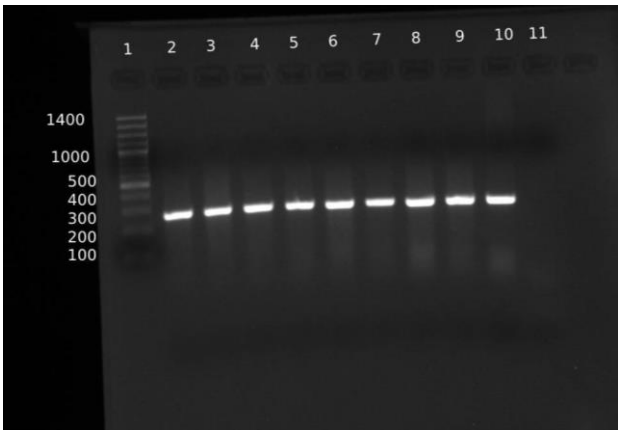



Figure 22 : Agarose gel electrophoresis of *V. vulnificus* samples from PCR for *vvhA* gene identification. Bands were visualized using 0.5 ug/mL of ethidium bromide. Lane 1 - 50 ng/uL Marker MW Ladder; Lanes 2,3 - ATCC 27562; Lanes 4,5 –BS 0607-5; Lanes 6,7 –CB 0607-02; Lane 8,9 – CB 1006-27, Lane 10 - Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 11- negative control.

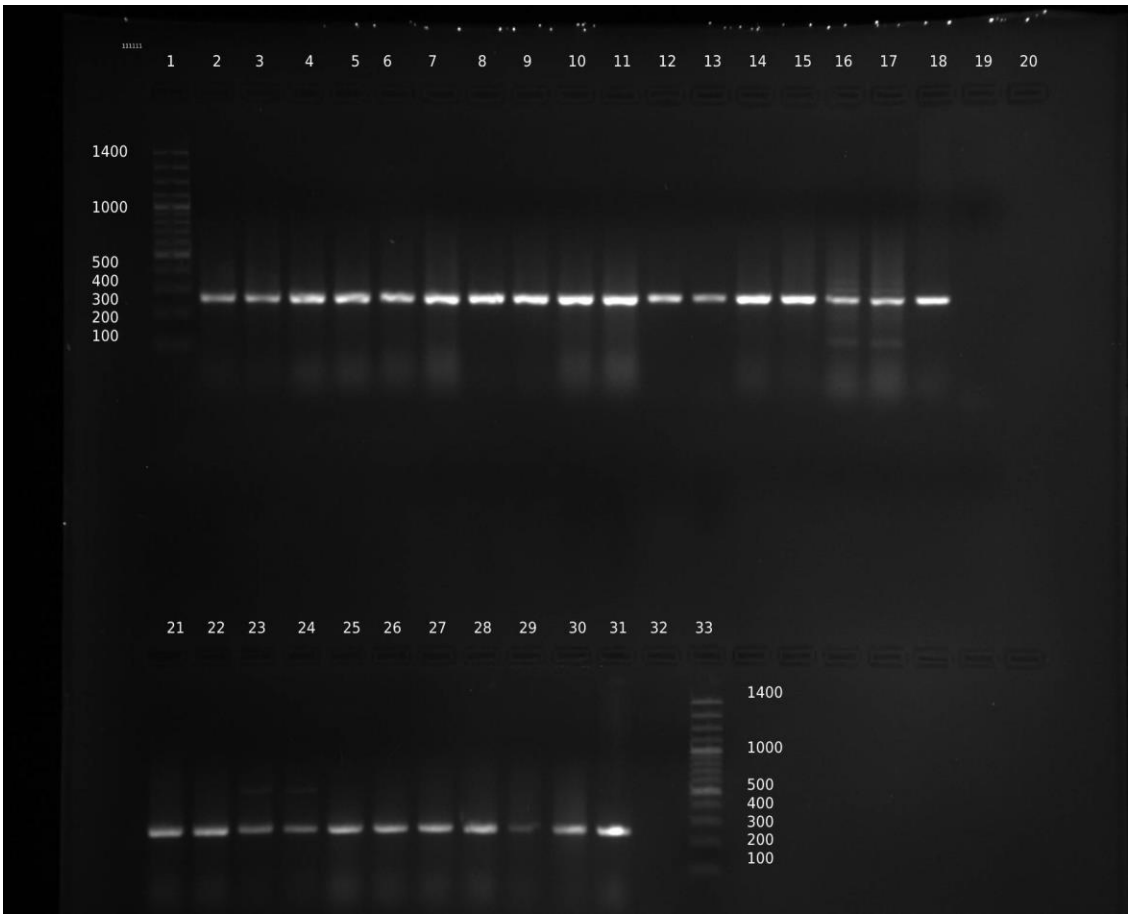



Figure 23 : Agarose gel electrophoresis of *V. vulnificus* samples from PCR for *vvhA* gene identification. Bands were visualized using 0.5 ug/mL of ethidium bromide. Lane 1 - 50 ng/uL Marker MW Ladder; Lanes 2,3 - ATCC 27562; Lanes 4,5 –ARA 0040407-39; Lanes 6,7 –BS 0607-5; Lane 8,9 – BS 0507-16; Lane 10,11 – CB 0507-16; Lane 12,13 – CB 0707-23; Lane 14,15 – CP 1006-37; Lane 16,17 – CB 0707-42; Lane 18 - Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 19 - negative control; Lane 20 – empty; Lane 21,22 – CB 0707-82; Lane 23,24 – NB 0507-8; Lane 25,26 – RB 0407-13; \Lane 27,28 – RB 0906-57; Lane 29,30 – NUE 040077-7; Lane 31 - Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 32 – Negative control; Lane 33 - 50 ng/uL Marker MW Ladder.

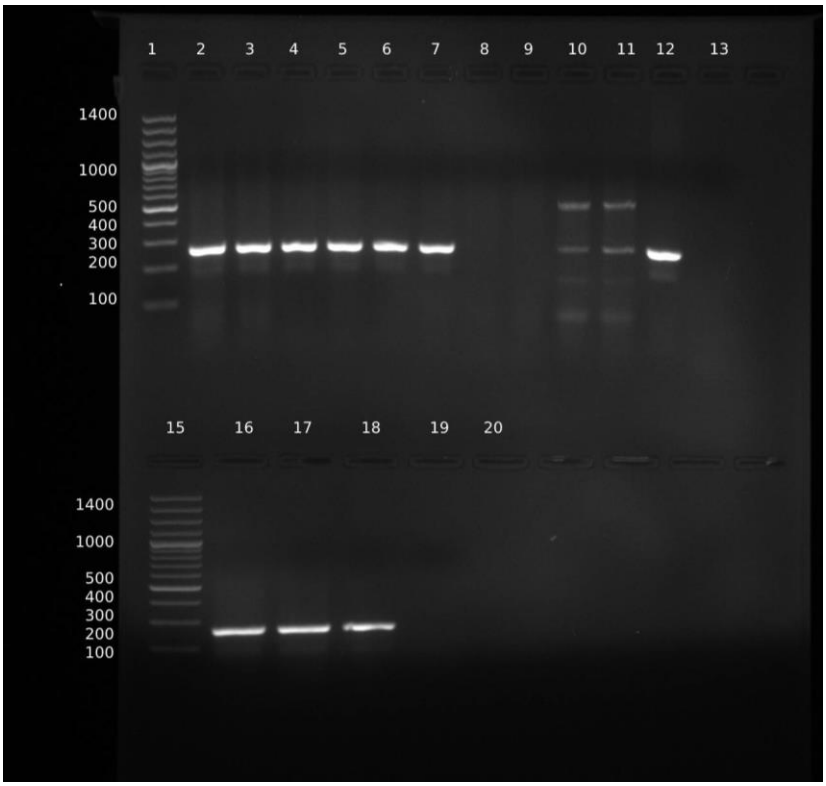


Figure 24 : Agarose gel electrophoresis of *V. vulnificus* samples from PCR for *vvhA* gene identification. Bands were visualized using 2 0.5 ug/mL of ethidium bromide. Lane 1 - 50 ng/uL Marker MW Ladder; Lanes 2,3 - ATCC 27562; Lanes 4,5 – BS 0507-11; Lanes 6,7 –BS 0607-31; Lane 8,9 – CB 0507- 20; Lane 10,11 – NB 0507-7; Lane 12 -Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 13 – Negative control; Lane 15 - 50 ng/uL Marker MW Ladder; Lane 16,17 – NB 0507-13, Lane 18 - Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 19 – Negative control; Lane 20 - 50 ng/uL Marker MW Ladder.

*rpoS* and *vvhA* identification through multiplex PCR:

A 2% agarose gel with 0.5 µg/mL Ethidium bromide was run at 7.5 V/cm and visualized. Lane 1 contained 50 ng/µL Marker MW Ladder; last two lanes contained the positive control (*V. vulnificus* ATCC 27562 DNA at 60 ng/µL), and the negative control as H2O. Polymerase chain reactions (PCR) from Figures 25 – 28 shows results with the primers of *rpoS* and *vvhA*, which yielded an amplicon of 393 bp for all experiments but did not give the predicted *vvhA* amplicon at 265 bp.

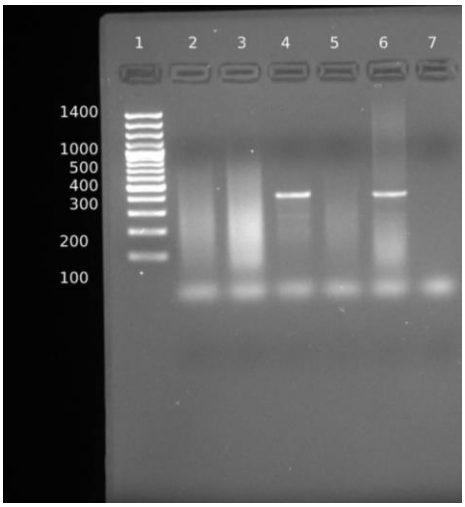


Figure 25 : Agarose gel electrophoresis of *V. vulnificus* samples from PCR for *vvhA* gene and *rpoS* identification. Bands were visualized using 0.5 ug/mL of ethidium bromide. Lane 1 - 50 ng/uL Marker MW Ladder; Lanes 2,3 - ATCC 27562; Lanes 4,5 – NB 0507-13; Lane 6 - Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 7 – Negative control.

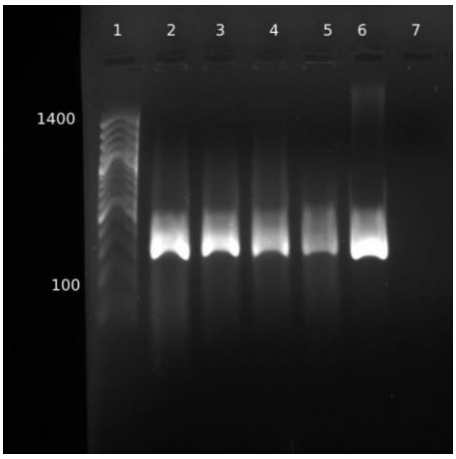


Figure 26 : Agarose gel electrophoresis of *V. vulnificus* samples from PCR for *vvhA* and *rpoS* gene identification. Bands were visualized using 2 0.5 ug/mL of ethidium bromide. Lane 1 - 50 ng/uL Marker MW Ladder; Lanes 2,3 - ATCC 27562; Lanes 4,5 – ARA 0040407-39; Lane 6 -Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 7 – Negative control.

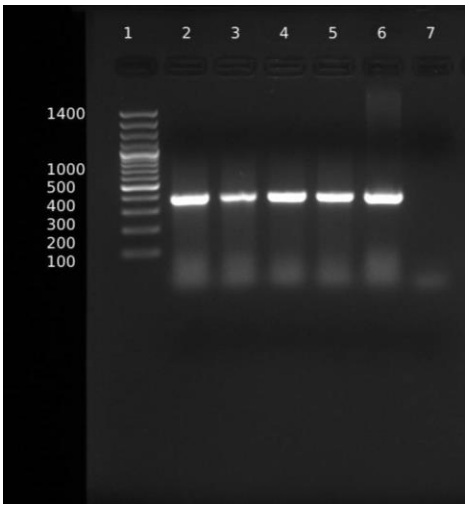


Figure 27 : Agarose gel electrophoresis of *V. vulnificus* samples from PCR for *vvhA* and *rpoS* gene identification. Bands were visualized using 2 0.5 ug/mL of ethidium bromide. Lane 1 - 50 ng/uL Marker MW Ladder; Lanes 2,3 - ATCC 27562; Lanes 4,5 – ARA 0040407-39; Lane 6-Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 7 – Negative control

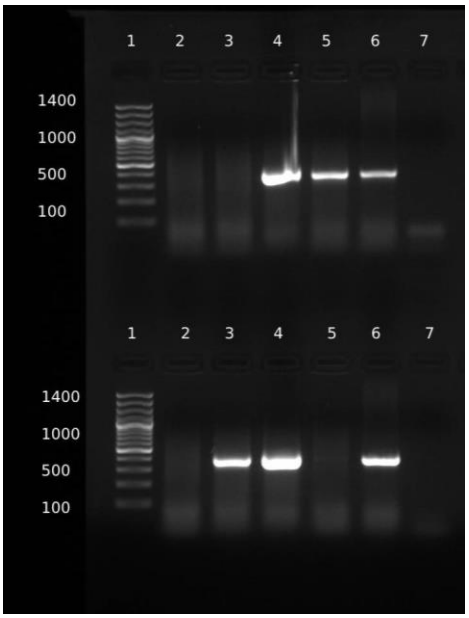


Figure 28 : Agarose gel electrophoresis of *V. vulnificus* samples from PCR for *vvhA* and *rpoS* gene identification. Bands were visualized using 2 0.5 ug/mL of ethidium bromide. Lane 1 - 50 ng/uL Marker MW Ladder Lanes 2,3 - ATCC 27562; Lanes 4,5 – ARA 0040407-39; Lane 6-Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 7 – Negative control. Bottom: Lane 1 - 50 ng/uL Marker MW Ladder Lanes 2,3 - ATCC 27562; Lanes 4,5 – ARA 0040407-39; Lane 6-Positive control *V. vulnificus* ATCC 27562 DNA 60 ng/uL; Lane 7 – Negative control

Table 11: Confirmation of presence of *vvhA* and *rpoS* in *Vibrio* isolates

Legend: YES – Presence of gene

NO – Absence of gene

| | | | | Multiplex | |
|---|---|---|---|---|---|
| Strain | WGS IDS | *vvhA* | *rpoS* | *vvhA* | *rpoS* |
| ATCC 27562 | | YES | YES | NO | YES |
| ARA 0040407-39 | VA-WGS-18022 | YES | YES | NO | NO |
| ARA 0040407-40 | | YES | YES | NO | YES |
| BS 0607-5 | VA-WGS-18031 | YES | YES | NO | NO |
| CB 1006-27 | VA-WGS-18058 | YES | YES | NO | NO |
| NB 0507-7 | VA-WGS-18 | YES | YES | NO | NO |
| BS 0507-11 | VA-WGS-18025 | YES | YES | NO | NO |
| BS 0507-16 | VA-WGS-18026 | YES | YES | NO | NO |
| BS 0607-31 | VA-WGS-18030 | YES | YES | NO | NO |
| CB 0507-20 | VA-WGS-18042 | NO | YES | NO | NO |
| CB 0507-16 | VA-WGS-18041 | YES | YES | NO | NO |
| CB 0507-30 | VA-WGS-18043 | YES | YES | NO | NO |
| CB 0607-2 | VA-WGS-18045 | YES | YES | NO | NO |
| CB 0607-9 | VA-WGS-18047 | YES | YES | NO | NO |

| | | | | | |
|---|---|---|---|---|---|
| CB 0707-35 | VA-WGS-18050 | YES | YES | NO | NO |
| CB 0707-42 | VA-WGS-18051 | YES | YES | NO | NO |
| CB 0707-82 | VA-WGS-18052 | YES | NO | NO | NO |
| CB 0707-91 | VA-WGS-18053 | YES | YES | NO | NO |
| CB 1006-12 | VA-WGS-18054 | YES | YES | NO | NO |
| CB 1006-49 | VA-WGS-18056 | YES | YES | NO | NO |
| CP 0607-10 | VA-WGS-18057 | YES | YES | NO | NO |
| CP 1006-27 | VA-WGS-18058 | YES | YES | NO | NO |
| CP 1006-37 | VA-WGS-18059 | YES | YES | NO | NO |
| NB 0507-13 | VA-WGS-18060 | YES | YES | NO | YES |
| NB 0507-8 | VA-WGS-18061 | YES | YES | NO | NO |
| NUE 0400707-7 | VA-WGS-18062 | YES | YES | NO | NO |
| RB 0407-13 | VA-WGS-18063 | YES | YES | NO | NO |
| RB 0906-57 | VA-WGS-18064 | YES | YES | NO | NO |

# CHAPTER VII

# DISCUSSION

*V. vulnificus* is a Gram-negative opportunistic human pathogen commonly found in fish, shellfish, and shrimp. (Jones and Oliver, 2009; Baker-Austin and Oliver 2018). Low salinity levels ranging from 5 to 25 ppt and temperature ranging between 20 – 30o C are the desired conditions for the optimal growth of *V. vulnificus* (De Paola *et al.* 2006). A person can be infected with *Vibrio* infections through water, uncooked seafood and consumption of raw seafood (Altekruse *et al.* 2000). This food and waterborne bacterial pathogen can kill patients within 72 hours if treatment is delayed (Bross *et al.* 2007). Prior research determined PCR amplicons for the *vvhA* and *rpoS* genes. This study initially determined that these PCR assays were not reproducible. After careful observations and literature review it was hypothesized that genomes of the *V. vulnificus* may have undergone mutations at the locations of the forward and reverse primer annealing sites of the *vvhA* and *rpoS* genes. Previous experiments also showed that the primers were not annealing to the target sequence in several strains of *V. vulnificus*. One strategy employed to check primer binding efficiency was the in-silico approach using the command line blast search. Blast search was used to retrieve the target *vvhA* and *rpoS* sequences using the initial *vvhA* sequences (Yamamato *et al.* 1990) and rpoS from NCBI database. It was observed that in strains WGS-ID 18022 (ARA 004040407-39 from Aransas Bay), WGS ID 18023 (BI 0607-1 from Bird Island, on the Laguna Madre side of the Padre Island National Seashore), WGS ID 18031 (BS0607-5 from Copano Bay), and ATCC 27562 ("wild-type" strain), the forward and reverse primer sequences were not exactly aligning to the target sequences. This simple study using the blast search tool to check the primer binding efficiency showed that the initial primer sequences cannot be used with these strains. Based on these in-silico results, new primers were designed to work for all the available (28

isolates) *V. vulnificus* strains. PCR assays were conducted with these newly designed primer sequences and appropriate-sized amplicons for *vvhA* and *rpoS* were obtained. This work was extended to initiate multiplex PCR using both re-designed primer types. While multiplex PCR assays were not as conclusive, future work will optimize those reactions with possible adjustments to melting temperatures.

The significance of this current study provides a more economical method of detecting pathogenic *V. vulnificus* isolates from the Coastal Bend region, and it employs more current genomic data that were not available when the Bacteriological Analytical Manual was originally written (Kaysner, DePaola and Jones 2004), and the data generated previously in this laboratory ( Planas-Costas 2014). *V. vulnificus* is subdivided into three biotypes and two eco-types (C and E). This study focused on the genomics of the biotypes. Among the three biotypes, Biotype 1 isolates are pathogenic to humans and Biotype 3 is the hybrid form of Biotypes 1 & 2 which is also pathogenic to humans but is only recognized in Israel (Thiaville *et al.* 2011). There are several biochemical tests to differentiate these biotypes, but confirmation may take from two to three days. It will be quicker, simpler, and less complicated to confirm identity using PCR technique (Neogi *et al.* 2010) instead of biochemical testing. Globally, aquaculture is one of the fastest food-producing sectors (Olsen and Hasan, 2012) (Naylor *et al.* 2000). Foodborne and waterborne causative agents are also increasing and causing disease both in humans and animals. This will directly affect the global economy as the quality and security in food assurance is critical (Olsen and Hasan, 2012). Shellfish and fish pathogens are most likely to cause *Vibrio*sis by three *Vibrio* spp., *V. vulnificus, V. parahaemolyticus,* and *Vibrio cholerae* (Sapkota *et al.* 2008; Chatterjiee and Haldar 2012, Ruwandeepika *et al.* 2012; Deng *et al.* 2020). Extensive research on virulence factors and genes has been studied in *Vibrio* species; RpoS or sigma -38 is the most common stress response

regulator in many Gram-negative bacteria, as compared to that of RpoD or sigma-70 (Hengge-Aronis 2000; Hulsmann et al 2003). The *rpoS* gene becomes activated in the stationary phase, during starvation, and other stressful conditions such as high temperature and high salinity. It also regulates the virulence factors (Hulsmann *et al.* 2003; Park *et al.* 2004; Schellhorn *et al.* 2014). VvhA, the hemolysin/cytolysin toxin, belongs to a pore-forming family of toxins and plays a role in the pathogenesis of *V. vulnificus*. The key role of this cytolysin activity is hemolysis/cytolysis of various mammalian cells in order to release iron, which is required by nearly all bacteria for growth (Yamamoto *et al.* 1990; Yuan *et al.* 2020). *V. vulnificus* deaths may be associated with seafood (Heng *et al.* 2017), or with necrotizing fasciitis caused by entry of *V. vulnificus* into wounds, leading to severe morbidity and high levels of mortality (Horseman and Surani, 2011). Media reports that persons contracting such infections results in high levels of public concern, which may have severe economic impact on tourism. A person who is infected with *V. vulnificus* may be diagnosed through biochemical tests, but growth and confirmation may take 50-60 hrs. For this reason, rapid methods of diagnosis may decrease morbidity and mortality. While qRT-PCR is one such method, use of this technique is expensive and requires sophisticated thermocyclers and operator expertise, which may not be cost-effective for small laboratories. In contrast, traditional end point PCR is direct, requires minimal level of expertise, can be directly performed in a few hours, and has reproducibility and reliability.

One impact of this study was to develop a technique to detect the pathogenic isolates of *V. vulnificus* consistently and effectively. In initial experiments with primers designed previously, all results were negative. Because these primers did work in Buck's laboratory with prior students, this gave rise to an in-silico approach to confirm that primers still annealed. The updated genomic sequence data would increase specificity of detection of *V. vulnificus* for diagnosis of pathogenic

bacterial infections and food safety. As shown in Table 3, these selected isolates were from the Gulf of Mexico, and this study demonstrated a potential method for more accurate identification of the pathogenic strains. While the Bacteriological Analytical Manual (BAM) developed by the US Food and Drug Administration initially developed a protocol for detecting *vvhA* using forward primer *vvhA* 785 and reverse primer *vvhA* 1303 (Kaysner, dePaola, Jones 2004), the earlier work in the Buck laboratory with these primers gave mixed results, so additional primers were developed for both *vvhA* and *rpoS* genes (Galvan 2009; Planas-Costas 2014). Few of these isolates resulted in amplicons, either due to confusion over forward and reverse primer annealing, technical aspects of performing the lysis procedure (Parvarthi *et al.* 2005), or changes in the genome or plasticity of the DNA (Quirke *et al.* 2006). Bacteria used in this study did undergo repeated freezing and thawing due to power outages, and the use of the cryo-preserved cultures for growing strains on slants. (Sprouffske *et al.* 2016) stated that archiving bacteria by cryo-preservation with glycerol does not lead to allelic variation in *E. coli*; however, little is reported in literature regarding repeated freezing and thawing of *Vibrio* species and whether this process induces mutations. For this rationale, this investigation aimed to combine in-silico analysis with re-design of PCR primers. The primers designed in this study are highly specific to the gene *vvhA* because the method is purely based on genomics, and all the genomes involved were subjected to the comparative genome analysis. Since *vvhA* is unique for *V. vulnificus*, such primers, whether for single-plex or multiplex PCR, may have increased probability of success for clinical diagnosis.

As shown in Appendix D, the primers did align to the *vvhA* gene of the strains described in the literature as shown by in-silico analysis, and Table 10 demonstrates that primers also annealed within the PCR assay to the local isolates (mostly Biotype 1) found in the Coastal Bend region. While additional confirmation through use of the primers in multiplex PCR did not hold true, the

explanation may be that the correct parameters for multiplex PCR may not have been properly optimized. While the most likely explanation for reproducing the assays is primer annealing and handling during the PCR assay, future work will be to develop specific parameters for multiplex PCR, and to optimize these conditions so that primers in single-plex PCR will also work for multiplex PCR.

The approach followed in this study utilizing single-plex, multiplex and genomics makes this study unique from other prior investigations. The novel aspect of this study on diagnosis of *V. vulnificus* infections with PCR techniques is that previous reports used strains from Biotype 1, which are most likely to result in human infections. Further studies should determine if primers are biotype specific. Initially in this study, all the available whole genomes (Schoch *et al.* 2020) were compared with the *vvhA* from ATCC 27562 and *vvhA* was found in all these genomes. Later in this study an in- silico PCR and a Blast search were performed on the strains mentioned in Appendix D with the vvhA primer set, and primers designed in this study annealed to strains which are of Biotype 1 (Mullis *et al.* 2019). In an unpublished report, these Biotype 1 isolates may be subdivided into two ecotypes, C and E (C. Saenz, pers.comm). Primers were also found to anneal to a biotype 2 strain, ATCC 33817, (Amaro *et al.* 1996) and to biotype 3 (VVyb1) (Efimov et al. 2013). However, Biotype 3 has many genes unique to this biotype compared to biotypes 1 and 2. VVyb1 strain has vvhA with 95% similarity with Biotype 1 (ATCC 27562). In this current study,1-2 nucleotides from the existing sequence primers developed here were switched; future aims will determine if these primers will work for Biotype 3. Future investigations will require a more complex in-silico analysis for Biotype 3.

Table 10 shows identified strains of *V. vulnificus* with *vvhA* and *rpoS*. One strain out of 28 strains tested did not show a positive result for *vvhA* or *rpoS*, as was seen in an earlier study with different primers (Planas-Costas 2014). From the current results of single-plex PCR, multiplex PCR should also give positive results. The unexpected lack of results of multiplex PCR in this study may be related to suboptimal annealing temperatures, since the melting temperature (Tm) for the primers were fairly close. The melting temperature needs to be optimized where both the target genes will be in the desired output. As shown above in the gel figures of multiplex PCR from Figures 25 – 28, both genes were not amplified. Prior literature reports (Bauer and Rorvik 2007; Rai *et al.* 2009) described multiplex PCR reactions and used the *toxR* gene to differentiate the three major *Vibrio* pathogens, but a limitation is that colonial morphology improved assay accuracy (Bauer and Rorvik 2007). Future experiments will require optimization of the multiplex method described here.

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# APPENDIX A

## Properties for end point primers

The basic guidelines to design an efficient primer for the end point PCR or the Multiplex PCR are that both forward and the reverse primers must be 18 – 25 nucleotides long with 40 – 60% of G-C content (for the end-point PCR) and 55 - 60% (for the multiplex PCR). Second, the melting temperature (Tm) value plays an important role in the PCR and the difference between the primers should be < 5 $^{O}$C (for end point PCR) and < 0.5 – 1 $^{O}$C for multiplex PCR. Third, primers should not align either to itself or the other primer. The nucleotides in the primers should not be complementary to each other. If there are any such regions, it should not be more than three nucleotides complementary to each other. Fourth, the final concentration of the primers must be 1 mM and should be diluted in water. Fifth, primers should be designed such that the last nucleotide of the 3' primer sequences should end with "G" or "C" and also multiple "T" nucleotides should not be present at the 3' end or 5' end. Lastly, the final product size should be ranging from 300 to 500 bp (for the end point PCR) and 100 – 300 bp (for the multiplex PCR) (Houghton, 1994; pers. comm.; Weiss, 1999, pers. comm).

APPENDIX B

Master-mix components for Multiplex reaction

| Components | Final Volume (n+2) n=4 |
|---|---|
| 5x Green Go Taq Reaction Buffer | 24 µL |
| dNTPs (deoxyribonucleotidetriphosphates) | 1.8 µL |
| *vvhA* 887F Forward Primer | 1 µL |
| *vvhA* 1152R Reverse Primer | 1 µL |
| *rpoS* 573F Forward Primer | 1 µL |
| *rpoS* 2004R Reverse Primer | 1 µL |
| dI Water | 89 µL |
| Go Taq DNA polymerase | 1.2 µL |

APPENDIX C

Thermal cycling parameters for Multiplex

| | Step 1 | Step 2 | Step 3 | Step 4 | Step 5 | Step 6 |
|---|---|---|---|---|---|---|
| Time | 10 min | 40 sec | 45 sec | 45 sec | 10 min | ∞ |
| Temperature | 94$^{o}$ C | 94$^{o}$ C | 54$^{o}$ C | 72$^{o}$ C | 72$^{o}$ C | 4$^{o}$ C |

APPENDIX D

In-silico analysis (*vvhA* primers alignment to the strains listed below)

| S .No | Strains | Primer Alignment |
|---|---|---|
| 1. | *Vibrio vulnificus* 06-2450 | NO |
| 2. | *Vibrio vulnificus* 96-11-17M | NO |
| 3. | *Vibrio vulnificus* A1402 | NO |
| 4. | *Vibrio vulnificus* B2 | YES |
| 5. | *Vibrio vulnificus* BAA87 | NO |
| 6. | *Vibrio vulnificus* CladeA-yb158 | YES |
| 7. | *Vibrio vulnificus* CMCP6 | NO |
| 8. | *Vibrio vulnificus* CN7 | NO |
| 9. | *Vibrio vulnificus* E64MW | NO |
| 10. | *Vibrio vulnificus* Env1 | NO |
| 11. | *Vibrio vulnificus* JY1305 | NO |
| 12. | *Vibrio vulnificus* JY1701 | NO |
| 13. | *Vibrio vulnificus* MO6-24/O | YES |
| 14. | *Vibrio vulnificus* NBRC 15645 = ATCC 27562 | YES |
| 15. | *Vibrio vulnificus* VV | NO |
| 16. | *Vibrio vulnificus* VVyb1(BT3) | NO |
| 17. | *Vibrio vulnificus* YJ016 | YES |
| 18. | *Vibrio vulnificus* ATCC33817 | YES |

Appendix E

Table of Isolates sequenced by VA Department of health

| WGS ID | Assumed Organism | Source | Receive Date | Collection date | Actual Organism | Submitter ID |
|---|---|---|---|---|---|---|
| VA-WGS-18022 | *Vibrio vulnificus* | Aransas Bay | 5/31/2018 | Jun-07 | *Vibrio vulnificus* | ARA0040407-39 |
| VA-WGS-18023 | *Vibrio vulnificus* | Bird Island | 5/31/2018 | Jun-07 | ***Vibrio ostreicida*** | BI0607-1 |
| VA-WGS-18024 | *Vibrio vulnificus* | Bayside | 5/31/2018 | Apr-07 | *Vibrio vulnificus* | BS0407-6 |
| VA-WGS-18025 | *Vibrio vulnificus* | Bayside | 5/31/2018 | May-07 | *Vibrio vulnificus* | BS0507-11 |
| VA-WGS-18026 | *Vibrio vulnificus* | Bayside | 5/31/2018 | May-07 | *Vibrio vulnificus* | BS0507-16 |
| VA-WGS-18027 | *Vibrio vulnificus* | Bayside | 5/31/2018 | May-07 | *Vibrio vulnificus* | BS0507-4 |
| VA-WGS-18028 | *Vibrio vulnificus* | Bayside | 5/31/2018 | Jun-07 | *Vibrio vulnificus* | BS0607-13 |
| VA-WGS-18029 | *Vibrio vulnificus* | Bayside | 5/31/2018 | Jun-07 | *Vibrio vulnificus* | BS0607-28 |
| VA-WGS-18030 | *Vibrio vulnificus* | Bayside | 5/31/2018 | Jun-07 | *Vibrio vulnificus* | BS0607-31 |

| | | | | | | |
|---|---|---|---|---|---|---|
| VA-WGS-18031 | *Vibrio vulnificus* | Bayside | 5/31/2018 | Jun-07 | *Vibrio vulnificus* | BS0607-5 |
| VA-WGS-18032 | *Vibrio vulnificus* | Bayside | 5/31/2018 | Jun-07 | *Vibrio vulnificus* | BS0607-9 |
| VA-WGS-18033 | *Vibrio vulnificus* | Bayside | 5/31/2018 | Sep-06 | *Vibrio vulnificus* | BS0906-13 |
| VA-WGS-18034 | *Vibrio vulnificus* | Bayside | 5/31/2018 | Sep-06 | *Vibrio vulnificus* | BS0906-130 |
| VA-WGS-18035 | *Vibrio vulnificus* | Bayside | 5/31/2018 | Oct-06 | *Vibrio vulnificus* | BS1006-273(Tr) |
| VA-WGS-18036 | *Vibrio vulnificus* | Bayside | 5/31/2018 | Nov-06 | *Vibrio vulnificus* | BS1106-92(Tr) |
| VA-WGS-18037 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Apr-07 | *Vibrio vulnificus* | CB0407-15 |
| VA-WGS-18038 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Apr-07 | *Vibrio vulnificus* | CB0407-18 |
| VA-WGS-18039 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Apr-07 | *Vibrio vulnificus* | CB0407-20 |
| VA-WGS-18040 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Apr-07 | *Vibrio vulnificus* | CB0407-5 |
| VA-WGS-18041 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | May-07 | *Vibrio vulnificus* | CB0507-16 |

| | | | | | | |
|---|---|---|---|---|---|---|
| VA-WGS-18042 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | May-07 | *Vibrio vulnificus* | CB0507-20 |
| VA-WGS-18043 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | May-07 | *Vibrio vulnificus* | CB0507-30 |
| VA-WGS-18044 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Jun-07 | *Vibrio vulnificus* | CB0607-12 |
| VA-WGS-18045 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Jun-07 | *Vibrio vulnificus* | CB0607-2 |
| VA-WGS-18046 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Jun-07 | *Vibrio vulnificus* | CB0607-21 |
| VA-WGS-18047 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Jun-07 | *Vibrio vulnificus* | CB0607-9 |
| VA-WGS-18048 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Jul-07 | *Vibrio vulnificus* | CB0707-23 |
| VA-WGS-18049 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Jul-07 | *Vibrio vulnificus* | CB0707-29 |
| VA-WGS-18050 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Jul-07 | *Vibrio vulnificus* | CB0707-35 |
| VA-WGS-18051 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Jul-07 | *Vibrio vulnificus* | CB0707-42 |
| VA-WGS-18052 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Jul-07 | *Vibrio vulnificus* | CB0707-82 |

| | | | | | | |
|---|---|---|---|---|---|---|
| VA-WGS-18053 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Jul-07 | *Vibrio vulnificus* | CB0707-91 |
| VA-WGS-18054 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Oct-06 | *Vibrio vulnificus* | CB1006-12 |
| VA-WGS-18055 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Oct-06 | *Vibrio vulnificus* | CB1006-27 |
| VA-WGS-18056 | *Vibrio vulnificus* | Copano Bay | 5/31/2018 | Oct-06 | *Vibrio vulnificus* | CB1006-49 |
| VA-WGS-18057 | *Vibrio vulnificus* | Cole Park | 5/31/2018 | Jun-07 | *Vibrio vulnificus* | CP0607-10 |
| VA-WGS-18058 | *Vibrio vulnificus* | Cole Park | 5/31/2018 | Oct-06 | ***Vibrio alginolyticus*** | CP1006-27 |
| VA-WGS-18059 | *Vibrio vulnificus* | Cole Park | 5/31/2018 | Oct-06 | *Vibrio vulnificus* | CP1006-37 |
| VA-WGS-18060 | *Vibrio vulnificus* | Nueces Bay | 5/31/2018 | May-07 | *Vibrio vulnificus* | NB0507-13 |
| VA-WGS-18061 | *Vibrio vulnificus* | Nueces Bay | 5/31/2018 | May-07 | *Vibrio vulnificus* | NB0507-8 |
| VA-WGS-18062 | *Vibrio vulnificus* | Nueces Bay | 5/31/2018 | Jul-07 | *Vibrio vulnificus* | NUE0400707-7 |
| VA-WGS-18063 | *Vibrio vulnificus* | Redfish Bay | 5/31/2018 | Apr-07 | *Vibrio vulnificus* | RB0407-13 |

| | | | | | | |
|---|---|---|---|---|---|---|
| VA-WGS-18064 | *Vibrio vulnificus* | Redfish Bay | 5/31/2018 | Sep-06 | *Vibrio vulnificus* | RB0906-57 |