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Detection and Characterization of *Vibrio vulnificus* and *Vibrio parahaemolyticus* Isolates: Pentaplex PCR Assay and its Application.

Nabanita Bhattacharyya

Louisiana State University and Agricultural and Mechanical College

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DETECTION AND CHARACTERIZATION OF *VIBRIO VULNIFICUS* AND
VIBRIO PARAHAEMOLYTICUS ISOLATES: PENTAPLEX PCR ASSAY AND ITS
APPLICATION

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

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by
Nabanita Bhattacharyya
B.Sc., University of Madras, India, 2002
M.Sc., University of Madras, India, 2004
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ABSTRACT

The aim of the study was to develop an effective multiplex PCR protocol for simultaneous, rapid detection and characterization of two potential pathogens, *Vibrio vulnificus* and *Vibrio parahaemolyticus* and to enumerate their abundance in the environment.

A Pentaplex PCR (pPCR) assay condition was developed with a combination of two species- and three pathogenic- specific PCR primer sets to simultaneously detect and characterize bacterial isolates for virulent/ non- virulent strains of *V. parahaemolyticus* and *V. vulnificus*. The pPCR assay was validated by three methods. First pPCR was tested on 300 bacterial isolates comprising of 7 reference strains, 117 *V. vulnificus*, 30 *V. parahaemolyticus* and 146 unknown bacterial species and results were compared with other reported PCR reactions. Second, 51 pPCR tested isolates were analyzed by 16S rDNA sequencing to confirm for any false negative/positive reaction. Finally, the effectiveness of the pooled five primer pairs to amplify specific genes in individual target species amongst a heterogeneous bacterial sample was validated. The pPCR assay conditions worked with 96.6 - 98.7% efficiency.

The pPCR assay was tested on 782 bacterial isolates from Breton Sound and Barataria Bay water samples from selected months of 2011. Our findings showed higher occurrence of *V. vulnificus* (~ 49%) than that of *V. parahaemolyticus* (~ 12 %) and their prevalences were influenced by temperature and salinity in the Gulf waters. The pPCR tested isolates showed frequent but lower occurrence of pathogenic strains (less than 1%), in cooler months.

This simple, rapid, and cost-effective assay can be applied to screen and confirm a large number of isolates from clinical/environmental samples which will help to detect, predict disease outbreaks and therefore, to develop better risk management strategies.

CHAPTER 1 INTRODUCTION

The genus of *Vibrio* includes a wide range of motile gram negative bacteria. *Vibrio vulnificus* and *Vibrio parahaemolyticus* are the two important pathogenic species in this genus. Due to their halophilic nature these bacteria are commonly found in marine and estuarine environments. Studies on human infections caused by *V. parahaemolyticus* (Fujino *et al.*, 1951) and *V. vulnificus* (Blake *et al.*, 1979) were reported as early as 1951 and 1979, respectively. Both species are now enlisted by agencies in the United States, including- Food & Drug Administration (FDA), Center for Disease Control & Prevention (CDC), Food Safety Inspection Service (FSIS), and National Institutes of Health (NIH), as potent human pathogens. The pathogenic strains of *V. parahaemolyticus* and *V. vulnificus* may cause human infections such as gastrointestinal illness and septicemia (Blake *et al.*, 1980; Tacket *et al.*, 1984; Klontz *et al.*, 1988; Daniels *et al.*, 2000). The primary mode of their transmission into human is by the consumption of raw or undercooked oysters (Blake *et al.*, 1980; Rippey, 1994; Potasman *et al.*, 2002). Other modes of transmission are through consumption of potable water or contact with seawater harboring them. Therefore, detection and monitoring of *V. parahaemolyticus* and *V. vulnificus* in marine and coastal waters, sediments and oysters have become important over the years to the oyster industries (particularly in the Gulf of Mexico) and public health authorities.

Various conventional methods have been developed for detection of *V. vulnificus* and *V. parahaemolyticus*, e.g., use of selective agar, differential agar media, biochemical tests and examination of their colony morphology. These conventional methods usually require a lengthy laboratory procedure and provide very limited knowledge about bacterial pathogenicity. (Kaysner and DePaola, 2004)

In recent years, PCR (Polymerase Chain Reaction) based techniques were explored to detect the occurrence of these species and their pathogenic strains by targeting the amplification of specific gene sequences with specific primers. Many species-specific and pathogenic genes have been found for both *V. parahaemolyticus* and *V. vulnificus*. The species of *V. parahaemolyticus* are commonly differentiated by the presence of thermolabile hemolysin (*tlh*) gene and *V. vulnificus* by cytotoxin gene (*vvhA* or *vvh*). The FDA emphasizes the detection of

pathogenic *V. parahaemolyticus* strains possessing thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) gene in the environment and seafood samples. The *viuB*⁺ (siderophore-encoding genes) *V. vulnificus* virulent strains expressing an iron acquisition factor is of clinical importance as it thrives better in blood serum with higher iron levels. (Wyckoff *et al.*, 1997; Panicker *et al.*, 2004b; Bogard & Oliver, 2007)

Several PCR protocols have been developed and use unique primers in various combinations for the detection of these five molecular biomarkers, i.e., *tlh*, *tdh*, *trh*, *vhA*, and *viuB* (Bej *et al.*, 1999; Panicker *et al.*, 2004a). However, the previously reported protocols have not combined the five pairs of primer sets in one PCR reaction, for the simultaneous detection of all five target genes (*tlh*, *trh*, *tdh*, *vhA* and *viuB*) associated with each species.

The purpose of this study was to develop a Pentaplex PCR (pPCR) assay for simultaneous, rapid detection of the five genetic markers, which includes three pathogenic (*tdh*, *trh* and *viuB*) and two non-pathogenic, species-specific (*tlh*, *vhA*) genes in *V. parahaemolyticus* and *V. vulnificus* in a single PCR reaction tube. It then aimed at applying the pPCR assay, to detect, monitor and differentiate several bacterial isolates collected from the sea-surface waters in the northern Gulf of Mexico for *V. parahaemolyticus* and *V. vulnificus* and their virulent strains.

The pPCR assay should be a cost-effective approach for screening a large number of putative bacterial isolates for virulent and non-virulent strains of these *Vibrio* species. It will largely reduce the cost of laboratory supplies and time required for processing when compared to separate detection methods for the two *Vibrio* species using previously published PCR methods. The results of the study would also provide a better understanding of the occurrence of these bacteria that are impacted by two influential environmental parameters such as temperature and salinity in the Gulf.

This thesis is organized into five chapters. Chapter 2 presents a literature review that summarizes the fundamental information on the ecology of *V. vulnificus* and *V. parahaemolyticus*, their roles in causing human infections and detection methods for these two species with an emphasis on the previously reported PCR assays. Chapter 3 shows the

development and assessment of the Pentaplex PCR (pPCR) assay. Chapter 4 presents the application of the pPCR for characterization of environmental isolates of *V. vulnificus* and *V. parahaemolyticus*. It also explores the effects of environmental variables (i.e., salinity and temperature) on the prevalences of *V. vulnificus* and *V. parahaemolyticus* in Breton Sound and Barataria Bay waters in the Gulf of Mexico. The conclusion is summarized in Chapter 5, in which the future research needs are discussed.

CHAPTER 2

LITERATURE REVIEW

2.1 *VIBRIO* COMMUNITY - ROLE AS AN ENVIRONMENT AND PUBLIC HEALTH RISK FACTOR

The genus of *Vibrio* under the family of Vibrionaceae, are gram negative, non-spore forming and motile, straight or curved rod shaped (0.5µm – 1 µm) bacteria (Bergey *et al.*, 2009). Most vibrios are oxidase positive and facultative anaerobes (Bergey *et al.*, 2009; Percival, 2004). *Vibrio* communities dwell in wide range of marine and fresh water environment. Salinity along with the degree of influx of fresh water due to heavy rainfall, tidal waves or stream diversions and temperature plays an important factor in their growth and prevalence. (Lund *et al.*, 2000)

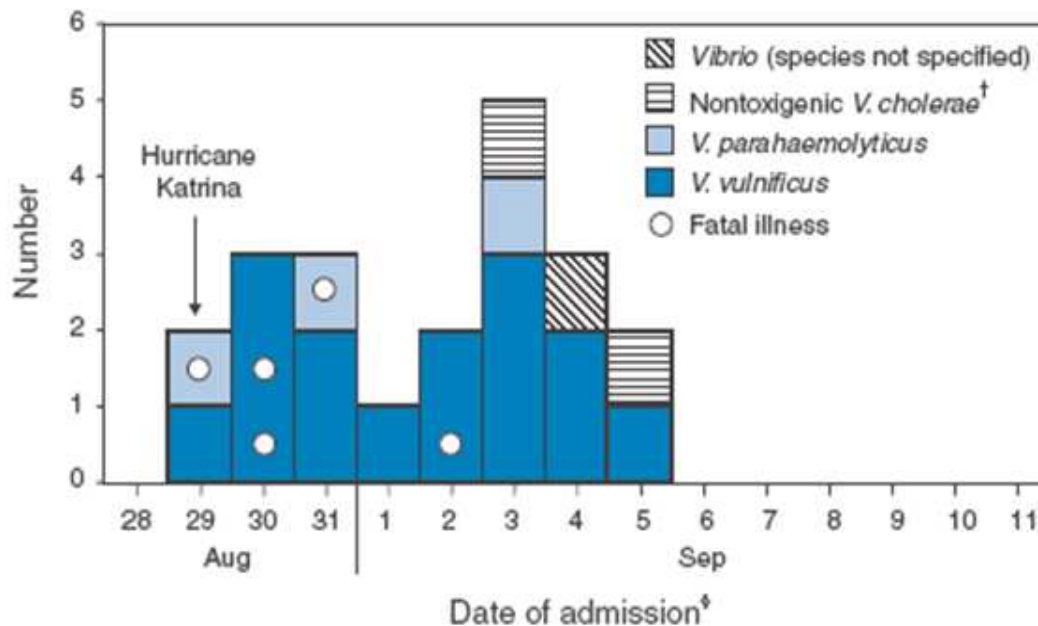
The major *Vibrio* species (spp.) causing food borne illnesses are *V. cholera*, *V. vulnificus* and *V. parahaemolyticus*. However, there are other less common *Vibrio* pathogens such as *V. fluvialis*, *V. furnissi*, *V. hollisae*, *V. metschnikovii*, *V. damsela*, *V. mimicus* and *V. alginolyticus*. Most vibrios express their pathogenicity by releasing various toxins, such as proteases, collagenases, enterotoxins, cytotoxin and mucinase which cause infections (Table 1). (Lund *et al.*, 2000)

It has been reported by CDC 2011 that approximately one in six people in the United States are affected with food borne illness every year (CDC, 2011; Elaine Scallan *et al.*, 2011) and vibrios play an important part in causing food borne illness. The increasing concern on *Vibrio* infections that pose a threat to human health had led CDC to initiate a voluntary surveillance system, i.e. “Cholera and Other *Vibrio* Illnesses Surveillance” (COVIS) for maintaining a database of *Vibrio* infections especially in the Gulf Coast regions of Alabama, Florida, Louisiana, Mississippi and Texas since 1988. The program was later on extended throughout the nation in 2007 due to increasing awareness of infections caused by *Vibrio* spp. (COVIS-CDC, n.d; Hoi Ho *et al.* on Medscape, n.d). Many probable cases of *Vibrio* infection are under-reported every year (MMWR, 2005).

Table 1: Infection caused by Non-cholera *Vibrio* Species, rate of infection and common symptoms associated with the infection type. (Source from: Hoi Ho *et al.* (Medscape), (no date) n.d; Lund *et al.*, 2000)

Infection Type	Clinical symptoms	Non-cholera <i>Vibrio</i> Species (Rate of Infection %)	Cytotoxins/Enzymes
Gastroenteritis	Diarrhea, Abdominal cramps, Nausea, Vomiting, Fever, Bloody stools, Headache, Myalgia	<i>V. parahaemolyticus</i> (59%) Non-01 <i>V. cholera</i> (67%) <i>V. fluvialis</i> (73%) <i>V. mimicus</i> (85%) <i>V. furnissii</i> (above 90%) <i>V. hollisae</i> (85%) <i>V. alginolyticus</i> (5-12%) <i>V. vulnificus</i> (5%)	Cytotoxin Hemolysin Heat stable/labile enterotoxins
Wound infection	Swelling, Pain, Erythema, Bullae, Necrosis, Gangrene	<i>V. alginolyticus</i> (71%) <i>V. vulnificus</i> (45%) Non-01 <i>V. cholera</i> (9%) <i>V. damsela</i> (>95%) <i>V. carchariae</i> <i>V. fluvialis</i> (10%) <i>V. parahaemolyticus</i> (34%) <i>V. mimicus</i> (3%)	Protease Hemolysin Lipase DNAase Cytolysin
Septicemia	Fever, Hypothermia, Hypotension, Tachycardia, Shock, Bullae, Acute respiratory distress syndrome, Multiple organ dysfunction	<i>V. vulnificus</i> (43%) <i>V. fluvialis</i> (6%) <i>V. damsela</i> (rare) Non-01 <i>V. cholera</i> (15%) <i>V. cincinnatiensis</i> (rare)	Proteases Endotoxic lipopolysaccharide

V. parahaemolyticus and *V. vulnificus* (apart from non-toxigenic *V. cholera*) are frequently reported in affected cases of **vibriosis** that peaks during the warmer months (CDC, 2011; CDC, 2008, CDC, 2009; COVIS (CDC), n.d). An outbreak of *V. parahaemolyticus* in July 2004 on a cruise ship in Alaska, affected nearly one third of the passenger with gastroenteritis illness. Outbreaks of *Vibrio* induced illness generally occur during the warmer months of the year. A more recent example is displayed with, the FDA News online release on September 26, 2011, that warned consumers to avoid the consumption of raw oysters from Hood Canal Area 4 in Washington State which were harvested between August 30 and September 19, 2011. At the time there were three confirmed cases and two possible cases associated with *V. parahaemolyticus* infection (FDA, 2011).



* N = 22; Alabama, a third state under surveillance, reported no cases.

[†] Nontoxigenic *V. cholerae* illnesses represent infections entirely distinct from the disease cholera, which is caused by toxigenic *V. cholerae* serogroup O1 or O139.

[§] Date of admission was not available for one Louisiana resident. In cases that did not require hospitalization, the date represents the first contact with a health-care provider for the illness.

Figure 1: Cases of *Vibrio* illness among the resident in Louisiana and Mississippi during Hurricane Katrina, in United States, August 29 – September 11, 2005 (Source from: MMWR, 2005)

After Hurricane Katrina on August 2005, nearly 80% of the 22 cases reported from Louisiana and Mississippi, were infected by *V. vulnificus* (MMWR, 2005), followed by *V. parahaemolyticus* and non-toxicogenic *V. cholera*. The pathogen gained entry into the bloodstream of the affected cases when the residents received skin and tissue injuries that were exposed to contaminated flood seawater during or following the hurricane. Figure 1 illustrates the illness caused by *Vibrio* spp. Five cases were fatal among these 22 cases. (MMWR, 2005)

As exemplified above, *V. parahaemolyticus* and *V. vulnificus* are a major threat to public health in the United States, and therefore monitoring their occurrence in the environment is critical to assurance of public health. The following sections discuss the occurrence, pathogenicity, factors that lower their abundance and detection methods for these two species.

2.2 OCCURRENCE OF *V. PARAHAEMOLYTICUS* AND *V. VULNIFICUS* IN THE ENVIRONMENT AND THEIR PATHOGENICITY

2.2.1 Occurrence of *V. parahaemolyticus*

V. parahaemolyticus has been isolated frequently from coastal waters and seafood in temperate zones all over the world. Although they prefer a halophilic condition they also have been reported to occur in lower numbers associated with freshwater planktons (Sarkar *et al.*, 1983). *V. parahaemolyticus* outbreaks have occurred worldwide, e.g. in United States (Barker, 1974), Britain (Barrow & Miller, 1972), Chile (Fuenzalida *et al.*, 2007), Bangladesh (Hughes *et al.*, 1978), Taiwan (Chiou *et al.*, 2000), Spain (Lozano-León *et al.*, 2003), Africa (Ansaruzzaman *et al.*, 2005).

The organism was first discovered by Fujino *et al.*, Osaka, Japan in the early 1950s, which caused food poisoning from eating boiled, half cooked fry of sardine “Shirasu” (Fujino *et al.*, 1951) and was later found in blue crabs (Krantz *et al.*, 1969) and other seafood (DePaola *et al.*, 1994). Interestingly, apart from posing a threat to human health, there were two virulent strains of *V. parahaemolyticus* (strain number: 880713 and 880915) reported as a zoonotic pathogen. These two strains are lethal to abalones (edible snails) and had the ability to transfer infection to humans following the consumption of infected abalones (Lee *et al.*, 2003).

Prevalence of these bacteria are strongly influenced by temperature and are abundant in the warmer months (Davis & Sizemore, 1982; DePaola *et al.*, 2003; Cook *et al.*, 2002; Daniels *et al.*, 2000; McLaughlin *et al.*, 2005; Sobrinho *et al.*, 2010). *V. parahaemolyticus* have been found to decline when the temperature in the water column drops to 11°C and proliferates when the temperature increases to 21°C (Watkins & Cabelli, 1985). Temperature related studies showed that *V. parahaemolyticus* cells become inactive when frozen at -80°C and survives for at least 3 weeks at 4°C (Johnson *et al.*, 1973). When frozen cells, are subjected to ambient temperatures 35±2°C they revive and grow (Kaysner and DePaola, 2004 - FDA-Bacteriological Analytical Manual, Chapter 9). *V. parahaemolyticus* survives 8 days even after incubation at -18°C (Vanderzant and Nickelson, 1972). These bacteria are destroyed when subjected to 100°C for one minute (Vanderzant and Nickelson, 1972).

Salinity has been related to strongly impact the abundance of *V. parahaemolyticus* (DePaola *et al.*, 2003). Although some studies have observed salinity as one of the secondary factors, along with nutrient stress (Roszak & Colwell, 1987; Oliver & Wanucha, 1989), turbidity and chlorophyll (Watkins and Cabelli, 1985; Lobitz *et al.* 2000; Deepanjali *et al.*, 2005; Phillips *et al.*, 2007; Zimmerman *et al.* 2007; Johnson *et al.*, 2010; Sobrinho *et al.* 2010) .

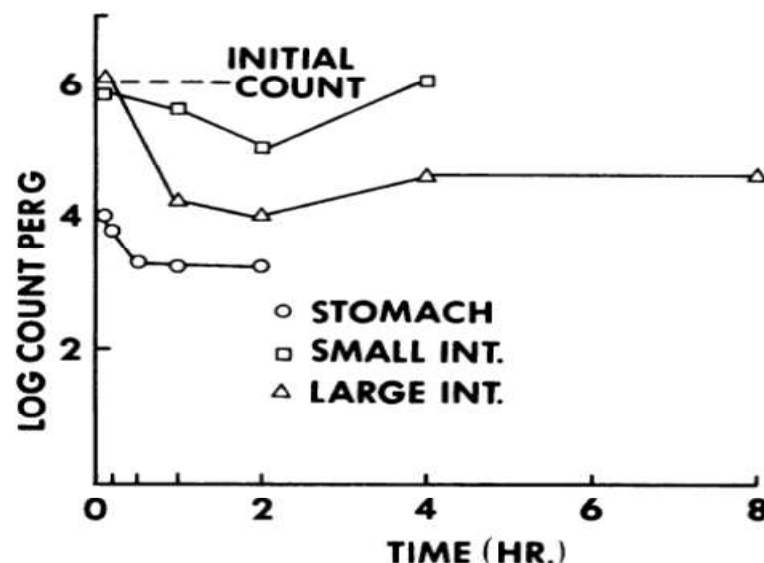


Figure 2: The change in population of *V. parahaemolyticus* inoculated into contents of three different parts of the porcine gastrointestinal tract. The pH value of the small intestine was 7.2 and the large intestine was 8.1.(Source from: Vanderzant and Nickelson, 1972)

V. parahaemolyticus prefers to grow in alkaline conditions in the gastrointestinal tract, and are susceptible to acidic pH lower than 6.0. Its survival for several hours in the porcine gastrointestinal tract demonstrates that viable cells in food upon ingestion may initiate its multiplication in small intestine (pH 7.2) and proliferation with increased pH in large intestine (pH 8.1). The alkaline pH of the small intestine favors the growth of *V. parahaemolyticus* (Vanderzant and Nickelson, 1972) as shown in Figure 2.

2.2.2 Pathogenicity of *V. parahaemolyticus*

People infected with this pathogen show symptoms of gastroenteritis such as diarrhea, abdominal cramps, nausea and vomiting (Blake *et al.* 1980; Joseph *et al.*, 1982; Janda *et al.*, 1988; Nishibuchi *et al.*, 1992) which occur within 24 hours of ingestion and the illness subsides after 3 days (Hunter, 2010).

All *V. parahaemolyticus* strains are designated to contain a thermolabile hemolysin gene that is species-specific. Among the various pathogenic traits, identification of two genetic markers *tdh* and *trh* are given importance, in this study. The virulent strains of *V. parahaemolyticus* may contain either a *tdh* or *trh* hemolysin gene or both. Pathogenic strains of *V. parahaemolyticus* have been associated in causing hemolytic activity (Joseph *et al.*, 1982), that can lyse erythrocytes (Honda *et al.*, 1988; Nishibuchi *et al.* 1989). The hemolytic strains were mostly isolated from human subjects affected by food poisoning (Chun *et al.* 1975; Miyamoto *et al.*, 1969). The *tdh* gene in *V. parahaemolyticus* strains express thermostable direct hemolysin (TDH) protein (Honda *et al.*, 1976; Joseph *et al.*, 1982). Another cytotoxic thermostable hemolytic toxin TDH-related hemolysin is encoded by *trh* gene (Carruthers, 1975; Honda, *et al.* 1976; Honda *et al.*, 1988) which is produced upon the entry of the microbe into the cell.

TDH above 5 µg per 430 g of body weight in rats proved to be a lethal cardiotoxin (Honda *et al.*, 1976). Through immunological studies, TRH and TDH of a Kanagawa hemolysin protein strain were found to be immunologically analogous to one another (Honda and Iida 1993; Honda *et al.* 1988). *V. parahaemolyticus* strains producing either TDH or TRH or even both have known to cause gastroenteritis symptoms in humans on consumption of contaminated seafood (Miyamoto *et al.*, 1969; Kim *et al.*, 1999).

Some of these pathogenic genes are present in the bacterial genomic DNA which contributes to evolution by horizontal gene transfer. These pathogenic genes are mobile genetic elements that are flanked with sequences which encode a bacteriophage-like integrase enzyme to facilitate its integration into genomic DNA of other microbial strains, thereby, leading to horizontal gene transfer. Integration of these pathogenic genes may impart a virulent character to non-pathogenic *Vibrio* spp. (Dobrindt *et al.*, 2004; Hurley *et al.*, 2006). For example, the hemolysin gene occurs in many bacterial species, the presence of hemolysin genes such as *tdh*, *trh1*, and *trh2* in *V. parahaemolyticus* were also found to occur in detectable amounts in 3 strains of *V. hollisae* or *Grimontia hollisae*, and 2 strains of *Vibrio mimicus* (Bej *et al.*, 1999; Yamazaki *et al.*, 2010) and a sucrose-positive strain of *V. alginolyticus* showed to contain a *trh* gene (Nordstrom *et al.*, 2007).

The FDA and ISSC (Interstate Shellfish Sanitation Conference) put these two genes *tdh* and *trh* under strict monitoring programs since 1997. The agencies delineated a sample based control plan where the levels of total *V. parahaemolyticus* containing *tlh* gene, should not surpass 10,000 viable cells per gram of oyster. The Interim Control Plan (ICP) in 1999 was revised in 2001 to include the stipulation that if *tdh*⁺ pathogenic strains are present at the level of 10,000 cells / g, the oysters should be re-checked. If the *tdh* gene was found to be present, then the harvest waters would be shut down and resampled, and the limit of the number of cells/g was reduced to 5000. Various models were analyzed and it suggested that if the limit was reduced to 5000 cells/g it could avert nearly 100% of the illnesses caused by *V. parahaemolyticus* in oysters at retail. (FDA, 2005)

2.2.3 Occurrence of *V. vulnificus*

V. vulnificus have similarities with *V. parahaemolyticus* in their occurrence, as both are halophilic (Thorsteinsson *et al.*, 1974; Morris, 1988). Recurrent outbreaks of *V. vulnificus* have occurred in United States due to the consumption of improperly handled oysters or by eating them raw from Gulf of Mexico (Rippey, 1994; Shapiro *et al.*, 1998; Wallace *et al.*, 1999). *V. vulnificus* in coastal environments have been reported to have a profound correlation to water temperature, salinity, biological and other physiochemical factors (Kaspar and Tamplin, 1993; Høi & Larsen *et al.*, 1998; Bryan *et al.*, 1999; DePaola *et al.*, 2006; Ralph and Currie, 2007).

Abundance of *V. vulnificus* has been reported to sustain in an optimal salinity range. The reported ranges vary between 5- 25 ppt or parts per thousand (Kaspar and Tamplin, 1993; Høi & Larsen *et al.*, 1998; Motes *et al.*, 1998; Randa *et al.*, 2004). Kaspar and Tamplin *et al.*(1993) proved in an experimental setup, that *V. vulnificus* abundance is observed when the temperature raises from 13 to 22°C when a constant salinity of 10 ppt was maintained and the numbers were found to decrease 100-fold when the temperature reached 0°C. However, a steep decline in their abundance occurs above 30 ppt (Kaspar and Tamplin, 1993) which may be due to plasmolyses of cells.

2.2.4 Pathogenicity of *V. vulnificus*

Out of 263 total known cases from 1989-2000, 138 deaths and 125 illnesses have occurred due to *V. vulnificus* pathogens (CSPI; CSPI- reports, n.d). They are known to cause wound infections - primary sepsis when ruptured skin comes in contact with the pathogen and gastroenteritis on consumption of contaminated or raw seafood (Blake *et al.*, 1979; Tacket *et al.*, 1984; Shirouzu *et al.*, 1985; Johnston *et al.*, 1986; Vollberg and Herrera, 1997).

V. vulnificus causes its pathogenicity by being extremely destructive to tissues causing necrotizing fasciitis, hence along with *V. parahaemolyticus*, these species are grouped under “Flesh eating Bacteria” (Woo *et al.*, 1984; Hung *et al.*, 1988; Green *et al.*, 1996; Hlady & Klontz, 1996; Fujioka *et al.*, 2003; Ralph & Currie, 2007). Other pathogenic traits (Linkous & Oliver, 1999) found in *V. vulnificus* strain include, capsular polysaccharide (Kreger *et al.*, 1981; Reddy *et al.*, 1992; Strom & Paranjpye, 2000) resistance to phagocytosis, express proteins that have iron acquisition ability (Wright *et al.*, 1981), secrete extracellular hemolysin protein (Oliver *et al.*, 1986) and the endotoxin- lipopolysaccharide (McPherson *et al.*, 1991).

In this study detection of *vvhA* and *viuB* for *V. vulnificus* was focused. The gene *vvhA* is species-specific. Initial studies reported that *vvhA* encodes a 50kDA protein that was cytolytic. This protein showed evidence of amino acid sequence homology also found in *V. cholera* non-O1, *V. mimicus* and *V. hollisae* (Yamamoto *et al.*, 1990). *In vivo* gene expression studies in mice infected with *V. vulnificus* has shown to produce an exotoxin that leads to septicemia, confirming the presence of hemolysin gene (Gray & Kreger, 1985; Lee *et al.*, 2004) and causing hypotension in rats (Kook *et al.*, 1996; Kook *et al.*, 1999; Lee *et al.*, 2004). Although this concept is still in

debate (Lee *et al.*, 2004), because upon inactivation of the *vvhA* gene, the cells still demonstrated pathogenicity (Wright & Morris, 1991). *V. vulnificus* may contain a pathogenic siderophore-encoding gene, *viuB*. The *viuB* gene encodes a homologous amino acid sequence 80% similar to a ferric vibriobactin utilization gene (Bogard & Oliver, 2007) in *V. cholera* (Litwin *et al.*, 1996). The presence of *viuB* prefers to thrive better in serum containing elevated levels of iron (Bogard & Oliver, 2007; Wyckoff *et al.*, 1997), therefore, *viuB* is more prevalent in clinical strains than environmental strains (Bogard & Oliver, 2007; Han *et al.*, 2009).

It has been reported that victims with elevated serum iron levels due to damaged iron metabolism are very vulnerable to *V. vulnificus* infections (Wright *et al.*, 1981). This shows that the presence of *viuB* is strongly dependent on serum iron availability (Bogard & Oliver, 2007). Iron plays an integral part constituting the structure of hemoglobin. Some of the red blood cells are destroyed intravascularly and others by the reticuloendothelial system (RES). The heme-moiety containing iron in the degraded hemoglobin is delivered to the liver or RES for iron reclamation for the body (Shils & Shike, 2006). Liver plays a main role in iron storage and regulation for the entire human body (Shils & Shike, 2006). In patients with liver diseases (Vollberg & Herrera, 1997), the iron storage capacity is impaired, which boosts the level of free hemoglobin in the plasma, thus increasing susceptibility of infection upon the consumption of *V. vulnificus* contaminated oysters. It has also been found that patients with AIDS or diabetes, β -thalassemia and hemochromatosis are prone to potential health risk when consuming raw oysters (Litwin & Calderwood, 1993; Litwin *et al.*, 1996; FDA Fact-Sheet, 2009; Han *et al.*, 2009; Hunter, 2010).

2.3 FACTORS LOWERING THE OCCURRENCE OF *V. vulnificus* AND *V. parahaemolyticus* IN THE ENVIRONMENT

2.3.1 Bacteriophages

V. vulnificus and *V. parahaemolyticus* have been reported to be susceptible to cell lysis by bacteriophages. Bacteriophages occur in lower densities in the water column than in oysters and are usually prevalent during the warmer months of the year (Baross *et al.*, 1978; DePaola *et al.*, 1997; DePaola *et al.*, 1998). Studies have reported that about nine phage isolates belonging under four morphological groups were isolated near Louisiana coastal waters (Pelon *et al.*,

1995). Phages have shown to infect *V. vulnificus* strains isolated from oysters, crab and human blood and they were found to occur in oyster tissues (DePaola *et al.*, 1997). About 87% of 60 *V. vulnificus* isolates tested by Pelon *et al.* (1995), were susceptible to infection caused by one or more bacteriophages (Pelon *et al.*, 1995). Therefore, these bacteriophages may be another means of lowering densities of *Vibrio* apart from temperature and salinity effects.

2.3.2 Bacteriocins

Many Gram negative and Gram positive bacterial strains secrete proteinaceous compounds that have been reported to be antagonists to other bacterial species residing in the same environment (Joerger, 2003; Riley & Wertz, 2002). These compounds are known as Bacteriocins and when produced by *Vibrio* spp. are termed “Vibriocins” (Farkas-Himsley & Seyfried, 1962). These antimicrobial compounds have been isolated from *V. cholera* (Wahba, 1965), *V. harveyi* (McCall & Sizemore, 1979) and *V. vulnificus* (Shehane & Sizemore, 2002) are found to inhibit a wide range of species, such as *V. vulnificus*, *V. parahaemolyticus* and other *Vibrio* spp. (Shehane & Sizemore, 2002).

Carraturo *et al.* (2006), identified Bacteriocin – like inhibitory substance (BLIS) produced by halophilic non-pathogenic *Vibrio* spp. *V. mediterranei* (Pujalte & Garay, 1986) has antimicrobial activity against *V. parahaemolyticus* which dwells in the same halophilic environment as that of the antagonist (Carraturo *et al.*, 2006). These bacteriocins may aid in reducing the *Vibrio* population and currently research is being carried out to determine the feasibility of using bacteriocins to reduce the risk of *V. vulnificus* and *V. parahaemolyticus* contamination in oysters.

2.3.3 VBNC stage

One interesting phenomenon is the ability of bacteria to enter a phase where they fail to grow in conventional growth culture medium but are still viable. This stage “Viable But Non-Culturable” VBNC is known to occur in *Vibrio cholera*, *V. parahaemolyticus*, *Campylobacter jejuni* and *Helicobacter pylori* (Barer, 1997; Coutard *et al.*, 2007). The VBNC state in *Vibrio* spp., usually occurs when the cells are under cold stress with saline conditions (Lyon, 2001) or under starvation such as carbon stress (Chen *et al.*, 2009). Electron microscopic studies by

Coutard *et al.*, 2007, have shown that, VBNC cells at cold temperatures remain viable and are able to undergo cell division once ambient temperature conditions are provided. This is an important public health issue once, these bacteria enter into VBNC state they cannot be detected by classical methods and their occurrence in the samples are difficult to detect especially in winter (Coutard *et al.*, 2007).

Studies have also shown that *V. vulnificus* enter into the VBNC state. One study showed (Oliver *et al.*, 1995) that *V. vulnificus* in estuarine waters enter into the VBNC state during the cooler months when temperature drops below 15°C and the average salinity is 14.5 ppt. The *Vibrio* cells revive back into cultivable state having vigorous metabolic activities only when the water temperature reaches an average of 21°C and a salinity of 24.5 ppt (Oliver *et al.*, 1995). Expression of the *vvh* gene was seen even after four months in the VBNC stage by detecting the mRNA transcripts (Fischer-Le Saux *et al.*, 2002). Therefore it is important to use the genetic information as a baseline in understanding the occurrence of the organisms and to detect prevalence of pathogenic strains.

2.4 METHODS OF DETECTION

2.4.1 Non - PCR based conventional methods

Screening for *V. parahaemolyticus* and *V. vulnificus* and identification of virulent strains using biochemical and colony hybridization methods as mentioned in the FDA-Bacteriological Analytical Manual is a time consuming process. Water, sediment and oyster samples when added to Alkaline Peptone Water (APW) by serial dilution for enumerating the Most Probable Number (MPN) produce only putative results, as other halophilic microorganisms in the samples may grow in the APW solution. These MPN tubes are further subjected to PCR analysis to validate the bacteria under detection. (Kaysner and DePaola, 2004)

Conventional detection methods such as growth on selective media have been used as a test for detection of microbes (Lyon, 2001). Thiosulfate-Citrate-Bile-Sucrose Agar (TCBS, BD™) agar, a selective growth medium is generally used for isolating *Vibrio* spp. from water, sediments and contaminated sea food (Kobayashi *et al.*, 1963). Most of the gram-positive bacteria are inhibited due to the presence of mixture of bile salts such as oxgall and sodium

cholate. Hydrogen sulfide producing strains can utilize sulfur in the form of Sodium thiosulfate with combination of ferric citrate. *Vibrio* can ferment and metabolize sucrose provided in the TCBS agar. The high pH of the medium enables recovery of salt loving *Vibrio* spp. Any change in pH of the medium is detected by the dyes thymol blue and bromothymol blue. Most of the *Vibrio* colonies give a hue of colors ranging from green to yellow on the TCBS agar. Although TCBS medium is widely used to isolate vibrios in general, it also supports growth of other non-*Vibrio* spp., such as *Pseudomonas* and *Aeromonas* (HARDY-DIAGNOSTICS).

Another widely used differential medium is Cellobiose-Colistin (CC) agar containing acceptable levels of the colistin antibiotic and cellobiose. CC agar is specifically used for the isolation of *V. vulnificus*, which are observed as flat yellow colonies that have a higher plating efficiency than when grown on cellobiose-polymyxin B-colistin (CPC) agar (Oliver *et al.*, 1992; Høi & Dalsgaard *et al.*, 1998).

Use of these selective agar media only assist in isolation of culturable *Vibrio*, but distinction between species, i.e. *V. parahaemolyticus* and *V. vulnificus* cannot rely solely upon identifying colony characteristics on agar surface. *Vibrio* spp., tend to give similar colony color or morphology and thus identification requires further confirmation (Kaysner and DePaola, 2004). Bacterial colonies on selective medium are further analyzed by genetic based methods (Hill *et al.*, 1991; Brauns *et al.*, 1991; Lee & Pan 1993; Tada *et al.*, 1992; Banerjee *et al.*, 2002), for the detection of specific species. Molecular genetics based analyses provide greater validity and more reliable results.

2.4.2 Other Non- PCR based methods

Immunological methods developed for identification of antigens produced by the pathogenic strains of *V. parahaemolyticus* using have been developed earlier (Honda *et al.*, 1980). The principle of this method was based on binding specificity of specific antigens to specific antibodies. However, antiserum against the toxins have shown to impart partial cross-reactivity with other immunologically similar antigens (Honda *et al.*, 1988), providing inconsistent results. Further genetic analyses of *tdh* and *trh* genes in *V. parahaemolyticus* virulent strains show that both genes encode a similar 165 amino acid residue (TDH and TDH-

related hemolysin) with a 50 – 70 % sequence homology (Kishishita *et al.*, 1992; Honda & Iida, 1993; Nishibuchi & Kaper, 1985 & 1995; Park *et al.*, 2000) . Enzyme linked immunosorbent assay developed for detection of *V. parahaemolyticus* gave false positive reactions with three other *Vibrio* spp., *V. tubiashii*, *V. campbellii* and *V. vulnificus* (Chen & Chang, 1995). Hence, this technique was not suitable to identify different pathogenic strains.

DNA probe hybridization with Alkaline- or Digoxigenin- labeled oligonucleotide probes for both *V. vulnificus* (*vvh* cytotoxin- hemolysin gene) and *V. parahaemolyticus* (*tlh*, *tdh* and *trh* hemolysin gene) have been developed (Morris *et al.*, 1987; Shirai *et al.*, 1990; Wright *et al.*, 1993; McCarthy *et al.*, 1999; Gooch *et al.*, 2001; Banerjee *et al.*, 2002; Nordstrom *et al.*, 2006; Zimmerman *et al.*, 2007). Although, the time taken to achieve the steps involved during the entire hybridization (McCarthy *et al.*, 1999) protocol retards the rapid detection, as one gene is targeted at a time for detection and interference of weak bands gives false positive results due to other *Vibrio* spp. Furthermore, the irregular distribution and lesser copy number of pathogenic genes, along with VBNC state in bacterial cells can escape detection by DNA probe hybridization method (Cook *et al.*, 2002). To improve the detection of a large number of virulent and non-virulent isolates, a number of PCR protocol has been developed.

2.4.3 Concept of PCR and PCR detection methods

Biotechnology based methods such as Polymerase Chain Reaction (PCR), a discovery made by Nobel laureate Kary B. Mullis in 1983, has proven to be a faster and more reliable method for the detection of microbes. PCR uses a set of temperature cycles which aid in denaturing the double stranded DNA molecule at 95⁰C to single stranded DNA, followed by the annealing of specific oligonucleotides called “primers” at ~55 – 60⁰ C. Finally the extension of primers specifically bound to sequences flanking the target DNA occurs with the addition of dNTPs (deoxynucleotide triphosphates) at ~72⁰C. (Joseph Sambrook, 2001)

The three steps (denaturation, annealing and extension) are repeated in ~ 30 cycles in the presence of an enzyme and a suitable buffer with all the cofactors involved in the amplification process. A heat stable enzyme *Taq* Polymerase purified from *Thermus aquaticus* (discovered by Thomas D. Brock) is one of the foremost components catalyzing the extension of unique primers

specifically designed to bind to the target gene. The *Taq* Polymerase enzyme also aids in extension and amplification of several thousand copies of the target gene of interest. In addition temperature and time must be modulated in order to avoid erroneous PCR results. Amplified products of the target gene of varying molecular size can be electrophoresed on agarose gel stained with a DNA intercalating fluorescent dye Ethidium bromide and visualized under a UV illuminator (Joseph Sambrook, 2001).

The PCR reaction offers a time and cost-effective means of detection of bacteria. In terms of rapid detection that avoids gel electrophoresis of the amplicons, real time PCR is effective. Although the cost of real time PCR machines can be much more expensive than a conventional PCR thermocyclers, depending upon the number of reaction wells and the number of signal detection channels.

2.4.4 PCR studies on *V. parahaemolyticus* identification

Due to the pathogenic effect of two main genes in *V. parahaemolyticus*, several PCR protocol for detection of the hemolysin genes, *tdh* and *trh*, were developed (Lee & Pan, 1993; Tada *et al.*, 1992). The presence of both of these genes acts as bio-markers to detect virulent type strains of *V. parahaemolyticus* (Shirai *et al.*, 1990; Tada *et al.*, 1992). These genes were later reported to occur in other *Vibrio* spp., as detected by DNA colony hybridization (Nishibuchi & Kaper, 1985 & 1995). Therefore, a primer pair to identify a species-specific gene was important to be included in the PCR protocol. Designing of primers for *tl* or *tlh* gene (450 bp amplicon) has able to resolved the identification of *V. parahaemolyticus* as it is present in both pathogenic and non-pathogenic strains (Taniguchi *et al.*, 1985; Taniguchi *et al.*, 1986). Furthermore, to reduce the time and cost of amplification of each of these genes individually, Bej *et al.* (1999) developed a **multiplex PCR** (mPCR) protocol. The primers for *tlh* gene has been used in collaboration with the primers for *tdh* gene (Nishibuchi & Kaper, 1985) and the *trh* gene (Honda *et al.*, 1991; Honda & Iida, 1993) to provide an alternative method for single PCR detection and analyses of the distribution of multiple (*tlh*, *tdh* and *trh*) genes in *V. parahaemolyticus* strains (Bej *et al.*, 1999), simultaneously in one PCR reaction tube (mPCR reaction). This protocol (Bej *et al.*, 1999) has been used to detect a *tdh* positive *V. parahaemolyticus* outbreak in Spain caused by consuming raw oysters (Lozano-León *et al.*, 2003).

With the advent of PCR studies, the earlier data reporting that urease positive strains of *V. parahaemolyticus* were correlated to the presence of *trh* gene (Suthienkul *et al.*, 1995; Osawa *et al.*, 1996) were later disproved (Nakaguchi *et al.*, 2003; Robert-Pillot *et al.*, 2004; Sujeewa *et al.* 2009). Thus erroneous results from various biochemical reactions could be eliminated with the help of PCR. The turbidity in the APW-MPN culture tubes or colonies on TCBS and CC agar could be further detected using multiplex PCR (Bej *et al.*, 1999) to screen pathogenic genes in water and organic samples (Jahangir Alam *et al.*, 2002; Hara-Kudo *et al.*, 2003).

Annealing temperature plays a key role in any PCR reaction. In mPCR where more than one primer pair is involved, it is necessary to set one annealing temperature which is optimal in directing proper hybridization of all the primer pairs and amplification of more than one primer-specific target gene sequence. Several multiplex PCR protocols have been designed. For example, a successful mPCR for the detection of *tdh* (amplicon size 263 bp) and the *toxRS/new* sequence (amplicon size of 651 bp) for O3:K6 strains was achieved at 45°C annealing temperature (Okura *et al.*, 2003). However, another reported protocol where occurrences of seven virulent biomarkers tested in *V. parahaemolyticus* and *V. cholerae* used different annealing temperatures for detection of the pathogenic genes, thereby prolonging the time of detection. These seven biomarkers were *tlh* (448 amplicon size; Genbank [AB012596.1](#)), *trh* (500 amplicon size; Bej *et al.*, 1999), *tdh* (534bp amplicon size; Genbank [D90238.1](#)), *tox R* (658 amplicon size; Genbank [L11929](#)) and *tox RS* for *V. parahaemolyticus* along with two other genes for *Vibrio cholera*. (Xie *et al.*, 2005).

Concentration of primers, PCR buffers, dNTPs (deoxynucleotide triphosphates) and enzymes are other important factors that has to be optimized in a PCR reaction, to enable amplification of target genes that are present in lower copy numbers. For example, the pathogenic genes *tdh* and *trh* have been reported to be present in lower copy numbers (1-2) on chromosomal DNA than the species- specific *tlh* gene, which interferes with the detection of the pathogenic genes causing false negative result (Brasher *et al.*, 1998; Dileep *et al.*, 2003; Iida *et al.*, 1998; Nordstrom *et al.*, 2007; Zimmerman *et al.*, 2007). Therefore, experiments in PCR have limited the primer concentration for *tlh* gene, which is known to be present in high copy numbers, to save enough PCR reaction components to enhance amplification and detection of pathogenic genes present in lower ratios (Nordstrom *et al.*, 2007).

2.4.5 PCR studies on *V. vulnificus* identification

A heat labile extracellular toxin showing hemolytic activity encoded by the *vvhA* gene (Gray & Kreger, 1985) is species-specific to *V. vulnificus* (Wright *et al.*, 1985; Morris *et al.*, 1987; Hill *et al.*, 1991). The first reported PCR study done on this gene was in contaminated oyster samples, which was achieved by designing specific primer sequences that would amplify 519 bp region (Hill *et al.*, 1991) in a previously sequenced cytotoxin hemolytic gene (Yamamoto *et al.*, 1990). This research initiated further PCR studies in the identification of *V. vulnificus*.

Primers amplifying 340 bp region of the hemolysin gene demonstrated that PCR could not only detect genes in viable cells but also in the VBNC state (Brauns *et al.*, 1991), which were undetected by the use of biochemical, immunological and DNA colony hybridization methods. Subsequently, the *viuB*, a virulence gene of *V. vulnificus* (504 bp amplicon size) related to iron acquisition (Litwin & Calderwood, 1993; Litwin *et al.*, 1996) was amplified in a **multiplex PCR** protocol along with the species-specific gene *vvhA* in shellfish (Panicker *et al.*, 2004a). The use of multiplex PCR (mPCR) in detection of *vvhA* and other pathogenic genes such as, *viuB*, *vcg* (virulence-correlated), 16S rRNA, and *CPS* (capsular polysaccharide operon), in one PCR reaction tubes enabled characterization and differentiation between environmental and clinical isolates, simultaneously (Han *et al.*, 2009; Han & Ge, 2010).

2.4.6 Simultaneous identification of both *V. vulnificus* and *V. parahaemolyticus* in other mPCR assays

Success with PCR and mPCR assay (as discussed above) has been achieved by combining more than one unique gene specific primer pairs in a single PCR reaction tube. Apart from applying suitable PCR reaction buffers and *Taq* polymerase enzyme, factors such as selection of unique primer sets to amplifying specific genes, testing primer pair combinations compatibility and finding an optimal annealing temperature must be optimized in mPCR assay. This would enable detection of a wide range of bacterial species simultaneously in one PCR reaction tube. For instance, Brasher *et al.* (1998) developed a rapid mPCR method to detect five bacterial pathogens in shellfish, such as, *Escherichia coli*, *Salmonella typhimurium*, *Vibrio vulnificus*, *V. cholera* and *V. parahaemolyticus* for the presence or absence of *uidA*, *cth*, *invA*, *ctx*, and *tl* genes simultaneously, at annealing temperature 55°C with five pairs of generic

species-specific primer set. A “**double**” multiplex PCR amplification approach by Brasher *et al.* (1998) aided to achieve maximum sensitivity of $\leq 10^1$ – 10^2 cells (Brasher *et al.*, 1998). Furthermore, Neogi *et al.* (2010) developed a multiplex PCR at 55°C annealing temperature, combining **novel** species-specific PCR primers to detect samples for three *Vibrio* spp., *toxR* gene for *V. cholerae* and *V. parahaemolyticus* and *vvhA* gene for *V. vulnificus* simultaneously and detection limit was 10 cells per PCR tube (Neogi *et al.*, 2010). Their methods can be applied for quick detection of multiple species coexisting in food, clinical and environmental samples. (Brasher *et al.*, 1998; Neogi *et al.*, 2010)

A multiplex PCR involving primers targeting simultaneous amplification of ten genes (*tlh*, *tdh*, *trh*, *orf8*, *vvhA*, *viuB*, *tcpI*, *ompU*, *toxR*, *hlyA*) in a single PCR reaction for detection of three *Vibrio* spp. reported earlier (Panicker *et al.*, 2004a), did not give reliable results, probably due to the complexity of the primer matrix. Dr. Feifei Han’s (a former LSU student from the Department of Food Science) dissertation, studied the detection of *V. vulnificus* and *V. parahaemolyticus* using two separate multiplex PCR reactions. The first multiplex targets the amplification of two genes *vvhA* and *tlh* on the *Vibrio* isolates for species identity - *V. vulnificus* or *V. parahaemolyticus*. A second separate multiplex was done for determination of pathogenic genes (*tdh*, *trh*) on confirmed *V. parahaemolyticus* isolates. The presence of *viuB* pathogenic gene was targeted along with other genes *vcg*, 16S rRNA and *CPS* for *V. vulnificus* in a separate multiplex reaction. (Han, 2010)

Multiplex PCR assays have been widely used, as mentioned above to detect diverse strains of organisms using a concoction of suitable primer pairs at optimal annealing temperature. However, no multiplex protocol has been developed to target the detection of bacterial isolates simultaneously, for the presence or absence of *V. vulnificus* and *V. parahaemolyticus* and their virulent strains in one PCR reaction by targeting *tlh*, *trh*, *tdh*, *vvhA* and *viuB* genes, as done in this study.

2.4.7 Identification of *V. vulnificus* and *V. parahaemolyticus* with 16S rDNA sequencing

The 16S rDNA sequencing technique has become a standard method for identification of various bacteria in environment and analysis of their phylogenetic relations. In addition, it gives an insight on the variation in sequences enabling us to analyze the evolutionary aspects, with

other closely-related species. Also, 16S rDNA sequencing is useful to validate a molecular detection method, by comparing the identity of the species tested in both the methods.

The 16S rRNA is present in 30S small subunit in Ribosomes (S stands for sedimentation rate measured in Svedberg). Ribosomes are complex cell organelles which comprises of RNA and proteins. In Figure 3, the prokaryotic 70S ribosome consists of a larger 50S subunit and a smaller 30S subunit (Lodish *et al.*, 1995; Clarridge, 2004; van Straalen & Roelofs, 2011). The steps involved in 16S rDNA sequencing, begins with the extraction of the DNA template from the cell. The template contains the 16S rDNA sequence which encompasses highly conserved domains ~1600 nucleotides (Buller, 2004) that can be amplified in a PCR thermal cyclor with the aid of specific forward and reverse 16S primers, dNTPs, *Taq*-polymerase enzyme and PCR buffer. The primers bind complementarily to the 5'- sequence flanking the 16S rDNA domains (Clarridge, 2004).

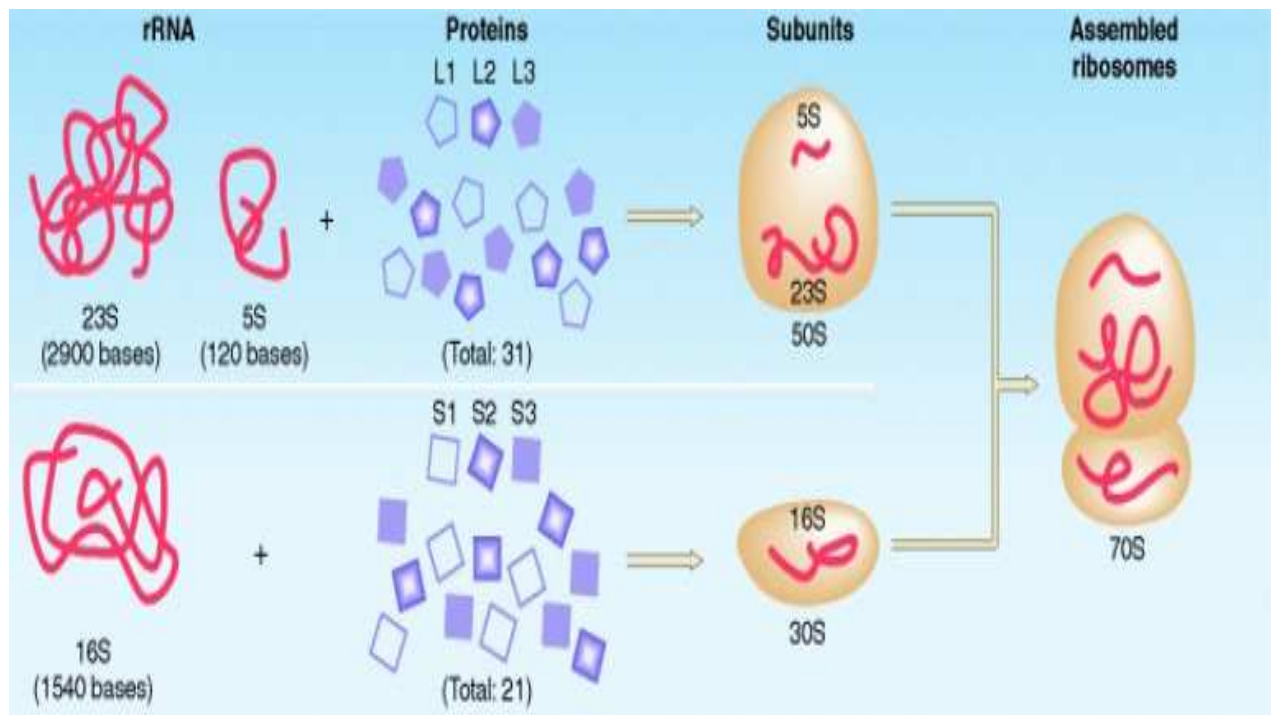


Figure 3: Entities making the prokaryotic ribosomal complexes. (Source from: Lodish *et al.* 1995 and Griffiths *et al.*, 1999)

The amplification of 16S rDNA can be restricted to first ~500bp with the use of suitable primers giving a satisfactory identity match. The first 500bp of the 16S DNA sequence contains hypervariable domains which enables rapid identification of species. These domains ranging from 20-30bp occurs nearly four times at the first 500bp region of 16S DNA (Bergey *et al.*, 2009). The amplified 16S rDNA gene products are sequenced in an automated DNA sequencer, using either a 16S forward or reverse primer.

The sequenced nucleotides are further analyzed to match and compare with published sequences stored in online databases such as NCBI- BLAST and FASTA which are publically accessible. At least, 500bp of the DNA in query must be sequenced with one of the specific forward or reverse 16S primers, in order to effectively attain a 97% or higher sequence identity with closely related species (Stackebrandt & Goebel, 1994; Tewari *et al.*, 2011). With the combination of PCR amplification and 16S primer, any microbial species culturable or non-culturable state can be studied for sequence homology (Amann *et al.*, 1995; Buerger *et al.*, 2012). Depending upon the interest whether to identify an organism at the genus- or species-level, specific primers at each level should be used respectively (Jonathan & Karlene, 2009).

CHAPTER 3

PENTAPLEX PCR ASSAY FOR SIMULTANEOUS DETECTION OF *V. VULNIFICUS*, *V. PARAHAEMOLYTICUS* AND THEIR VIRULENT STRAINS

3.1 INTRODUCTION

In the United States, *V. vulnificus* and *V. parahaemolyticus* are the leading causes of seafood-related illnesses. These bacteria reside in sea water column in association with phytoplankton and get trapped by mucus in oyster gills during their filter feeding mechanisms and bioaccumulate in oyster tissues (NSSP Guide, 2007). Thus there is an increased risk of food poisoning through consumption of raw seafood. These bacteria are also abundant in sediments (Johnson *et al.*, 2010). *V. vulnificus* and *V. parahaemolyticus* are reported as flesh eating bacteria, which gain entry into the human soft tissues when skin lesions are in contact with contaminated waters during recreational activities (Woo *et al.*, 1984; Hung *et al.*, 1988; Hlady & Klontz, 1996; Green *et al.*, 1996; Fujioka *et al.*, 2003). Therefore, it is crucial to detect and monitor these microorganisms in the environment (Blake *et al.*, 1980; Tacket *et al.*, 1984; Klontz *et al.*, 1988; Daniels *et al.*, 2000; Kaysner and DePaola, 2004; McMeekin, 2003).

Species-specific molecular biomarkers widely used for detection of *V. vulnificus* and *V. parahaemolyticus* are, *vvhA* and *tlh* genes, respectively. The virulent strains of these species are marked by the presence of pathogenic genes which upon entry into the host cell are expressed to produce proteins that have cytotoxic effects. (Lund *et al.*, 2000)

The pathogenic strains of *V. parahaemolyticus* contain either a *tdh*- encoding Thermostable Direct Hemolysin (TDH) or *trh*- encoding TDH- related hemolysin (TRH), gene or sometimes both in one strain. The strains with these genes are mostly isolated from clinical samples (Shirai *et al.*, 1990). The proteins encoded by them have proven to cause gastroenteritis and can lyse red blood cells (Miyamoto *et al.*, 1969; Honda *et al.*, 1988; Honda *et al.*, 1991; Honda & Iida, 1993; Kim *et al.*, 1999; Nishibuchi & Kaper, 1995).

A clinically important gene found in *V. vulnificus* is the *viuB*, a siderophore-encoding gene associated with iron acquisition. Hence it presents a risk factor to patients with elevated iron levels in serum (Wyckoff *et al.*, 1997; Panicker *et al.*, 2004b; Bogard & Oliver, 2007). *V.*

vulnificus infections are a greater threat to people who fall under higher risk groups, i.e., patients with diabetes, liver diseases, AIDS, β -thalassemia or hemochromatosis (Bogard & Oliver, 2007; Hunter, 2010).

The FDA, NSSP (National Seafood Sanitation Program) and ISSC have strict regulations to screen oysters and other seafood products for these species and their pathogenic strains (Kaysner and DePaola, 2004; FDA, 2005; FDA, 2011). Several multiplex PCR assays have been developed for detection of *V. vulnificus* and *V. parahaemolyticus*, in which relevant genes can be detected in one reaction. The sensitivity of these PCR assays varied. For example, one assay could detect 10^2 to 10^3 CFU or Colony Forming Units of *V. vulnificus*/ml without enrichment (Panicker *et al.*, 2004a) and enrichment process was suggested to increase the level of detection (Panicker *et al.*, 2004b). For *V. parahaemolyticus* 10 to 100 CFU / 10g of APW enriched oyster tissue homogenates has been reported which is within the limit of NSSP guideline reported as 10^4 cells per g of shell stock (Bej *et al.*, 1999).

This study aimed at developing a fast, cost-effective multiplex PCR (termed Pentaplex PCR) assay to simultaneously detect the three pathogenic genes (i.e. *tdh*, *trh* and *viuB*) and two species-specific genes (i.e. *tlh* and *vvhA*) (Bej *et al.*, 1999; Panicker *et al.*, 2004a) in putative bacterial isolates for *V. parahaemolyticus* and *V. vulnificus*, using 5 pairs of known primers in a single PCR reaction tube.

3.2 MATERIALS AND METHODS

3.2.1 Reference strains and environmental isolates

Six *Vibrio* reference strains (Table 2), with known genotype (*V. parahaemolyticus* FII-3A, *V. parahaemolyticus* AQ4037, *V. parahaemolyticus* TX2103, *V. parahaemolyticus* FIHES98, *V. vulnificus* 1007 and *V. cholera* ATCC 14035) and one environmental isolate confirmed as *V. vulnificus* (V.v 3138-001) were used initially to develop the Pentaplex PCR assay, for the optimization of annealing temperature. The strains were checked and reconfirmed with individual primers listed in Table 3, and were revived on Luria-Bertani (LB) agar, Miller composition (Atlas, 2004) at 35 ± 2 °C for 24hrs.

Additional 293 environmental isolates were obtained from water samples collected from Breton Sound, Barataria Bay and Bay Jimmy, in 2011 and 2012. These putative *V. parahaemolyticus* and *V. vulnificus* strains were isolated on BD™ Difco TCBS (Kobayashi *et al.*, 1963) and Cellobiose-Colistin (CC) Agar (Bacteriological Analytical Manual- BAM Media M189) with 24hrs of incubation at 35 ± 2 °C. They were used to evaluate the specificity and efficiency of the pPCR assay. The revived reference strains and environmental isolates were stored in 2 X LB broth Miller, composition (Atlas, 2004) with 20% glycerol (Woods *et al.* 1993) at -80°C for further use in this study.

3.2.2 DNA template preparation

The *Vibrio* strains stored in LB broth with 20% glycerol were directly used for preparation of crude DNA templates via lyses of cells with heat. Twenty microliter (μl) of cell suspension in glycerol stock was pipetted in a 96-well PCR reaction plate and placed in a thermocycler (BIO-RAD iCycler™ MyiQ™ optical module). The cells were subjected to a boil step at 98°C for 10 min to lyse the cell wall and release the DNA contents. After the boil step, 1μl of the crude DNA (i.e. the boiled cell suspension without purification) was used as template in PCR reactions.

To check for any interference by the nuclease free water and autoclaved LB glycerol stock contained in the DNA template preparation, these ingredients were prepared in the same way as the crude DNA templates but without any DNA and used as negative controls (Table 2). Boiled cells of *Vibrio cholera* ATCC 14035 was used as another negative control to determine if there is an interference by non-target bacterial DNA.

3.2.3 Primer selection and concentration

The primers (Table 3) used for this experiment were previously published by Panicker *et al.*, Bej *et al.* and reported by the FDA manual (Bej *et al.*, 1999; Kaysner and DePaola, 2004; Panicker *et al.*, 2004a). Since the primer concentration used by Panicker *et al.* (2004a) did not produce clear bands in this study, we used an increased primer concentration to overcome this problem. This increased primer concentration was then used throughout the experiment although it has not been further optimized.

Table 2: Positive and negative controls used for the PCR annealing temperature optimization study.

Reference strains as positive control	Genotype	Source/ Origin
<i>V. parahaemolyticus</i> F11-3A	<i>tlh</i> ⁺ <i>trh</i> ⁺ <i>tdh</i> ⁺	¹ Washington clams (Bej <i>et al.</i> , 1999)
<i>V. parahaemolyticus</i> AQ 4037	<i>tlh</i> ⁺ <i>trh</i> ⁺	¹ A. DePaola, US FDA, Gulf Coast Seafood Lab (Nordstrom <i>et al.</i> , 2007; Noriega Iii <i>et al.</i> ,2010)
<i>V. parahaemolyticus</i> Tx-2103	<i>tlh</i> ⁺ <i>tdh</i> ⁺	¹ A. DePaola, US FDA, Gulf Coast Seafood Lab(Nordstrom <i>et al.</i> , 2007; Noriega Iii <i>et al.</i> , 2010)
<i>V. parahaemolyticus</i> FIHES98	<i>tlh</i> ⁺	¹ A. DePaola, US FDA, Gulf Coast Seafood Lab (Nordstrom <i>et al.</i> , 2007; Noriega Iii <i>et al.</i> , 2010)
<i>V. vulnificus</i> 1007	<i>vvh</i> ⁺ <i>viuB</i> ⁺	¹ Blood, Louisiana Department of Health and Hospitals (Ge, 2008)
Environmental isolate as positive control	Genotype	Source
<i>V. vulnificus</i> (Vv3138-001)	<i>vvh</i> ⁺	^{2*} Isolated from Breton Sound water sample
Negative controls - (absence of <i>tlh</i> , <i>trh</i> , <i>tdh</i> , <i>vvh</i> , <i>viuB</i> gene)		
<i>V.cholera</i> ATCC 14035	Negative control	¹ Clinical isolate
Nuclease free water	Negative control	Provided by Promega Corp.
Autoclaved LB glycerol stock	Negative control	Prepared in Lab

¹ Isolates were obtained from Dr. Beilei Ge, Dept. of Food Science, Louisiana State University, Baton Rouge, LA.

^{2*} Isolated and Confirmed in Microbial Ecology Lab3138, Dr. Aixin Hou, Department of Environmental Science, Louisiana State University, Baton Rouge, LA.

Table 3: The primer sequence used to amplify target genes in the pentaplex PCR assay.

Organism	Target gene	Primer sequence ^{*1 & *2} (F- Forward and R- Reverse)	Reported Multiplex PCR annealing °C	FDA reported annealin g °C (Kaysner and DePaola, 2004)	Ampli- con size (bp) ^{*1 & *2}
<i>V. vulnificus</i>	<i>vvhA</i> Species– specific gene	F-5'- TTC CAA CTT CAA ACC GAA CTA TGA C -3'	65 ^{*1}	62	205
		R-5'-ATT CCA GTC GAT GCG AAT ACG TTG-3'			
	<i>viuB</i> Pathoge- nic gene	F- 5'-GGT TGG GCA CTA AAG GCA GAT ATA-3'		Not tested by FDA	504
		R- 5'-CGG CAG TGG ACT AAT ACG CAG C-3'			
<i>V. parahaemolyticus</i>	<i>tlh</i> Species – specific gene	F- 5'-AAA GCG GAT TAT GCA GAA GCA CTG-3'	55 ^{*2}	60	450
		R- 5'-GCT ACT TTC TAG CAT TTT CTC TGC-3'			
	<i>tdh</i> Pathoge- nic gene	F-5'-GTA AAG GTC TCT GAC TTT TGG AC-3'		60	269
		R- 5'-TGG AAT AGA ACC TTC ATC TTC ACC-3'			
	<i>trh</i> Pathoge- nic gene	F- 5'-TTG GCT TCG ATA TTT TCA GTA TCT-3'		58	500
		R- 5'-CAT AAC AAA CAT ATG CCC ATT TCC G-3'			

^{*1} (Panicker *et.al*, 2004a) Primer sequences (*vvhA* and *viuB*) and temperature with PCR sensitivity of 10³ CFU/ ml Unenriched cultures.

^{*2} (Bej. *et.al*, 1999) Primer sequences (*tlh*, *tdh*, *trh*) and temperature with PCR sensitivity of 10-100 CFU /10 g of APW enriched with oyster tissue.

°C – temperature in Celsius

3.2.4 Optimization of pPCR annealing temperature

An optimal annealing temperature allows the primers to bind specifically to the flanking sequence near the target gene locus. An optimization study was performed with 55°C, 57°C, 59°C, 62°C, and 65°C to determine the best annealing temperature that would allow a specific binding of the 5 pairs of primers (Table 3) so as to enable amplification of all the five target genes simultaneously in one PCR reaction. The optimization effort employed the six reference strains and one confirmed environmental isolate as positive controls and included the negative controls listed in Table 2. To check the reproducibility of the assay, the PCR reaction was carried out five times for each of the five different annealing temperatures with PCR reaction mixtures prepared fresh at the beginning of each experimental day and using the PCR conditions described below. In addition, the selected optimal annealing temperature was further tested with individual primers pairs for all the positive and negative controls in Table 2.

3.2.5 PCR conditions for Pentaplex assay

The PCR reaction mixture contained 1 µl of the boiled crude DNA sample, 12.5 µl 1X GoTaq® Hot Start Green Master Mix (Promega Corporation, Madison, USA), 1.35 µM of each of the primers F-vvhA, R-vvhA, 1.40 µM of each F-tlh, R-tlh, F-trh, R-trh and 1.35 µM of F-tdh, R-tdh and 3 µM of each of F-viuB and R-viuB (See **Note 1**). The nuclease-free water (Promega Corporation, Madison, USA) was used to make up a final reaction volume of 25 µl. The primers were manufactured by the IDT (Integrated DNA technologies Inc., Iowa, USA). All PCR amplifications were performed on a BIO-RAD iCycler™ MyiQ™ optical module and the PCR conditions are listed in Table 4.

3.2.6 Validation of Pentaplex PCR assay

After the optimization of annealing temperature, the pPCR assay was validated with 3 approaches. First, the 7 reference strains used as positive control in the annealing temperature optimization (Table 2) and 293 environmental isolates from the Gulf water were assayed with both the pPCR assay (developed in this study) and previously published PCR protocols (Bej *et al.*, 1999; Kaysner and DePaola, 2004; Panicker, *et al.*, 2004a) and the results were compared.

Positive and negative controls (Table 2) were included in every PCR run. The efficiency of the pPCR was calculated using the number of false positive bands, false negative bands, and spurious/phantom bands, if any were recorded.

Table 4: PCR conditions for this study

No. of Cycles	Steps in each cycle	Temperature (°C) & Time
Cycle 1 (1X) :	Step 1 : Initial Denaturation	94°C for 5:00 min ^{*1}
Cycle 2 (30X) :	Step 1 : Denaturation of the template DNA	94°C for 01:00 min
	Step 2 : Primer Annealing	57°C ^{*2} for 01:00 min
	Step 3 : Primer Extension	72°C for 01:00 min
Cycle 3 (1X)	Step 1 : Final Extension	72°C for 06:50 min

^{*1} When the time for Initial Denaturation increased to 10 min as a pre-amplification heating (D'Aquila *et al.*, 1991), it produced the best results for direct PCR of colonies (data not shown), i.e., the boiled step for crude DNA preparation can be avoided and isolates picked from TCBS and CC agar plates can be directly used for the PCR reaction.

^{*2} For optimization of annealing temperature, the temperature were varied according to the temperatures listed in Table 3; 57°C was selected as the optimal.

(Note 1: Integrated DNA technologies SciTools - Resuspension Calculator was used to calculate the amount of buffer required to re-suspend the primer stock to the desired concentration. The calculation used molecular weight of primers and its quantity in milligrams.)

Second, 51 out of 293 environmental isolates were selected for species identification with 16S partial DNA sequencing (discussed separately in **Section 3.2.8**). Third, the specificity of the pPCR assay was further evaluated by using different combinations of reference strains *V. parahaemolyticus* F11-3A and *V. vulnificus* 1007, and 7 environmental (16s rDNA sequenced) isolates of *Vibrio* species other than *V. parahaemolyticus* and *V. vulnificus* (referred as non- *V.v* / *V.p* strains; Table 5). The combinations included 7 non-*V.v* / *V.p* strains, (2) 7 non- *V.v* / *V.p* strains and *V. parahaemolyticus* F11-3A, (3) 7 non- *V.v* / *V.p* strains and *V. vulnificus* 1007, *V. parahaemolyticus* F11-3A, and (5) *V. vulnificus* 1007. Each PCR reaction was repeated twice.

Table 5: Seven environmental isolates used to test pPCR specificity

Isolates ¹	Number of isolates	Genotype ²
<i>Vibrio cholerae</i> / <i>Vibrio mimicus</i>	2	Negative for all the five genes* <i>tlh</i> , <i>trh</i> , <i>tdh</i> , <i>vvh</i> and <i>viuB</i>
<i>Vibrio ordalii</i> / <i>Listonella anguillarum</i>	2	
<i>Vibrio ponticus</i>	2	
<i>Providencia rettgeri</i>	1	

¹ Isolates were obtained from the Gulf water samples using TCBS agar. Species identification of the isolates was performed by 16S rDNA Sequencing (see below section 3.2.8 16S rDNA Sequencing).

² Genotype of the isolates was determined by using individual primers with previously published PCR protocols.

3.2.7 Analysis of PCR amplicons

PCR amplicons were analyzed by electrophoresis. Four microliters (μl) of the amplified gene products were electrophoresed at 100 V for ~ 60 minutes in 2% Agarose Gel (ISC Bio Express, Gene Pure Sieve GQA Agarose) that contained 1.25 μl Ethidium bromide (Bio-Rad 10mg/ml stock). The fluorescent bands were visualized using BIO -RAD Molecular Imager Gel Doc System Universal hood II S.N 76S/02980. A DNA marker 50 bp (New England Biolabs. Inc.) was used as reference points to measure the size of the PCR amplicon in the Agarose gel.

3.2.8 16S rDNA sequencing

Among the 293 environmental isolates examined with the pPCR designed in this study, 51 isolates were selected for species identification with 16S partial DNA sequencing, and 7 isolates randomly selected for the pPCR assay specificity evaluation (as discussed above, **Section 3.2.6**). The PCR cycle conditions for 16S rDNA amplification of the isolates were provided by Functional Biosciences Inc. (Madison, WI). Primers used were 16S-forward 5'-AGA GTT TGA TCC TGG CTC -3' and 16S-reverse 5'- TAC CGC GGC TGC TGG CAC -3' primers (<http://functionalbio.com/web/primers.php>) and the PCR reaction cycle steps were as follows: 96°C for 5min, followed by 35 cycles with each cycle involving 94°C for 30sec, 60°C

for 45 sec, and 72°C for 1min 30sec. The PCR reaction mixture contained 12.5µL of Go-*Taq* Hot Start Green *Taq* polymerase (Promega Corp.), 0.4 µM of each 16S primer and 2 µl (~ 20-30ng) of template DNA. The final reaction volume was made to 25µl with nuclease free water (Promega Corp.).

After amplification 4 µl of the PCR amplicon was electrophoresed in 1% Agarose gel along with a standard DNA marker (50bp DNA Step Ladder, Promega Corporation). The PCR amplicon size was expected to be ~500 bp. The PCR products were then processed and sequenced at Functional Biosciences using the 16S – F primer having sequences similar to the 8F universal primers (Amann *et al.*, 1995; Liu *et al.* 1997). Clean sequences with a sequence length ranging between 400 and 489 bp were blasted with Standard Nucleotide NCBI BLAST inquiry tool (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Nucleotides>).

3.3 RESULTS

3.3.1 Optimal annealing temperature

Among the 5 tested temperatures (55°C, 57°C, 59°C, 62°C, and 65°C), 57°C proved to be the optimal one. With this annealing temperature, all the five target genes including species-specific and virulent genes (Table 3) for *V. parahaemolyticus* and *V. vulnificus* were able to amplify with the pPCR assay protocol (see gel image in Figure 4).

The negative controls including *V. cholera* ATCC 14035, autoclaved LB glycerol stock, and nuclease free water gave no interfering bands (Figure 4). When individual primers pairs were tested separately, they worked perfectly at 57°C, without producing any spurious bands (gel image not shown).

The other tested temperatures were unable to amplify all the 5 target genes. For instance, the lower temperature 55°C did not amplify *viuB*, while, *trh* and *tlh* genes were not amplified at 62°C and 65°C (gel image not shown).

Table 6 summarizes the results of pPCR assay and individual primers pairs test for all positive and negative controls used in this study.

3.3.2 Validation of pPCR assay

Table 7a summarized the results of the Pentaplex PCR assay, individual primer test, and previously published protocols with 300 isolates that included 7 reference and 293 environmental strains.

Out of the 293 environmental isolates, 117 isolates were determined to contain *vvh* gene and identified as *Vibrio vulnificus* by the pPCR assay, which was consistent with the results of individual *vvh* test and multiplex PCR protocol by Paniker *et al.*, (2004a). The pPCR assay identified 30 environmental isolates as *Vibrio parahaemolyticus* with the presence of *tlh* gene, which was also in agreement with the individual *tlh* primer pair test and multiplex PCR protocol by Bej *et al.* (1999). The rest of the environmental isolates contained none of the five target genes by the pPCR assay; the individual primer pair tests and the multiplex protocols could not detect any of the target genes in these genus-unknown bacteria, either. Several of these isolates, however, generated faint but detectable phantom bands, which appeared either near the location of the target gene or distant from the target gene location in Agarose gel (examples shown in Figure 5a and 5b). The phantom bands appeared in both pPCR assay and the multiplex PCR assays (Table 7a).

All the 7 reference strains showed perfectly matching results between the pPCR assay and the other PCR protocols, except that each of the multiplex PCR protocols generated one phantom band (Table 7a). The virulent genes in all the reference strains were detected by 100% efficiency with the pPCR assay.

To further confirm that there were no false negative results, 41 out of 43 environmental isolates that presented none of the five target genes by the pPCR assay were analyzed by 16S rDNA sequencing. The results (Table 7b) showed that these isolates belong to five *Vibrio* spp. (*V. orientalis*, *V. natriegens*, *V. rotiferianus*, *V. ponticus* & *V. aestuarianus*) and five of the non-*Vibrio* spp. (*Bacillus gibsonii*, *Bacillus aerophilus*, *Providencia rettgeri*, *Photobacterium rosenbergii* (also reported by Thompson *et al.* (2005), to grow on TCBS agar), *Exiguobacterium indicum* (Chaturvedi and Shivaji (2006), reported that it is resistant to colistin)).

Eight environmental isolates (Table 7a) which were positive for *V. vulnificus* (n=4) or *V. parahaemolyticus* (n=4) showed a similarity of 99.81% and 97.7% to these two species, respectively.

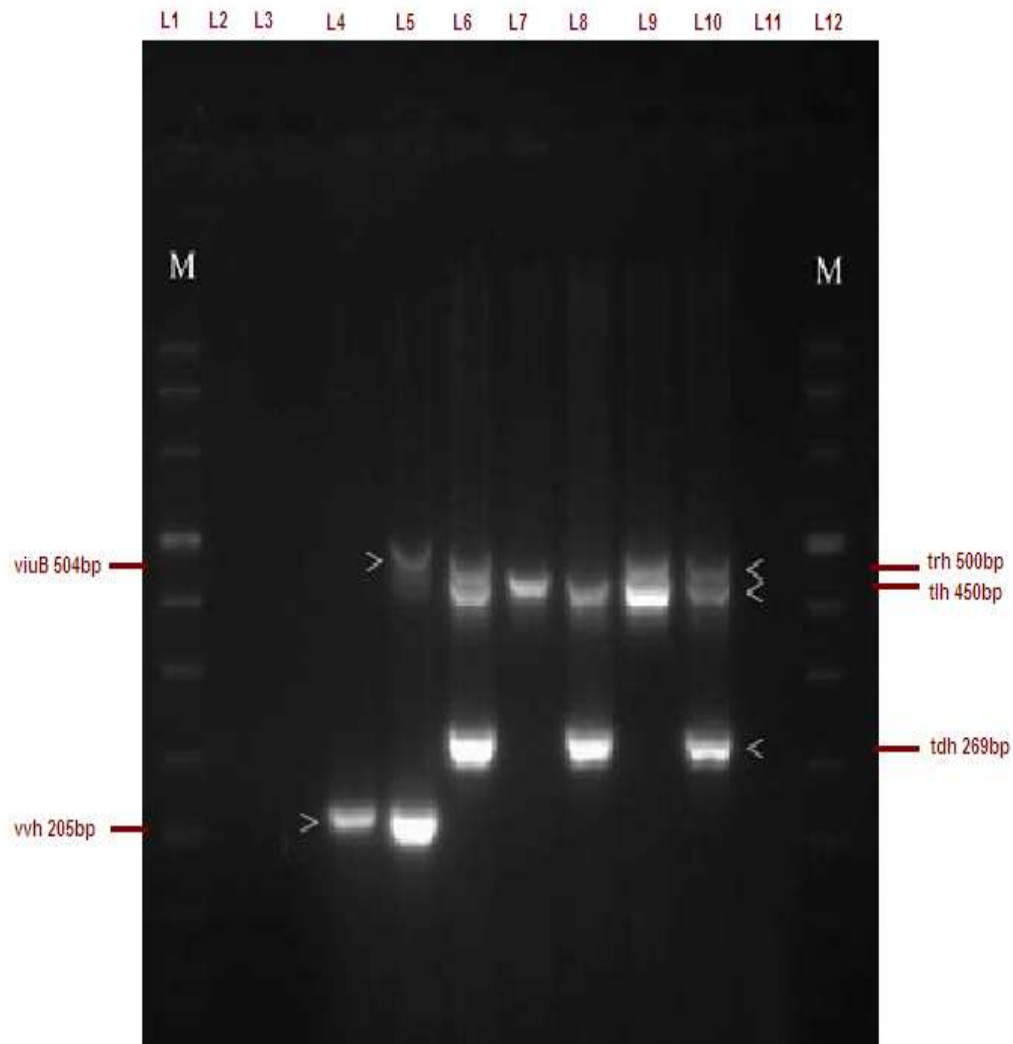


Figure 4: Gel image of five target genes (*tlh*, *trh*, *tdh*, *vvh*, and *viuB*) in the positive and negative controls listed in Table 2, examined with the Pentaplex PCR assay at 57°C. L = Lane where, L1 & L12: 50 bp DNA marker; L2: Negative control - *V. cholera* ATCC 14035, L3 : Negative control - Autoclaved LB glycerol stock; L4: *V. vulnificus* (Vv3138-001) lab isolate (*vvh*⁺) ; L5: *V. vulnificus* 1007 (*viuB*⁺ *vvh*⁺) ; L6 & L10: *V. parahaemolyticus* F11-3A (*tlh*⁺ *trh*⁺ *tdh*⁺) ; L7: *V. parahaemolyticus* FIHES98 (*tlh*⁺) ; L8: *V. parahaemolyticus* Tx-2103 (*tlh*⁺ *tdh*⁺) ; L9: *V. parahaemolyticus* AQ 4037 (*tlh*⁺ *trh*⁺) ; L11 : Negative control - Nuclease free water. The white arrow (> and <) signs represents the base pair of the PCR amplicon mentioned on the left- & right - hand side of the gel image)

Table 6: Results of pPCR assay compared to PCR using individual primers pairs for all positive and negative controls used in this study

Reference strain	PCR using individual primer pair					Pentaplex PCR (pPCR) (in this study)
	tlh	trh	tdh	vvhA	viuB	5 primer pair mix (tlh, trh, tdh, vvh and viuB)
V.p FIHES'98 (positive control)	<i>tlh</i> +	-	-	-	-	<i>tlh</i> +
V.p AQ 4037 (positive control)	<i>tlh</i> +	<i>trh</i> +	-		-	<i>tlh</i> + <i>trh</i> +
V.p Tx-2103 (positive control)	<i>tlh</i> +	-	<i>tdh</i> +	-	-	<i>tlh</i> + <i>tdh</i> +
V.p FII-3A (positive control)	<i>tlh</i> +	<i>trh</i> +	<i>tdh</i> +	-	-	<i>tlh</i> +, <i>trh</i> + , <i>tdh</i> +
V.v 1007 (positive control)	-	-	-	<i>vvh</i> +	<i>viuB</i> +	<i>vvh</i> +, <i>viuB</i> +
V.v 3138-001 (positive control)	-	-	-	<i>vvh</i> +		<i>vvh</i> +
<i>V. cholera</i> ATCC 14035 (negative control)	-	-	-	-		-
Nuclease free water (negative control)	-	-	-	-		-
Autoclaved LB glycerol stock (negative control)	-	-	-	-		-

Sign (-) represents no bands observed

Sign (+) bands represents presence of the respective PCR amplicon location.

V.p refers to *V. parahaemolyticus*

V.v refers to *V. vulnificus*

Table 7a: Results of 300 isolates tested using different PCR assays

Isolates	No. of Isolates tested	Pentaplex PCR (in this Study)	Multiplex PCR		PCR test with individual primers				
		Results	vvh & viuB primers (Panicker <i>et al.</i> , 2004a)	Tlh, trh and tdh primers (Panicker <i>et al.</i> , 2004a; Bej <i>et al.</i> , 1999)	tlh	trh	tdh	vvhA	viuB
Environmental Isolates (n= 147)									
<i>Vibrio vulnificus</i>	117 ^a	117 ^a	117 ^a	-	-	-	-	117 ^a	1
<i>Vibrio parahaemolyticus</i>	30 ^{b, c}	30 ^{b, c}	1 ^c	30 ^{b, c}	30 ^b	2	2	- ^c	-
Genus Unknown Environmental Isolates (n=146)									
Unknown spp.	146	4 ^{*E1} & 6 ^{*E2}	3 ^{*E1}	1 ^{*E1}	-	-	-	-	-
Reference strains (n =7) of <i>V. vulnificus</i> (V.v), <i>V. parahaemolyticus</i> (V.p) and <i>V. cholera</i>									
V.v 1007 (vvh ⁺ viuB ⁺)	1	1	1	- ^{*E1}	-	-	-	1	1
V.v 3138-001 (vvh ⁺)	1	1	-	-	-	-	-	1	-
V.p TX- 2103 (tlh ⁺ tdh ⁺)	1	1	1	-	1	-	1		-
V.p AQ4037 (tlh ⁺ trh ⁺)	1	1	1	- ^{*E1}	1	1	-	-	-
V.p FII-3A (tlh ⁺ trh ⁺ tdh ⁺)	1	1	-	1	1	1	1	-	-
V.p FIHES98 (tlh ⁺)	1	1	-	1	1	-	-	-	-
<i>V. cholera</i> ATCC 14035 (vvh ⁻ viuB ⁻ & tlh ⁻ trh ⁻ tdh ⁻)	1	-	-	-	-	-	-	-	-

Sign (-) indicates that absences of bands or amplicons

^{a, b} four environmental strains of each *V. vulnificus* ^a and *V. parahaemolyticus* ^b which have been tested with PCR methods and 16 S rDNA sequencing confirming that our target genes were present. The 16S rDNA sequencing showed 99 – 100% matches for the four *V. vulnificus* and 97% match with four *V. parahaemolyticus* strains.

^c one of the environmental isolates positive for pathogenic strain of *V. parahaemolyticus* showed a band at the vvh location. On repetition of the experiment the band did not appear.

*E1 and E2 refers to phantom bands. E1 are the PCR amplicon product showing faint bands near the target gene location of Agarose gel. E2 are the PCR amplicon product showing faint bands away from the target gene location of Agarose gel.

Sequencing results (Table 7b) showed that the 4 bacterial isolates which produced spurious or phantom band at the 400 – 420 bp range did not belong to *V. vulnificus* or *V. parahaemolyticus*, they showed a maximum similarity to *Vibrio cholera/Vibrio mimicus*. The spurious band near the 510-550 bp did not interfere with the observation of the bands of the target gene *viuB* or *trh* in the pPCR assay.

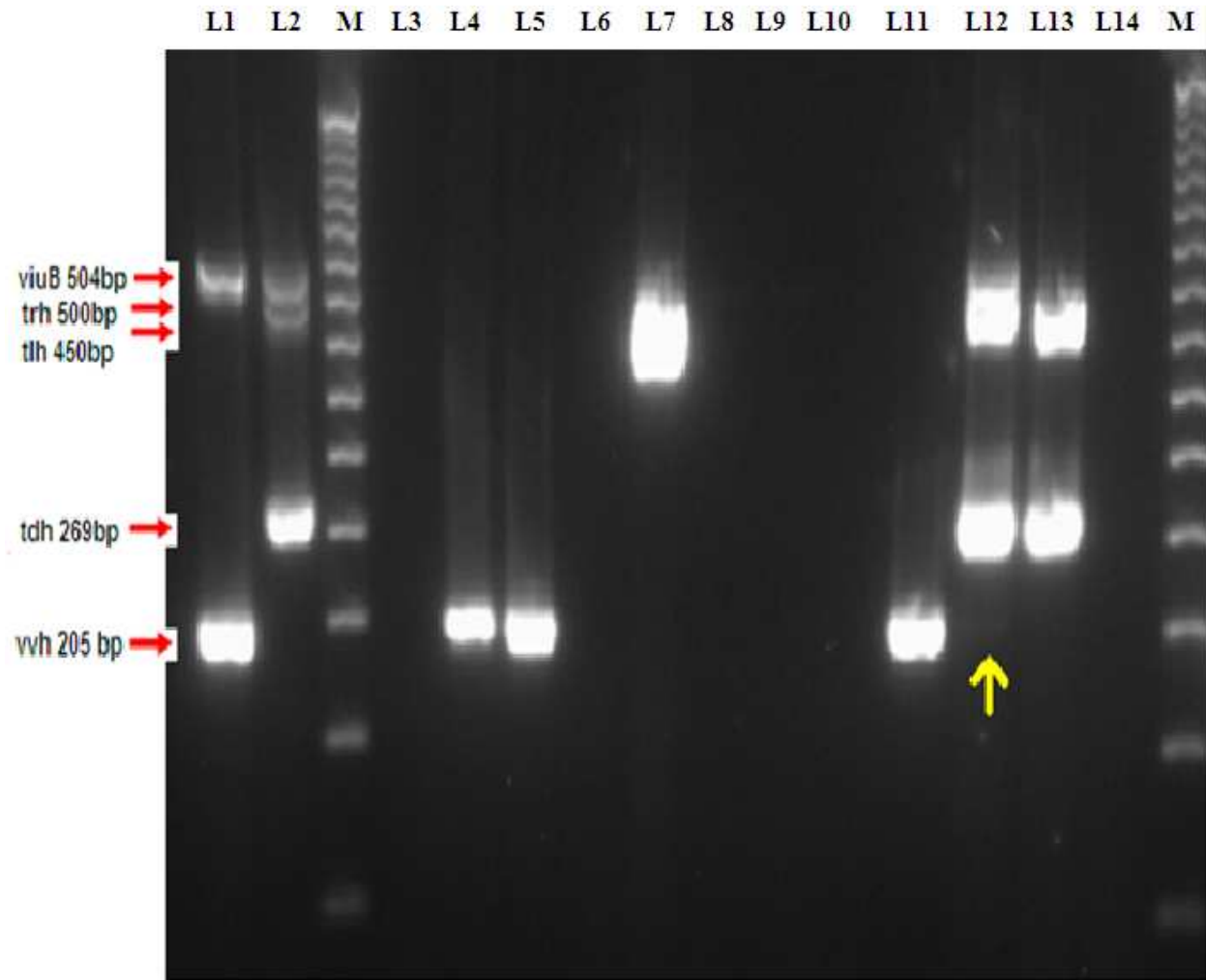


Figure 5a: The Pentaplex PCR assay for specificity on environmental isolates. The gel image shows (L = Lane), L1: *V. vulnificus* 1007 & L2: *V. parahaemolyticus* F11-3A as positive controls; followed by 50 bp DNA marker; L3: Negative control – *V. cholera* ATCC 14035; L4, L5 & L11: *V. vulnificus* environmental isolates possessing *vvh*⁺ gene fragment; L7: *V. parahaemolyticus* *tlh*⁺ gene; L6, L8, L9, L10 & L14: Environmental bacterial isolates negative for all the genes. L12 & L13: *V. parahaemolyticus* isolates with *trh*⁺, *tlh*⁺ and *tdh*⁺ gene. The yellow arrow sign in L12 represents the faint but detectable phantom band near the 206-205 bp location.

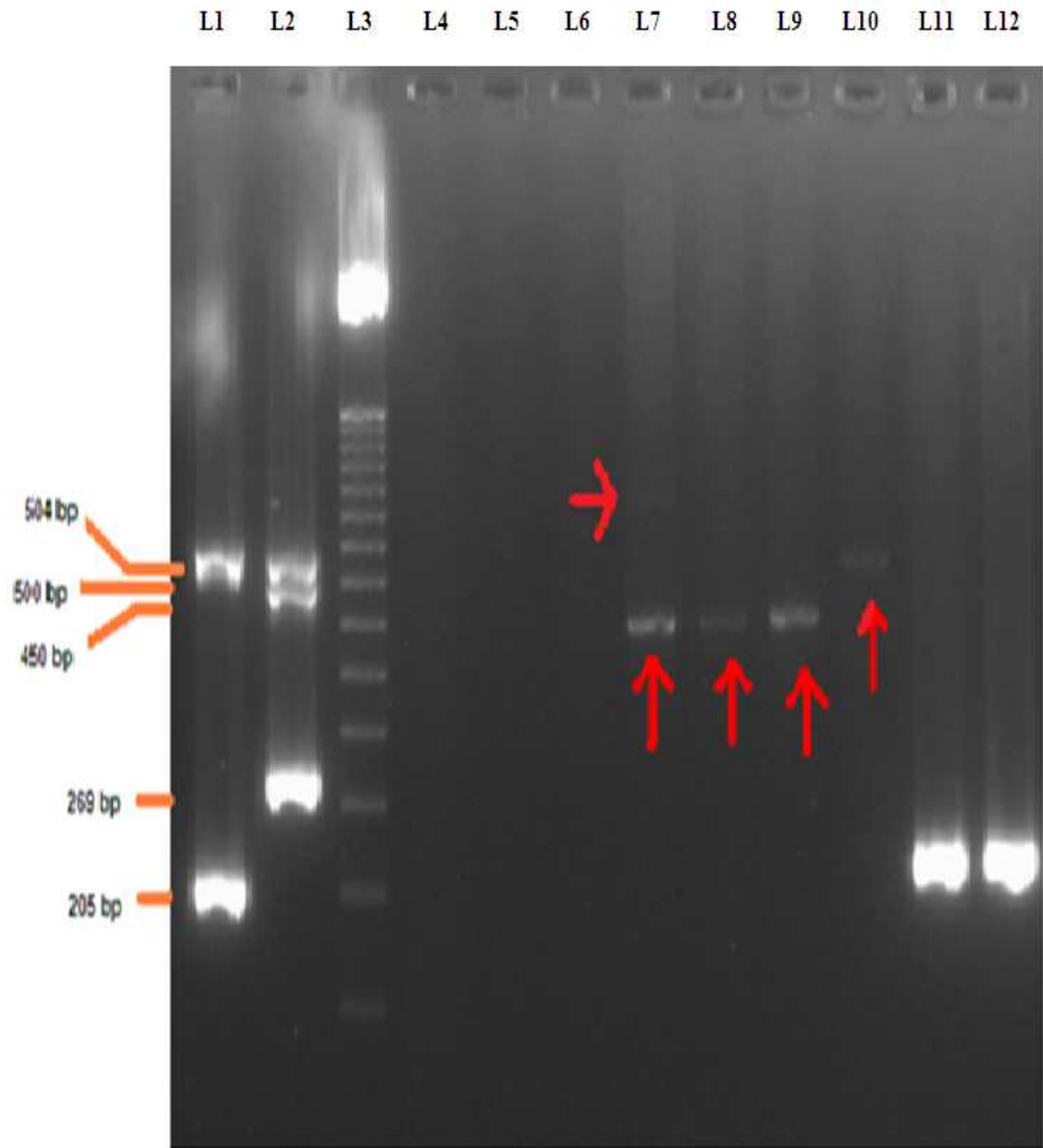


Figure 5b: The Pentaplex PCR assay for specificity on environmental isolates showing spurious bands. The gel image shows (L = Lane), L1: *V. vulnificus* 1007 & L2: *V. parahaemolyticus* F11-3A as positive controls; followed by L3 : 50 bp DNA marker; L4: Negative control - *V. cholera* ATCC 14035; L5 & L6: Environmental isolates negative for all the gene; L7 , L8 & L9: There are three red arrow sign indicating spurious/phantom bands in the range 380-400 bp and one in L7 showing a band 510-520 bp; L10: the red arrow indicating a false positive faint band at the *trh* location; L11 & L12 environmental isolates of *V. vulnificus* showing *vvh*⁺.

Table 7b: Results of 43 unknown isolates from Table 7a, sequenced with 16S rDNA and compared to different PCR primer mixes at 57°C.

Species	No. of Isolate	Results of Pentaplex PCR (in this Study)	Results of PCR test with Multiplex PCR		PCR test with individual primers				
			vvh & viuB primers (Panicker <i>et al.</i> , 2004a)	tlh, trh and tdh primers (Panicker <i>et al.</i> , 2004a; Bej <i>et al.</i> , 1999)	tlh	trh	tdh	vvhA	viuB
Other <i>Vibrio</i> spp. Environmental Isolates (n= 24)									
<i>Vibrio orientalis</i>	3	-	-	-	-	-	-	-	-
<i>Vibrio natriegens</i>	2	-	-	-	-	-	-	-	-
<i>Vibrio rotiferianus</i>	8	-	-	-	-	-	-	-	-
<i>Vibrio ponticus</i>	9	-	-	-	-	-	-	-	-
<i>Vibrio aestuarianus</i>	2	-	-	-	-	-	-	-	-
Non <i>Vibrio</i> spp. Environmental Isolates (n=6)									
<i>Bacillus gibsonii</i>	2	-	-	-	-	-	-	-	-
<i>Bacillus aerophilus</i>	1	-	-	-	-	-	-	-	-
<i>Providencia rettgeri</i>	1	-	-	-	-	-	-	-	-
<i>Photobacterium rosenbergii</i>	1	-	-	-	-	-	-	-	-
<i>Exiguobacterium indicum</i>	1	-	-	-	-	-	-	-	-
Other species (showing similar maximum % identity after 16 S rDNA sequence BLAST) Environmental Isolates (n=13)									
<i>Listonella anguillarum</i> / <i>Vibrio ordalii</i>	3	-	-	-	-	-	-	-	-
<i>Vibrio cholera</i> / <i>Vibrio mimicus</i>	6	2 ^{*E2}	-	-	-	-	-	-	-
<i>Vibrio mediterranei</i> / <i>Vibrio neptunius</i> / <i>Vibrio orientalis</i>	1	-	-	-	-	-	-	-	-
<i>Bacillus safensis</i> / <i>Bacillus pumilus</i>	1	-	-	-	-	-	-	-	-
<i>Aeromonas hydrophila</i>	2	-	-	-	-	-	-	-	-

Sign (-) indicates that no bands were found

*E² refers to Phantom bands where the PCR amplicon product showing faint bands away from the target gene location of Agarose gel.

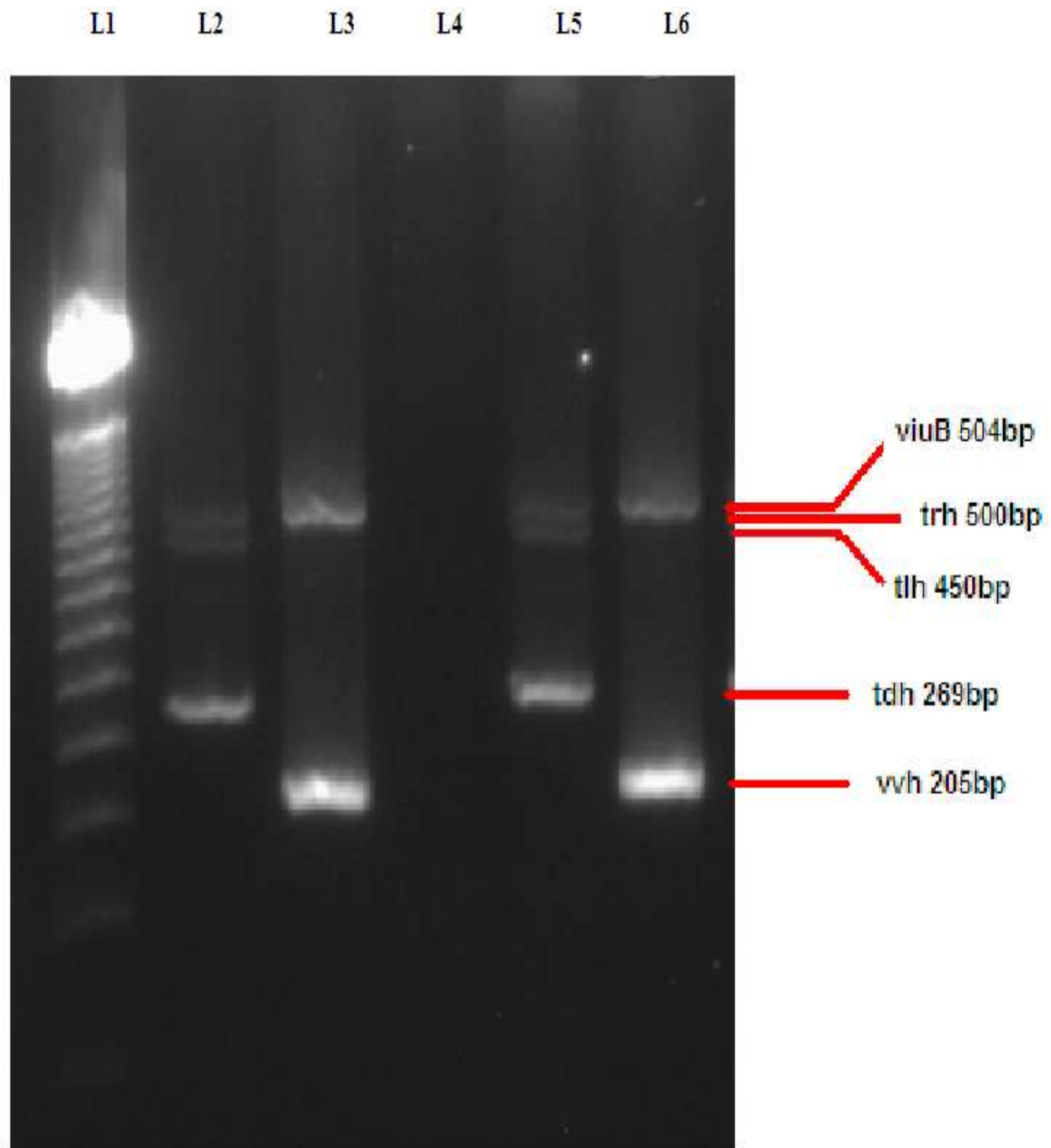


Figure 6: pPCR assay specificity test on different combinations of pooled non-V.v/V.p environmental isolates with reference strain. *V. parahaemolyticus* F11-3A (*trh*⁺ *tlh*⁺ *tdh*⁺) and *V. vulnificus* 1007 (*viuB*⁺ *vvh*⁺). L1: DNA marker, L2: Positive control with *V. parahaemolyticus* F11-3A mixed with random environmental isolates; L3: Positive control with *V. vulnificus* 1007 mixed with random environmental isolates; L4: Negative control showing no bands with unmixed random isolates; L5: Positive control unmixed *V. parahaemolyticus* F11-3A; L6: Positive control unmixed *V. vulnificus* 1007.

The pPCR assay specificity assessment with different combinations of pooled non- *V.v* / *V.p* environmental isolates and reference strains (Table 7c) showed that the pPCR assay was able to detect the target genes and did not produce any spurious/phantom bands (Figure 6), revealing that the assay was able to successfully detect the target genes in a complex matrix. The *V. parahaemolyticus* F11-3A and *V. vulnificus* 1007 were pooled for PCR detection (data not shown), which enabled the amplification for all the five target genes. However, the resolution between *viuB* (504 bp amplicon) and *trh* (500bp amplicon) was not clear in a 2% Agarose Gel Electrophoresis Image, due to the close proximity of the amplicon product size.

Table 7c: Seven randomly pooled environmental samples used to test pPCR specificity.

Organisms	pPCR analysis	Spurious Bands
Pooled bacterial isolates* from five different species (n=7) <i>(Vibrio cholerae/Vibrio mimicus, Vibrio ordalii /Listonella anguillarum , Vibrio ponticus , Providencia rettgeri)</i>	Negative for all the five genes** <i>tlh, trh, tdh, vvh and viuB</i>	-
Pooled bacterial isolates & <i>V. vulnificus</i> 1007	<i>vvh⁺ viuB⁺</i>	-
Pooled bacterial isolates & <i>V. parahaemolyticus</i> F II 3A	<i>tlh⁺ trh⁺ tdh⁺</i>	-
<i>V. vulnificus</i> 1007- positive control	<i>vvh⁺ viuB⁺</i>	-
<i>V. parahaemolyticus</i> F II 3A- positive control	<i>tlh⁺ trh⁺ tdh⁺</i>	-

*Isolates were obtained from the Gulf water samples on TCBS agar. 16S rDNA sequencing was used for species identification of the isolates.

**These isolates were tested with use of individual primers in a PCR reaction to analyze the presence or absence of the target genes.

Sign (-) represents no bands observed

Sign (+) bands represents the presence of PCR amplicons respective to specific genes

The spurious bands were taken into consideration while evaluating the specificity of the Pentaplex PCR assay. The efficiency of the pPCR was 98.6% for 293 environmental isolates excluding six phantom bands located distant from the target gene (Table 8).

Table 8: Efficiency of Pentaplex PCR assay in 293 environmental isolates.

Description	Number of isolates showing spurious bands	Efficiency of pPCR *C
Efficiency of pPCR in environmental isolates (n=293) tested		
Isolates producing a phantom band at a location near target gene; false positive result	4 ^{*E1}	289/293 = 98.6%
Isolates producing false negative result	0 ^{*ae}	0/293 = 0%
Isolates producing a phantom band not distant from the location of the target gene	6 ^{*E2}	287/293 = 98%
Total isolates producing false results	10	283/293 = 96.6%

^{*ae} one of the environmental isolates positive for pathogenic strain of *V. parahaemolyticus* showed a band at the *vvhA* location. On repetition of the experiment the band did not appear.

^{*E1} refers to Phantom bands where the PCR amplicon product showing faint bands near the target gene location of Agarose gel.

^{*E2} refers to Phantom bands where the PCR amplicon product showing faint bands away from the target gene location of Agarose gel.

^{*C} refers to pPCR efficiency % formula = {(293 environmental isolates – No. of isolates showing spurious bands)/293 isolates tested} x 100

3.4 DISCUSSION

The objective of this study was to develop a fast, cost-effective PCR protocol for simultaneous detection of the 3 pathogenic genes (*viuB* in *V. vulnificus*, *tdh* and *trh* in *V. parahaemolyticus*) and 2 species-specific genes (*vvhA* in *V. vulnificus* and *tlh* in *V. parahaemolyticus*). The pPCR assay developed in this study employed an optimal annealing temperature of 57°C and an increased primer mixture concentration. Other annealing temperature were not able to amplify all the five target genes simultaneously, may be due to the variation in the melting temperature (T_m) value of each primer sets causing instability in annealing to target

genes (White B.A., 1993). Lower anneal temperature 55°C was not suitable for the amplification of *viuB*, as the T_m value requires a higher annealing temperature above 60°C and this temperature did not support amplification of the *trh* and *tlh* genes as their primer sets requires a much lower temperature. Thus temperature of 57°C suited best to anneal all the primer sets specifically, to amplify the five target genes in our study.

The assay was validated by using 7 reference strains with known genotype and 293 environmental isolates obtained from the Gulf water samples on TCBS and CC agar and by comparing the results among the pPCR assay, individual primer pair tests, and previously published multiplex PCR protocols (Bej *et al.*, 1999; Panicker *et al.*, 2004a). The pPCR assay was able to detect the 5 target genes in the reference strains with 100% efficiency and with an efficiency of 96.6% for 293 environmental isolates when the spurious bands were taken into account (Table 8). These rates of efficiency were comparable with those of previously published multiplex protocols (Bej *et al.*, 1999; Panicker *et al.*, 2004a), which targeted the genes in each species (*V. vulnificus* or *V. parahaemolyticus*) separately. The pPCR assay was clearly able to differentiate between the strains used in Table 6 & 7a, shown in Figure 4 & 5a, from *V. cholera* ATCC 14035, indicating the importance of this protocol as a valuable detection system in food/water quality testing laboratories.

It is worth noting that when we used the published protocols (Panicker *et al.*, 2004a) in our laboratory, they produced spurious/phantom bands in the negative controls (Figure 7 and Figure 8). This might be because the base modification at the 5'- end of the primers used by Panicker *et al.* (2004a) was not included in the primer sets used in this study. When we changed the annealing temperature to 57⁰ C, these spurious bands disappeared. Therefore, we modified the published protocols with an annealing temperature of 57⁰C in our study. This observation further confirmed that 57⁰C works well for specifically amplifying all 5 target genes. The reference strains used in this study gave no spurious bands in the pPCR assay. However, out of the 293 isolates tested, 4 isolates showed the occurrence of faint but detectable phantom/spurious bands near the location of the target genes. This limitation could be resolved when the number of PCR cycles was decreased from 30 to 27 (data not shown). The increase in number of PCR cycle has been reported to cause phantom bands due to non-specific amplification (Bell & DeMarini, 1991; Roux, 2009).

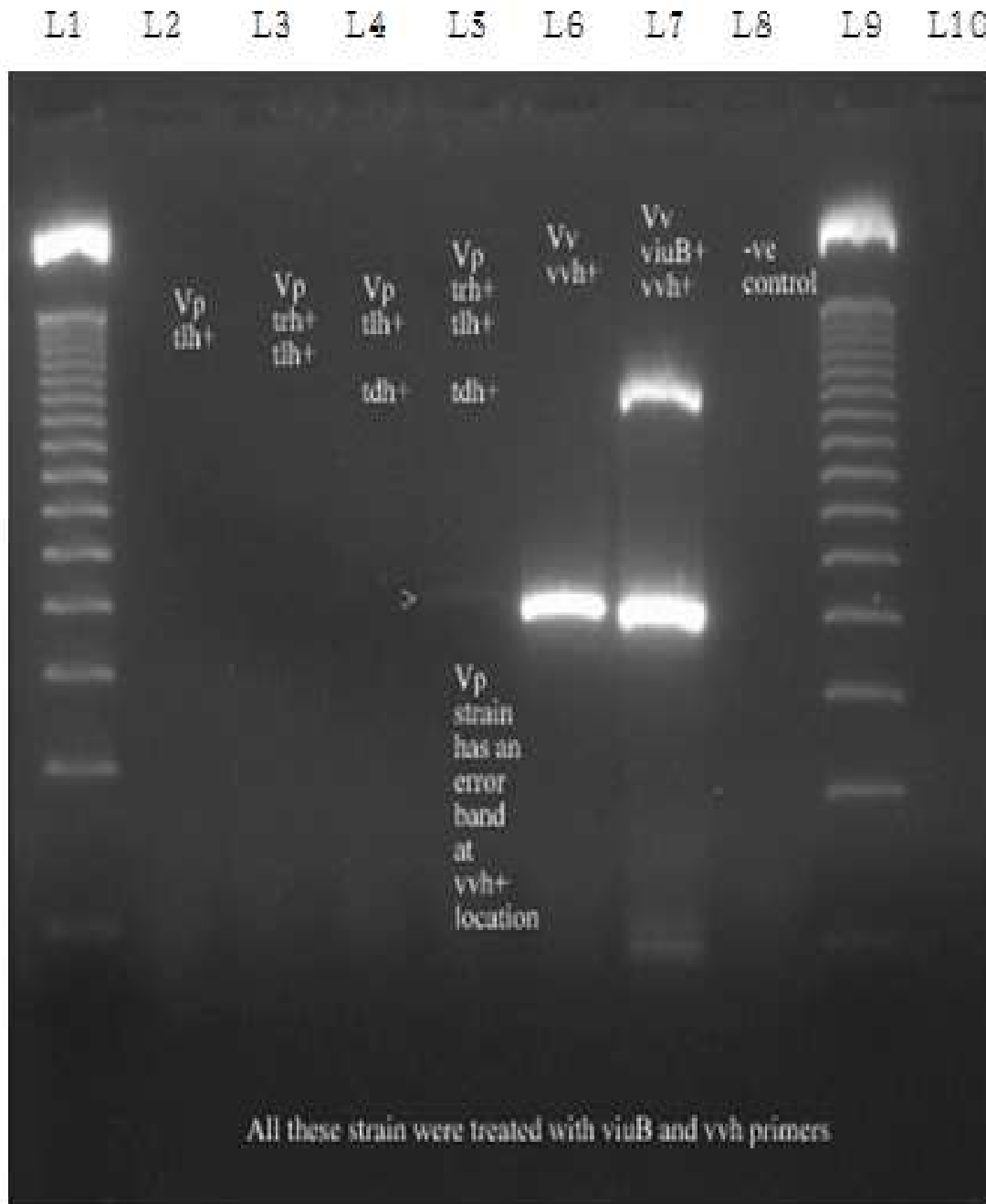


Figure 7: The PCR assay for positive and negative controls used in Table 2; is shown in the gel image (L = Lane) a spurious band formation at ~ 205 bp position at L5 *V. parahaemolyticus* F11-3A, when two primers vvhA and viuB were added in the reaction mix to detect the genes . Primer tested at 57 °C and 65° C. Gel image for 65° C is shown, L1 & L9: 50 bp DNA marker; L2: *V. parahaemolyticus* FIHES98 (*tth*⁺); L3: *V. parahaemolyticus* AQ 4037 (*tth*⁺ *trh*⁺); L4: *V. parahaemolyticus* Tx-2103 (*tth*⁺ *tdh*⁺); L5: *V. parahaemolyticus* F11-3A (*tth*⁺ *trh*⁺ *tdh*⁺); L6: *V. vulnificus* (Vv3138-001) lab isolate (*vvh*⁺) ; L7: *V. vulnificus* 1007 (*vvh*⁺ *viuB*⁺); L8: Negative control - *V. cholera* ATCC 14035. The white arrow (>) sign represents the spurious band.

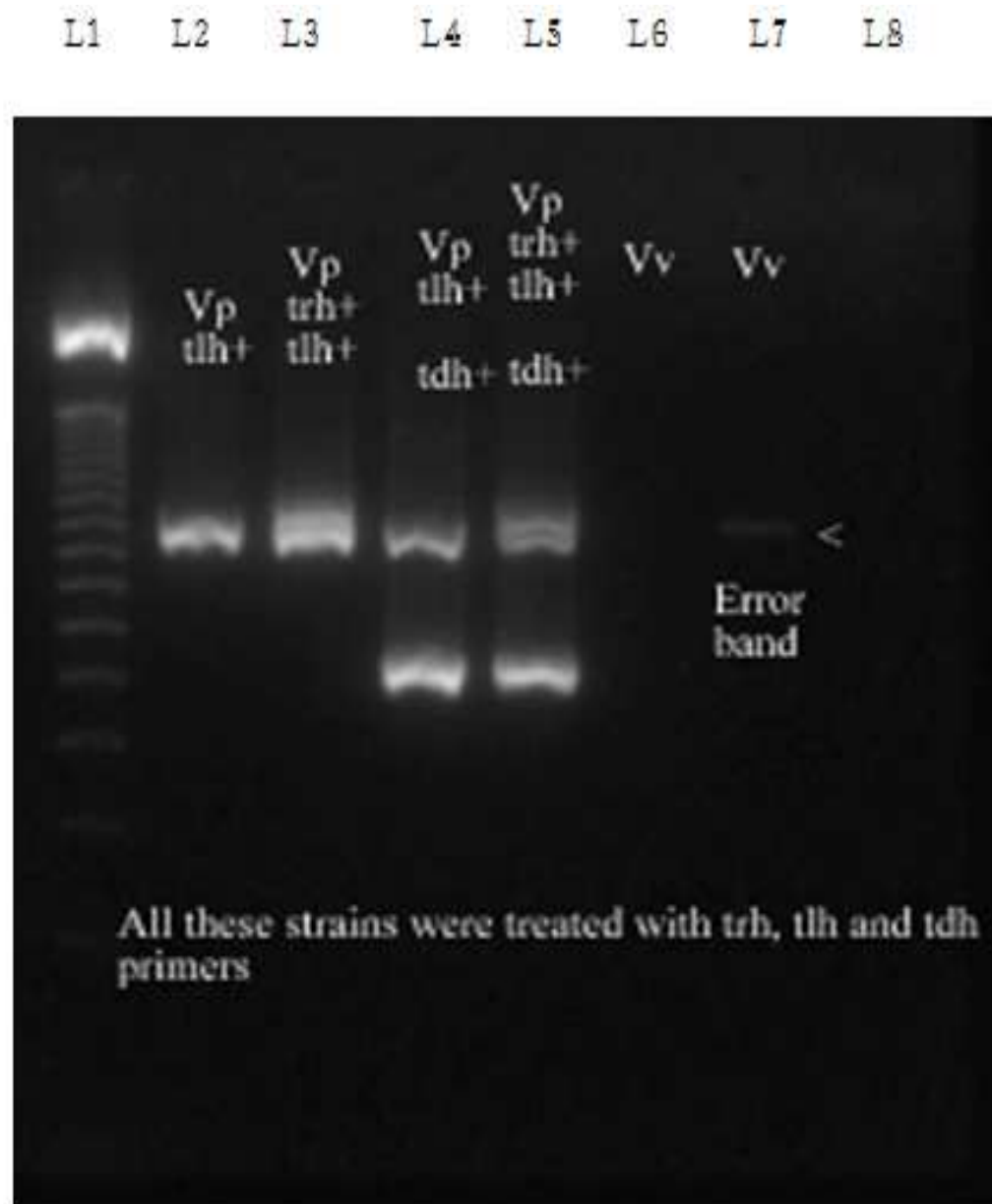


Figure 8: The PCR assay for positive and negative controls used in Table 2; is shown in the gel image (L = Lane) is a spurious band formation at ~ 500 bp position at L7: *V. vulnificus* 1007 (*vvh*⁺ *viuB*⁺), when three primers *tlh*, *tdh* and *trh* were used in the PCR master mix to detect the genes. Primer tested at 57 °C and 55° C. Gel image for 55° C is shown where, L1: 50 bp DNA marker; L2: *V. parahaemolyticus* FIHES98 (*tlh*⁺); L3: *V. parahaemolyticus* AQ 4037 (*tlh*⁺ *trh*⁺); L4: *V. parahaemolyticus* Tx-2103 (*tlh*⁺ *tdh*⁺); L5: *V. parahaemolyticus* F11-3A (*tlh*⁺ *trh*⁺ *tdh*⁺); L6: *V. vulnificus* (V.v 3138-001) lab isolate (*vvh*⁺); L7: *V. vulnificus* 1007 (*vvh*⁺ *viuB*⁺); L8: Negative control - *V. cholera* ATCC 14035. The white arrow (<) sign represents the spurious band.

As we used a pre-made PCR mixture from Promega Corporation, optimization of other factors such as varying the concentration of magnesium ions, pH, *Taq* polymerase or dNTPs concentration might have to be governed to minimize the non-specific amplification, which causes the appearance of spurious / phantom bands. Although the Hot start green PCR mix (Promega Corporation) had dyes incorporated by the manufacturer for ease to load in agarose gels, which may result in PCR inhibition in some cases (Hoppe *et al.*, 1992), in our study there was no inhibition and no false negative reactions observed. Incorporation of glycerol (5% - 10%) has been proved to be suitable to enhance the specificity of the PCR (Pomp & Medrano, 1991). This may have led our study to achieve a high 98.6% efficiency rate, as we used a suspension of isolates stored in LB 20% glycerol stock for direct preparation of crude DNA template. Moreover, to validate this result in the pPCR study, the negative controls did not give any interfering bands.

Six out of 300 isolates (Table 7a & 7b) showed a phantom band at other locations that did not interfere with the detection of the target genes on the Agarose gel. Overall, if the number of occurrence of these distant spurious bands is included, the efficiency of the pPCR is 96.6% .

Several observations help to further confirm the validity (i.e. high specificity and efficiency) of the pPCR assay. First, there were no false negative results in all the pPCR experiments carried out in this study. Second, the 4 isolates that were identified as *V. vulnificus* or *V. parahaemolyticus* by the pPCR assay showed a 16S rDNA sequence similarity of 98.9 – 99.9 % to known strains of *V. vulnificus* or *V. parahaemolyticus*. Third, the 41 environmental isolates that were determined as non- *V.v* / *V.p* species by the pPCR assay were confirmed to be other *Vibrio* species that were not *V. vulnificus* and *V. parahaemolyticus* and other non- *Vibrio* species (Table 7b). Finally, the pooled non- *V.v* / *V.p* environmental isolates showed no interference with the target genes in a complex matrix.

The pPCR was also able to detect the clinical strains of *V. vulnificus* and *V. parahaemolyticus* for *viuB* and *tdh* and *trh* genes, which gave comparable result when primers were tested individually. The primers designed by Panicker *et al.* (2004a) and Bej *et al.* (1999) proved to be able to work in a more complex matrix like the pentaplex PCR in our study than in the previously published work (Panicker *et al.*, 2004a).

Another observation made during the study was the hemolysin genes *trh* and *tdh* appeared in a few isolates which were negative for *tlh* gene. This could be attributed to the fact that these pathogenic genes are mobile genetic elements. It has been reported that members of other *Vibrio* species could contain *trh* and *tdh* hemolysin genes (Bej *et al.*, 1999; Dobrindt *et al.*, 2004; Hurley *et al.*, 2006) due to horizontal gene transfer.

The lowest detection limit of the pPCR was 30 cells/ ml in this study for pathogenic strains (data not shown), lying within the range reported by Panicker *et al.* (10^2 to 10^3 CFU/ml) and Bej *et al.* (10 to 100 CFU / 10 g APW enriched oyster tissue homogenates) (Bej *et al.*, 1999; Panicker *et al.*, 2004a).

To our best knowledge, the pPCR assay is the first that simultaneously, can detect a microbial isolate for *V. vulnificus* or *V. parahaemolyticus* as 5 pairs of gene-specific primers (two species-specific and three virulent) are added with other PCR reaction components in the single reaction tube. A multiplex PCR involving ten primers pairs was tried by Panicker *et al.* but did not give reliable results (Panicker *et al.*, 2004a). Our study suggests that the pentaplex PCR can be adopted into the current FDA manual for rapid screening of environmental bacterial isolates for *V. vulnificus* and *V. parahaemolyticus* and their virulent strains. The assay for detection of the 5 target genes can be completed at a laboratory within 5-6 hours after collection of isolates.

We have applied the assay to detect 782 isolates collected from the Breton Sound and Barataria Bay water samples for a study of the distribution and occurrence of *tlh*, *trh*, *tdh*, *vvh* and *viuB* genes as impacted by salinity and temperature, which will be presented in Chapter 4.

CHAPTER 4

DETECTION AND CHARACTERIZATION OF *V. VULNIFICUS* AND *V. PARAHAEMOLYTICUS* FROM THE GULF OF MEXICO WATERS USING PENTAPLEX PCR

4.1 INTRODUCTION

V. vulnificus and *V. parahaemolyticus* are well-known human pathogens that cause food poisoning through consumption of raw oysters (Kaysner and DePaola, 2004; FDA, 2005; MMWR, 2005, MMWR 2006; Ralph & Currie, 2007; FDA, 2011) and wound infection through contact of skin lesions with contaminated waters (Inoue, 2006; Ralph & Currie, 2007; Tena *et al.*, 2010). Their population in the environment is greatly influenced by seasonal dynamics. Warmer water temperature has been strongly correlated with increase in *V. vulnificus* and *V. parahaemolyticus* population in estuarine environment (Zimmerman *et al.*, 2007; Drake *et al.*, 2007, Johnson *et al.* 2010). Due to their inhabitance in halophilic conditions, salinity also plays a pivotal role in their growth and survival (Tamplin *et al.*, 1982; Motes *et al.*, 1998). In recent years, one of the major efforts to develop practical risk management strategies for *V. vulnificus* and *V. parahaemolyticus* has been to establish a relationship for predicting the levels of these microbes in oysters and seawater based on seasonal/regional variations of water temperature and salinity (DePaola *et al.*, 2006). Furthermore, the state of Louisiana ranks “first in U.S in the production of shrimp, crawfish, blue crabs and oysters” (The Wallstreet Journal by Esterl, M. 2010). Breton Sound and Barataria Bay are the two important Louisiana coastal estuaries in the Gulf of Mexico, which provide the main breeding grounds for offshore charter fishing, shrimp harvest and oyster industries. For climatic reasons, these estuaries are hotspots of *V. vulnificus* and *V. parahaemolyticus* inhabitance.

The objective of this study was to determine the impacts of temperature and salinity on the occurrence of *V. vulnificus* and *V. parahaemolyticus* in Breton Sound and Barataria Bay. Water samples were collected from various stations along two salinity gradient transects during warmer and cooler months in the year 2011. The pPCR assay developed in Chapter 3 was used for detection and characterization of *V. vulnificus* and *V. parahaemolyticus* isolates from the Gulf water samples.

4.2 MATERIALS AND METHODS

4.2.1 Sampling location

The sampling locations in Barataria Bay and Breton Sound were selected according to their salinity regimes.

Four sampling sites including Site 2 (Barataria Pass), Site 8 (Upper Barataria), Site 16 (Little lake), and Site 23 (Lake Salvador) were chosen in Barataria Bay (Figure 9a). Site 2 and 8 are higher saline stations, followed by site 16, which is frequently interfered with saltwater intrusion. These three stations are popular grounds of natural oyster cultivation (Van Sickle, 1976). Site 23 is a shallow freshwater system with the lowest salinity among the selected sites.

In Breton Sound (Figure 9b), samples were collected from Site 4 (Lake Lery), 8 (Grand lake), 11 (Oak River Bay), 12 (Four Horse Lake), 15 (Bayou Terre aux Boeufs), 16 (Black Bay) and 18 (Gallega islands). Site 4 (Lake Lery) is a characteristic bowl shaped shallow lake that is located southeast of Caernarvon and known to be a popular game-fishing spot for bass during spring and was selected for its low saline condition (Price 2000; Smith 2004). Site 8 (Grand lake) is one of the largest basins in the Atchafalaya Basin, known for two important commercial freshwater fishes, catfish and buffalo fish (Fishinglouisiana.com, n.d. ; Conservation, 1916). Site 11 (Oak River Bay), Site 16 (Black Bay) and Site 18 (Gallega islands) are surrounded by saline marshes and Site 12 (Four Horse Lake) and 15 (Bayou Terre aux Boeufs) by brackish marshes.

4.2.2. Sample collection

Water samples were collected using 1-L sterile Nalgene bottles during warmer and colder months, i.e., Jan 2011, May 2011, July 2011 and Nov 2011 for both bays and an additional warmer month June 2011 for Barataria Bay (BB) and August 2011 for Breton Sound (BS). Water samples from BS Site 18 were collected for the months after May 2011. The samples were stored in an ice chest containing blue ice packs. The ice packs were wrapped with bubble wrap to avoid direct contact with samples. All samples were collected in duplicates. The samples were processed within 5-6 hours after collection. Sea-surface water salinity and temperature was recorded using YSI-63 CTD meter (Yellow Springs Instrument Co.).

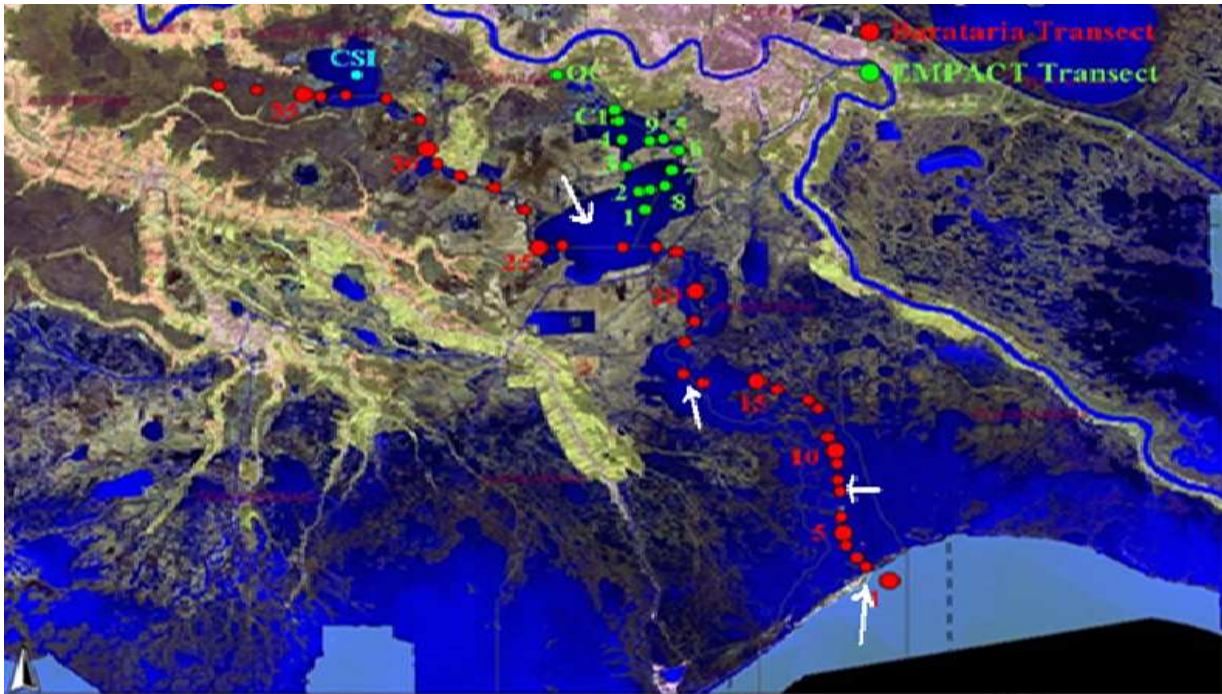


Figure 9a: Map of sampling sites (site marked in red circles indicated with a white arrow) in Barataria Bay. (Source obtained from Department of Oceanography, Louisiana State University)

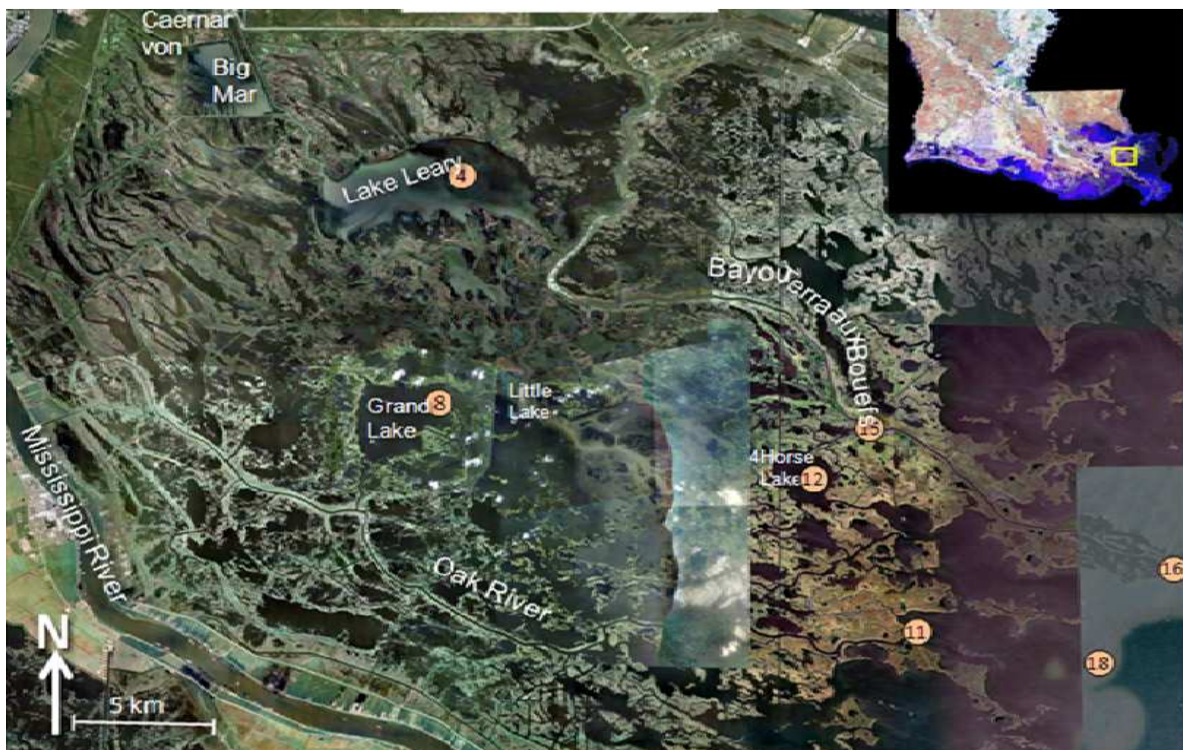


Figure 9b: Map of sampling sites in Breton Sound. (Source obtained from Google Earth by Dr. Robert Lane, Department of Oceanography, Louisiana State University)

4.2.3. Counts for Putative *V. vulnificus* and *V. parahaemolyticus* and Total *Vibrio*

Water samples were mixed by vigorously shaking the sample containers, at least 20 times. A preferred amount of water sample (1ml during warmer months and 5ml during the cooler months) from each container was then mixed with 50 ml of phosphate buffer saline (PBS) with a pH of 7.4 (Baker-Austin *et al.*, 2009) and filtered through a Millipore Mixed Cellulose Esters membrane (0.45 μ m pore size). Two duplicate membrane filters were obtained for each sample and one was placed on a BD™ Difco TCBS agar plate (Kobayashi *et al.*, 1963) and another on a CC agar (Bacteriological Analytical Manual- BAM Media M189, Kaysner and DePaola, 2004) plate and incubated at 35°± 2° C for 24 hrs.

After incubation the colonies on the TCBS and CC agar plates were counted for putative *V. vulnificus* and *V. parahaemolyticus* colonies and total *Vibrio*.

4.2.4. Colony preservation

Putative *V. vulnificus* and *V. parahaemolyticus* colonies were purified by streaking technique using another TCBS plate and incubated at 35°± 2° C for 24 hrs. The cells of purified isolates were stored in 2 X LB broths with 20% glycerol at -80°C (see Chapter 3 for details).

4.2.5. Detection and Characterization of bacterial isolates for *V. vulnificus* and *V. parahaemolyticus*

For each water sample, 2 to 24 putative *V. vulnificus* and *V. parahaemolyticus* colonies grown on TCBS and CC agar were selected for further detection and characterization of *V. vulnificus* and *V. parahaemolyticus* by targeting species- specific and pathogenic genes using the Pentaplex PCR assay developed in Chapter 3. DNA template preparation and the pPCR assay conditions were followed as described in Chapter 3.

About 2 to 10 uncharacteristic bacterial colonies (i.e., presumably non- *V. vulnificus* or *V. parahaemolyticus*) per water sample were also tested with the pPCR assay since some strains may change their colony color from green to yellow (Furniss *et al.*, 1978; HARDY - DIAGNOSTICS).

4.2.6. Data analysis

Counts of total *Vibrio*, total numbers of putative colonies on agar medium, numbers of the putative colonies tested with pPCR, and numbers of the putative colonies confirmed positive for *V. vulnificus* and *V. parahaemolyticus* with pPCR for each sample were used to calculate their prevalence in the environment. To determine the overall impacts of temperature and salinity on the prevalence of *V. vulnificus* and *V. parahaemolyticus*, these two variables were grouped into three ranges for statistical analysis, which was performed in Minitab 16 Statistical Software 2010, Minitab Inc.

4.3 RESULTS AND DISCUSSION

4.3.1. Salinity and Temperature

The highest temperatures occurred in July and August 2011, with an average of 32°C and the lowest were in January 2011 at 13°C. The salinity varied from station to station, with 0.2 ppt being the lowest and 28.6 ppt being the highest. There were no significant differences in sea surface water temperature between Breton Sound and Barataria Bay. Variations in salinity among different sites for different sampling months for both the bays are shown in Figure 9c. The lowest salinities were observed at Site 4 (Lake Lery) in Breton Sound for all sampling months, followed by Site 23 (Lake Salvador) in Barataria Bay. Higher salinities were found in cooler months at Site 2 (Barataria Pass) and 8 (Upper Barataria) in Barataria Bay, with a peak in November 2011.

4.3.2 Total Counts of *Vibrio* on TCBS agar per sampling site

The counts of total *Vibrio* on TCBS were lowest (~ 10 CFU or Colony Forming Units/100 ml) at Site 4 in Breton Sound and highest (2860 CFU/100ml) at site 2 in Barataria Bay. Overall, the combination of lower salinity and temperature drastically reduced the counts (Figure 9c and 9d). The warmer temperature did not seem to increase the total counts, for instance, higher numbers were observed in January at ~13°C.

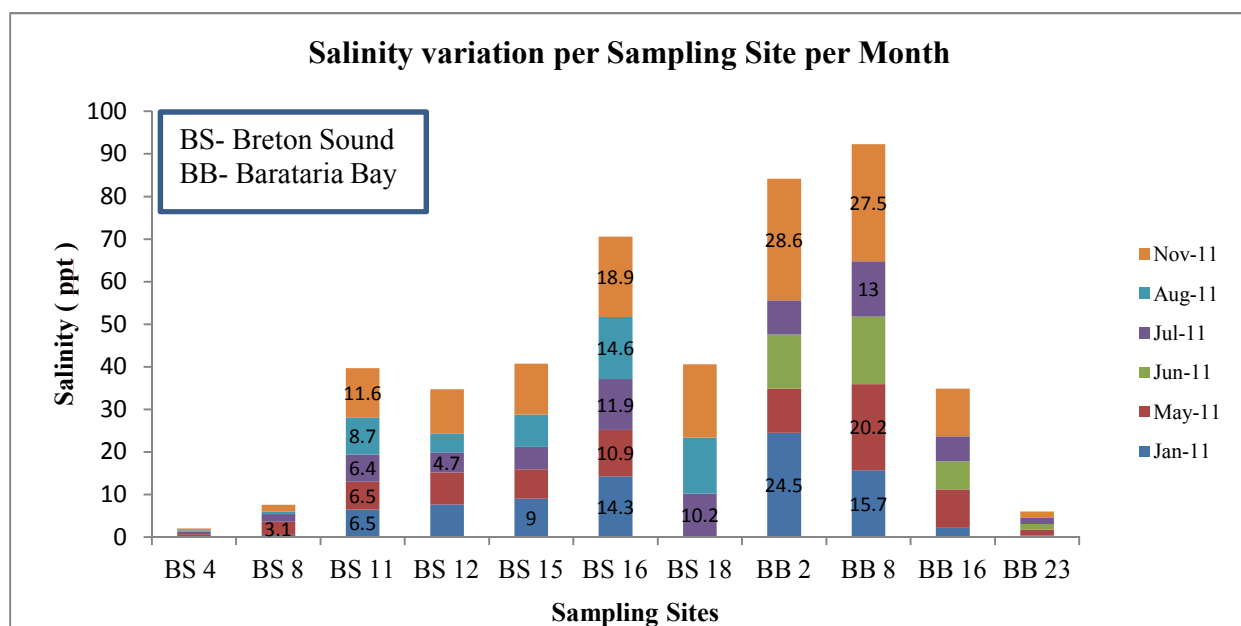


Figure 9c: Variations of salinity among sampling sites and sampling months. (See **Note 2**)

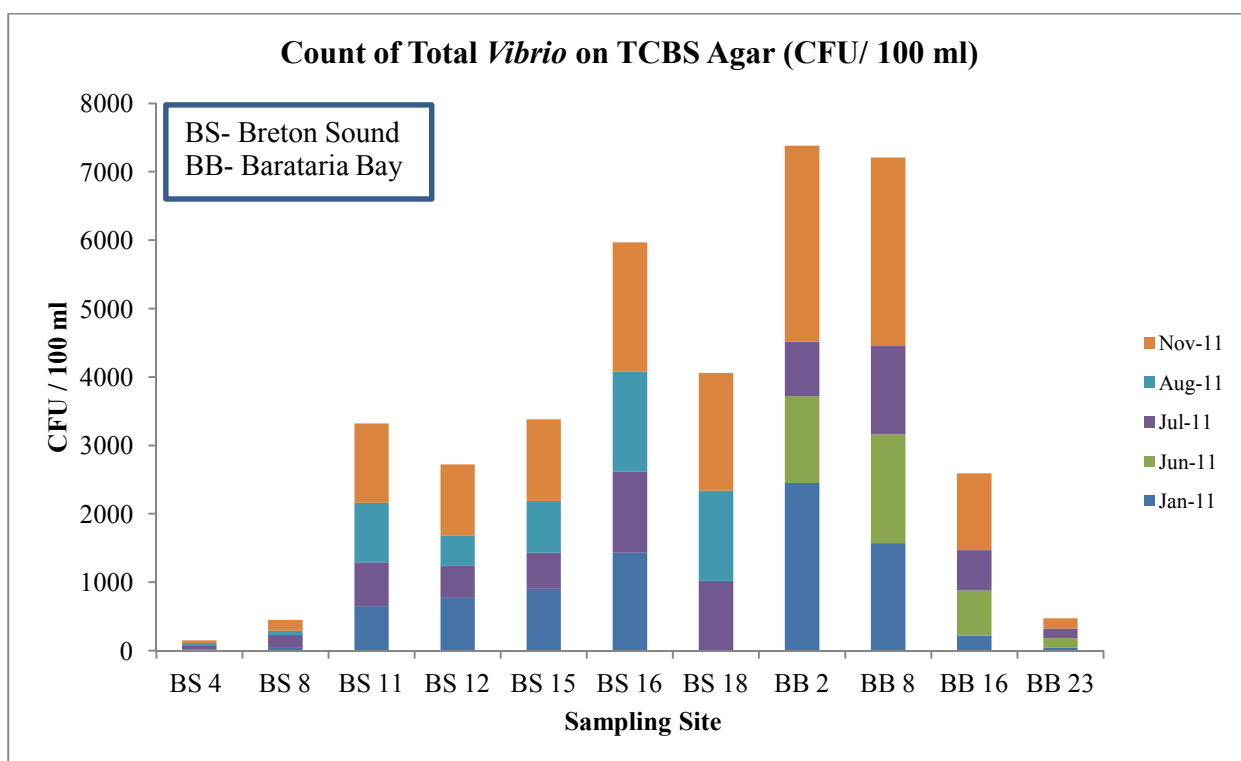


Figure 9d: Counts of total *Vibrio* on TCBS agar per site per sampling month. (See **Note 2**)

(**Note 2:** Water samples from BS Site 18 were collected only after May 2011. Data collected for BS Site 18 for three months- July, August & November 2011 are shown)

4.3.3 Colony characteristics of Putative *V. vulnificus* and *V. parahaemolyticus*

The culturable putative *V. vulnificus* and *V. parahaemolyticus* appeared as round 2- 3mm diameter bluish, green colonies on TCBS (Figure 10a) and *V. vulnificus* as flat yellow colonies on CC agar (Figure 10b). The CC agar has been specifically designed for better recovery of *V. vulnificus*, that typically appears as flat dark yellow colonies resembling fried eggs (Harwood *et al.*, 2004).

4.3.4 Detection and Occurrence of *V. vulnificus* and *V. parahaemolyticus* using pPCR assay

A total of 782 putative bacterial colonies (inclusive of all the samples) were tested for *V. vulnificus* and *V. parahaemolyticus* by pPCR assay (Table 9). Among the 782 isolates, 439 isolates were collected from TCBS agar and 343 from CC agar. Out of the 439 TCBS isolates, 214 (48.75 %) isolates were identified as *V. vulnificus* and 53 (12.07%) as *V. parahaemolyticus*. The 343 CC agar isolates had 167 (48.69%) positive for *V. vulnificus*. The occurrence of *V. vulnificus* on TCBS and CC agar were similar, i.e. 48.75% and 48.69%, respectively.

The occurrence of *V. parahaemolyticus* (12.07%) on TCBS was lower than that of *V. vulnificus* (48.75 %). Lower occurrences of *V. parahaemolyticus* than *V. vulnificus* observed using pPCR assay with isolates from water samples was in agreement with a previous study using colony hybridization method (Johnson *et al.* 2010) .

The occurrence calculated in Table 9, for both *V. vulnificus* (48.72%) from TCBS and CC and *V. parahaemolyticus* (12. 07%) on TCBS were comparable to 482 isolates collected from sediments and oysters (data not shown) in Breton Sound that were culturable on agar medium were also tested by pPCR indicating similar overall occurrences for both the *Vibrio* species.

The results for occurrence of *V. vulnificus* and *V. parahaemolyticus* from the putative colonies can be used to find the overall prevalence among total *Vibrio* population. However, their occurrence may change over the years due to various other coastal, geographical and ecological factors. Also, their occurrences are susceptible to change for individual sampling sites apart from other factors such as different months or salinity regime. (Drake *et al.*, 2007; Zimmerman *et al.*, 2007)

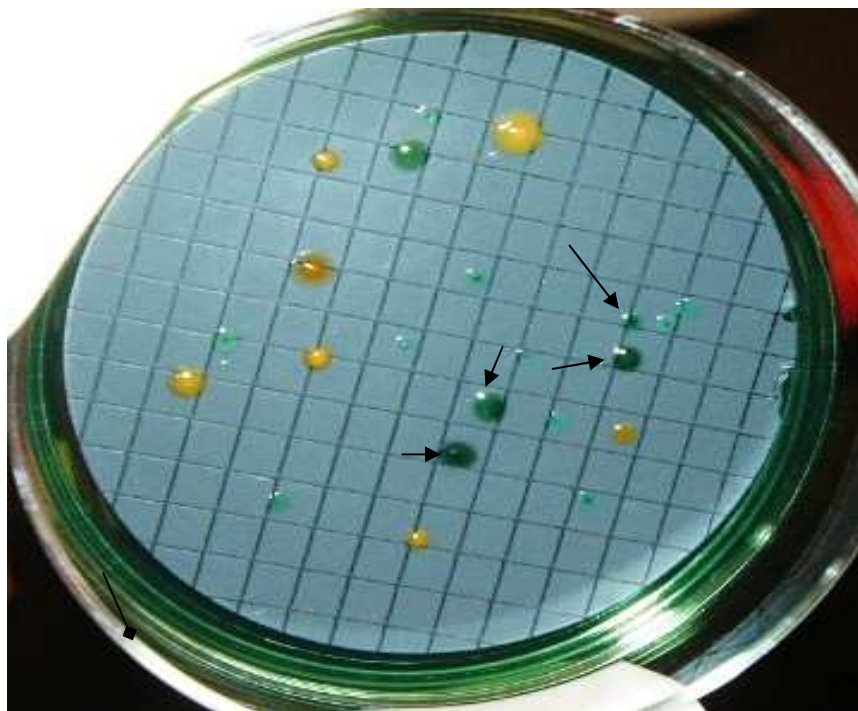


Figure 10a: Colonies of Putative *V. vulnificus* and *V. parahaemolyticus* colonies (dark green color) on TCBS agar. (Colonies indicated with an arrow sign →)

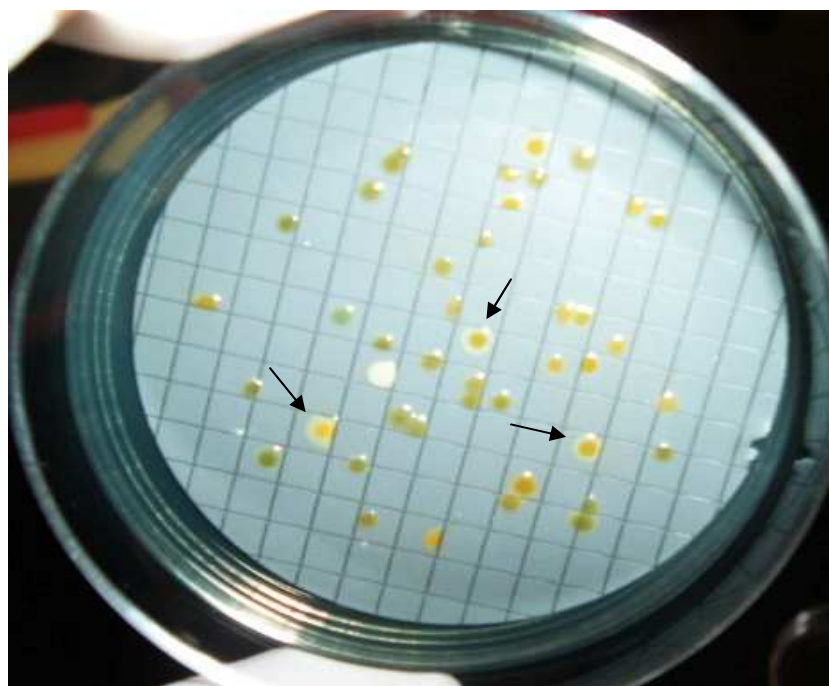


Figure 10b: Colonies of Putative *V. vulnificus* colonies (yellow, flat) on CC agar. (Colonies indicated with an arrow sign →)

Table 9: Overall number of isolates tested by pPCR to determine the positive *V. vulnificus* (V.v) and *V. parahaemolyticus* (V.p) from water samples collected from Breton Sound and Barataria Bay.

Sample Location	Total no. of Putative isolates on TCBS agar tested by pPCR	No. of pPCR confirmed V.v isolates on TCBS agar	No. of pPCR confirmed V.p isolates on TCBS agar	Total no. of Putative isolates on CC agar tested by pPCR	No. of pPCR confirmed V.v isolates on CC** agar
Barataria Bay from 4 salinity station	140	75 (53.6 %) ^a	16 (11.4 %) ^a	137	66 (48.2 %) ^a
Breton Sound from 7 salinity stations	299	139 (46.5%) ^a	37 (12.4 %) ^a	206	101 (49.1 %) ^a
Total for Both Bays	439	214 (48.75%) ^a	53 (12.07%) ^a	343	167 (48.69%) ^a

** Although CC agar is selective for V.v, 7.5% of V.p isolates were observed in the CC agar.

^a Occurrence of culturable V. v and V. p on TCBS and CC agar.

4.3.5 Detection and Occurrence of pathogenic strains by pPCR

Out of the 782 isolates ~ 0.3% were pathogenic strains of *V. parahaemolyticus* containing both *tdh*⁺ and *trh*⁺. Approximately, ~ 0.1 % of *viuB*⁺ and *trh*⁺ in *V. vulnificus* and *V. parahaemolyticus* were observed respectively (data not shown). Two percent of *trh*⁺ gene were observed, but were negative for *tlh*⁺ indicating that other species may have obtained the pathogenic gene *trh*⁺ through horizontal gene transfer in the environment (Bej *et al.*, 1999; Chen *et al.*, 2003; Dobrindt *et al.*, 2004; Hurley *et al.*, 2006; Nordstrom *et al.*, 2007; Yamazaki *et al.*, 2010).

4.3.6 Detection of uncharacteristic bacterial isolates on TCBS and CC agar by pPCR

The number of pPCR confirmed uncharacteristic bacterial isolates tested positive for *V. vulnificus* (n=2) on TCBS and *V. parahaemolyticus* (n= 26) on CC agar were not used in data analysis. These observations have been reported to occur on TCBS agar (HARDY-DIAGNOSTICS; Furniss *et al.*, 1978) and on CC agar (María Carmen Macián, 2000).

4.3.7 Prevalence of *V. vulnificus* (V.v) and *V. parahaemolyticus* (V.p)

In this study, one major effort was to determine the prevalence of *V. vulnificus* (V.v) and *V. parahaemolyticus* (V.p) in each sample. The ratio of V.v. or V.p counts to total *Vibrio* counts (referred as Prevalence of V.v or V.p) was calculated as follows:

Initial Occurrence of V.v or V.p = (Number of colonies confirmed positive for V.v or V.p by pPCR / number of putative colonies tested by pPCR)

Counts of V.v or V.p = (Initial Occurrence of V.v or V.p * Number of total putative colonies on Agar medium)

Prevalence of V.v or V.p = (Counts of V.v or V.p / Counts of total *Vibrio*)

The prevalence of V.v or V.p was calculated because the availability of putative isolates was inconsistent among samples. For instance, during May to August at 10 ppt salinity for a location, samples with higher numbers of putative colonies were observed. Among those 6 putative colonies tested using pPCR method, a lower initial occurrence (~66 %) for V.v or V.p was observed. In contrast, for the same salinity station in cooler months where the numbers of putative colonies tested were ~2, both tested positive, giving a higher initial occurrence (~100%) for either of the two *Vibrio* spp. This could falsely represent the occurrence of the two species in total *Vibrio* population. Hence, to calculate the **prevalence** of *V. vulnificus* and *V. parahaemolyticus* out of the total *Vibrio* population seemed the ideal solution, instead of using their initial occurrence for each samples.

The prevalence of pathogenic genes of *viuB*, *tdh* and *trh* were detectable during November 2011 at site 15 of Breton Sound, having salinity 11.9 ppt at 19.2 °C. From this site n= 21 isolates tested were 10 % positive for both *tdh* and *trh* and 5% for *trh*. Among these isolates 5% *viuB*⁺ *V. vulnificus* strains were observed. During the warmer months 30-32°C, the presence of the pathogenic strains was not detected in the pPCR confirmed V.v or V.p isolates. However, the prevalence of *V. vulnificus* and *V. parahaemolyticus* was higher in the summer than the cooler months (Table 11). It has been reported that pathogenic strains with *tdh* and *trh* were isolated more frequently during cooler months (DePaola *et al.*, 2003). The occurrence of *tdh*⁺ and *trh*⁺ *V. parahaemolyticus* strains following a seasonal trend is not clear (DePaola *et al.*, 2003). However, the prevalence of pathogenic (*tdh*⁺ and *trh*⁺) *V. parahaemolyticus* (Cook *et al.*, 2002; DePaola *et al.*, 2003; Hara-Kudo *et al.*, 2003) and *V. vulnificus* (*viuB*⁺) (Bogard & Oliver, 2007) have been reported to be low in the environment, which correlates with this study.

The number of months tested in this study is only an overall representative of cooler and warmer months. Therefore, the number of putative bacterial colonies tested per sample sites, by pPCR must be increased to ~ 50, to resolve any discrepancies in calculating the prevalence of *V. vulnificus* and *V. parahaemolyticus* (DePaola *et al.*, 2003) between seasonal months or salinity regime.

Based on the prevalence of *V. vulnificus* and *V. parahaemolyticus*, the data for each, salinity and month were grouped separately in Table 10 and Table 11.

4.3.8 Effect of Salinity on Prevalence of *V. vulnificus* and *V. parahaemolyticus*

The prevalence of *V. vulnificus* and *V. parahaemolyticus* was grouped based on the salinity ranges in Table 10.

The salinity 10 to 15 ppt in this study indicates that an optimal salinity range positively influences the prevalence of *V. vulnificus* (TCBS 39 % and CC agar 40 %). When the salinity deviates from 10 – 15 ppt, a gradual decline in prevalence of *V. vulnificus* occurs, i.e., their lowest prevalence is found at extreme saline conditions similar to previously reported studies (Rivera *et al.*, 1989; Lipp *et al.*, 2001; Randa *et al.*, 2004). The prevalence of *V. vulnificus* versus salinity groups on TCBS and CC agar are comparable to each other.

Overall the prevalence of *V. parahaemolyticus* is lower than *V. vulnificus* on TCBS agar, but they seem to be more tolerant to higher salinity range above 25 ppt than *V. vulnificus*. Prevalence of *V. parahaemolyticus* is highest at 10-15 ppt among the other salinity ranges in this study, which is in contrast from a previous study (Rivera *et al*, 1989) reporting 20ppt.

Table 10: The sampling from both Breton Sound and Barataria Bay, grouped into salinity regimes (exclusive of temperature factor) showing *Vibrio* counts and prevalence TCBS and CC agar.

Salinity groups (Parts per thousand or ppt)	Total count on TCBS (CFU/100ml)	No. of V.v on TCBS (CFU/100 ml)	No. of V.p on TCBS (CFU/100ml)	Total count on CC (CFU/ 100ml)	No. of V.v on CC (CFU/ 100ml)
0 - 5	52330	1088 (2 %)*	50 (0.1%)*	36070	1331 (3.6%)*
5 to 10	36040	6996 (19%)*	1358 (4%)*	37800	6241 (17%)*
10 to 15	51810	20372 (39%)*	6492 (13)*	40495	16128 (40%)*
15 to 20	20300	2388 (12%)*	507 (3 %)*	12600	2227 (18 %)*
20 to 25	770	0 (0%)*	18 (2 %)*	440	0 (0%)*
25 <	1125	25 (2%)*	50 (4%)*	5050	34 (1%)*

* Prevalence of V.v (*V. vulnificus*) and V.p (*V. parahaemolyticus*) for each of the salinity regimes.

Above 20 ppt the prevalence of *V. parahaemolyticus* is to some extent higher than that of *V. vulnificus*, suggesting that majority of *V. vulnificus* strains cannot withstand salinity stress that reduces its cultivability due to the VNBC state (Wong & Wang, 2004; Coutard *et al.*, 2007). The occurrence of *V. parahaemolyticus* in high saline conditions could also be due to genetic variation between strains, which enables the bacteria to adapt and survive in extreme conditions (Dworkin & Falkow, 2006). However, it has to be noted that the environmental conditions during 2011 were such that the number of sampling stations above 20 ppt were very less (U.S. Army Corps of Engineers, 2011; NOAA, 2011). Therefore, more number of higher saline stations from other years may give consistent results for the prevalence of the two *Vibrio* spp. and their virulent strains.

4.3.9 Seasonal Effect on Prevalence of *V. vulnificus* and *V. parahaemolyticus*

The prevalence of *V. vulnificus* and *V. parahaemolyticus* was grouped by the month of sampling in Table 11.

Prevalence of *V. vulnificus* (~0 %) and *V. parahaemolyticus* (~2%) is the lowest during the month of January 2011. A gradual increase in the prevalence of *V. vulnificus* (20 – 26 %) during the month of May, June, July and August on the TCBS agar was observed that was comparable to the CC agar counts. Prevalence of *V. parahaemolyticus* increased during the summer and decreased in winter. *V. vulnificus* and *V. parahaemolyticus* counts are highly susceptible to enter the VBNC state as the temperature drops in January. This drastically reduces their cultivability on agar medium (Oliver, 1995). In November, the moderately cooler temperature 20°C marks the decline in the prevalence of *V. vulnificus* (4%) and *V. parahaemolyticus* (2%). The month of July 2011 and May 2011 had lower occurrence of *V. parahaemolyticus*. It has to be noted that salinity in the Gulf of Mexico during 2011 was greatly affected in May 2011 by Mississippi River flooding (U.S. Army Corps of Engineers, 2011) and July 2011 marked as hurricane season recording 6.39 inches of precipitation (NOAA, 2011), which may have caused a temporary layer of freshwater over the denser saline waters (Osborne, 2000) lowering the salinity, thereby, lowering the prevalence of *V. parahaemolyticus*, but these conditions did not affect the counts of *V. vulnificus*.

Table 11: The sampling from Breton Sound and Barataria Bay, grouped by monthly average temperature °C (exclusive of salinity factor) showing *Vibrio* counts and prevalence on TCBS and CC agar. (The time of collection on the day of sampling per month was done from early morning to noon, so the temperature varied by 1-2°C between station in both Breton Sound and Barataria Bay).

Sampling Month	Av. °C per month	Total count on TCBS (CFU/100ml)	No. of V.v on TCBS (CFU/100ml)	No. of V.p on TCBS (CFU/100ml)	Total count on CC (CFU/100ml)	No. of V.v on CC (CFU/100ml)
January 2011	13	1020	0 (0 %)*	18 (2 %)*	6620	0 (0%)*
May 2011	28	47600	9643 (20 %)*	738 (2 %)*	59650	14766 (25 %)*
June 2011 only for Barataria Bay Sites	30	6800	1396 (21 %)*	1117 (16 %)*	7150	3016 (42 %)*
July 2011	30	38750	9510 (25 %)*	535 (1.3 %)*	28000	6414 (23 %)*
August 2011 only for Breton Sound Sites	32	34000	8964 (26 %)*	5340 (16 %)*	Samples not tested by pPCR on CC agar	
November 2011	20	36325	1355 (4 %)*	729 (2 %)*	33675	1764 (5 %)*

*Prevalence of V.v (*V. vulnificus*) and V.p (*V. parahaemolyticus*) for each of the month.

Av. °C refers to Average temperature in Celsius.

Through 16 S rDNA sequencing (Chapter 3), there was one isolate showing 96.65% similar sequence identity to *Vibrio mediterranei* in Barataria Bay Site 2 near Grand Isle, which gives insight to previous reports (Carraturo *et al.*, 2006; Pujalte & Garay, 1986) that strains of these species releases antagonistic compounds that could consequently decline the number of *V. parahaemolyticus* in the environment. The trend of *V. parahaemolyticus* prevalence is the same for June and August at 16 %. June and August 2011 were recorded to have lowest precipitation, overall representing that the warm and saline condition positively influences the increase in prevalence of *V. parahaemolyticus*.

4.3.10 Statistical Analysis for Effect of Temperature and Salinity on the Prevalence of the two *Vibrio* spp.

Based on the above results, **prevalence** of *V. vulnificus* and *V. parahaemolyticus* from each sample locations were grouped into 3 levels of salinity and temperature (Table 12). The main effects and interaction of these two factors were analyzed at each levels based on the **means of prevalence** for each species.

Table 12: Temperature and salinity data grouped into three levels.

Temperature Group	Temperature (°C)	Salinity Group	Salinity (Parts per thousand or ppt)
A	< 15	L	< 2
B	15-25	M	2 to 14
C	>25	H	> 14

The main effects plot is based on the study of magnitude of the effect of multiple factors on a response, i.e. in this study the “factors” are **temperature** and **salinity** and the “response” is **mean prevalence** of the two *Vibrio* spp. The point on the line in the main effect plots represents the **mean prevalence** of the two *Vibrio* spp., at each level for each factor. A horizontal line indicates no effect and steeper slope in the line represents the presence of an effect. The grand **mean prevalence** of each *Vibrio* spp., are indicated by a reference line. The interaction plot

studied, allowed us to visualize the interaction of both salinity and temperature on the response. The highest degree of interaction is represented when the slopes between the lines are greater. If the lines are parallel it indicates no interaction. Usually both the main effects and interaction plots are used in combination with ANOVA and Design of Experiments. To ascertain that the plots are statistically significant, a General Linear Model-Factorial Plots was tested on main effects and interaction plot. Statistical tool MINITAB 16 software was used. (The statistical analysis and its explanation were obtained from “help” menu in the MINITAB 16 software)

4.3.11 Main Effects Plot for Prevalence of *V.vulnificus* on both TCBS and CC agar

The Main effects plot for **mean prevalence** of *V.vulnificus* on TCBS as well as on CC agar Figure 11a and Figure 11b, clearly shows that there is a significant increase in mean prevalence as the temperature increases from group A (< 15°C) to group C (>25°C). Each temperature groups (A, B, C) show significantly different response from each other on TCBS ($p=0.007$, $\alpha=0.05$) and CC ($p=0.006$, $\alpha=0.05$) agar, respectively. As previously reported (Kaspar and Tamplin, 1993; Johnson *et al.*, 2010), the prevalence of *V.vulnificus* has shown to strongly correlate to water temperature. When the temperature gradually increases to 32°C a positive effect on the mean prevalence of *V.vulnificus* was also observed in our study.

A comparable trend in the main effects plot for salinity on the response in both TCBS and CC agar, were noticed. An optimal salinity range group M plays a considerable role in increasing the mean prevalence of *V. vulnificus* at warmer temperatures. Deviation from the salinity group M shows a steep decline in the response. The GLM results show that each of the salinity groups have significant response, both on TCBS ($p=0.001$, $\alpha=0.05$) as well as on CC ($p=0.048$, $\alpha=0.05$) agar. The p value for salinity effect for the mean prevalence of *V.vulnificus* on the CC agar is larger than that of TCBS. This is possibly due to the difference in plating efficiency of the CC and TCBS agar for isolation of *V. vulnificus* (Green *et al.*, 1996; Høi & Dalsgaard *et al.*, 1998, Kaysner and DePaola, 2004).

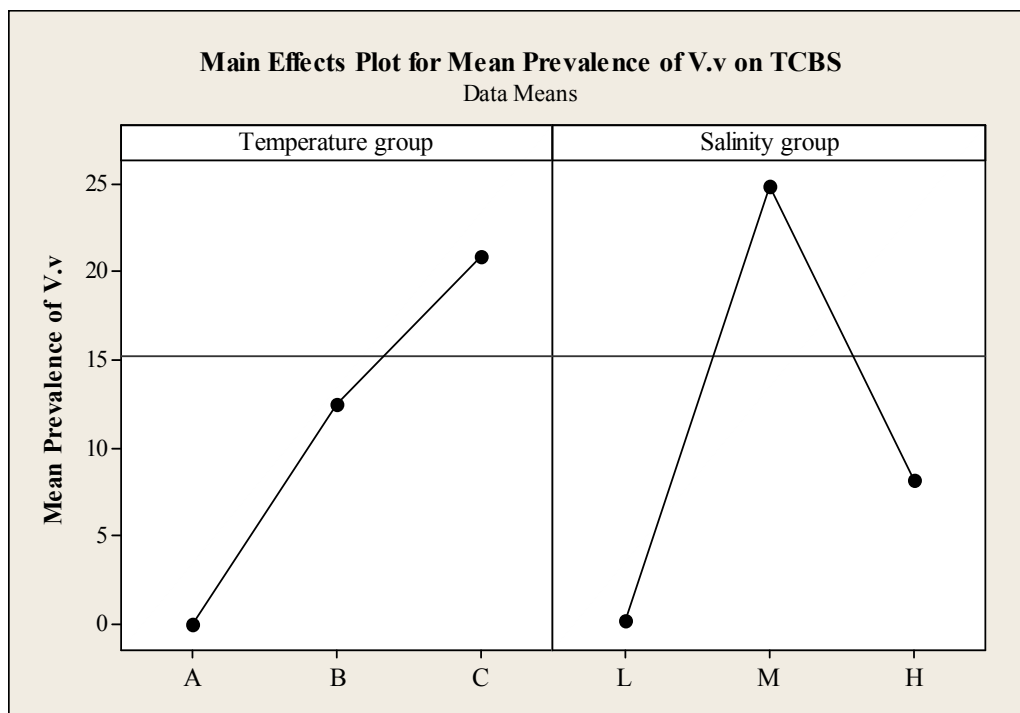


Figure 11a: Main Effects Plots for Mean Prevalence of *V.vulnificus* (V.v) on TCBS agar.

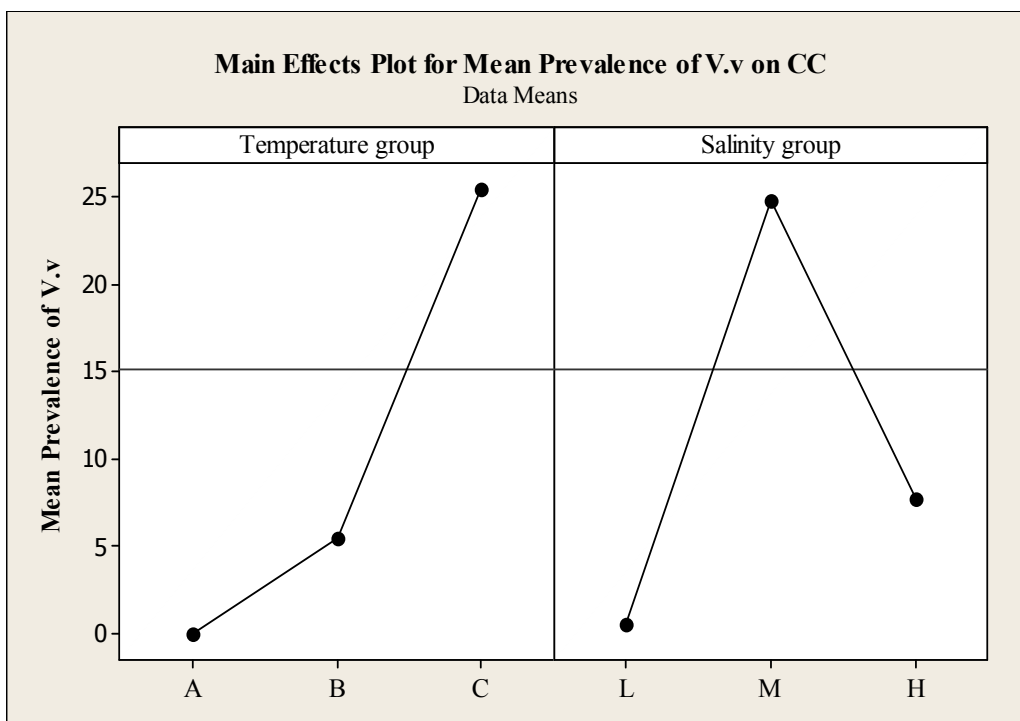


Figure 11b: Main Effects Plots for Mean Prevalence of *V.vulnificus* (V.v) on CC agar.

4.3.12 Interaction Plot for Prevalence of *V.vulnificus* on both TCBS and CC agar

In the interaction plot as the lines are not parallel, the effect of temperature- and salinity levels show significant interaction ($p = 0.039$, $\alpha = 0.05$) affecting the mean prevalence of *V.vulnificus* growing on TCBS agar (Figure 12a). GLM analysis for the interaction of both the factors on the response for CC agar ($p = 0.91$, $\alpha = 0.05$) represents (Figure 12b) that there is no significant evidence of interaction at 5% confidence level, but there is a moderate evidence at 10% confidence level. The lowest salinity group L shows no or low mean prevalence at all temperature groups, implying that the lowest salinity station below 2 ppt are not suitable for the abundance of *V.vulnificus* population at any temperature. As recorded in the original data (not shown) at 1.8 ppt at 29.7°C during the month of July, for Breton Sound Site 8, least occurrence of *V. vulnificus* was observed on both TCBS and CC agar. The mean prevalence of *V.vulnificus* is lower at cooler temperatures below 15°C, at all salinity levels probably due to cold stress (Oliver *et al.*, 1995; Randa *et al.*, 2004; Johnson *et al.*, 2010).

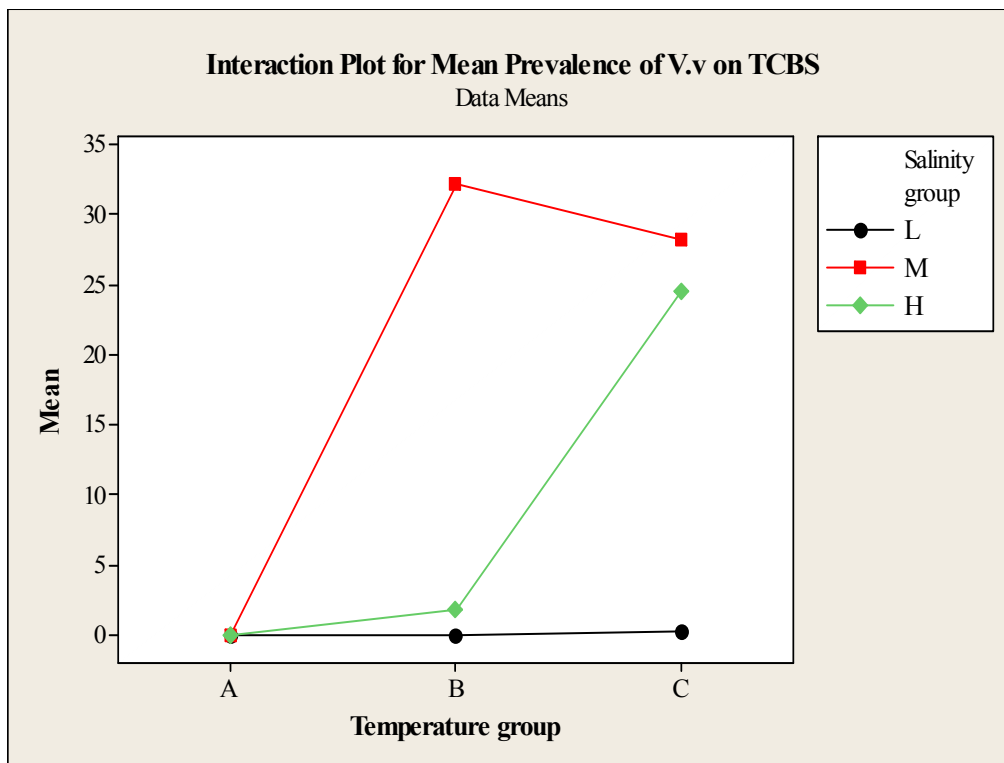


Figure 12a: Interaction Plot for Mean Prevalence of *V.vulnificus* (*V.v*) on the TCBS agar.

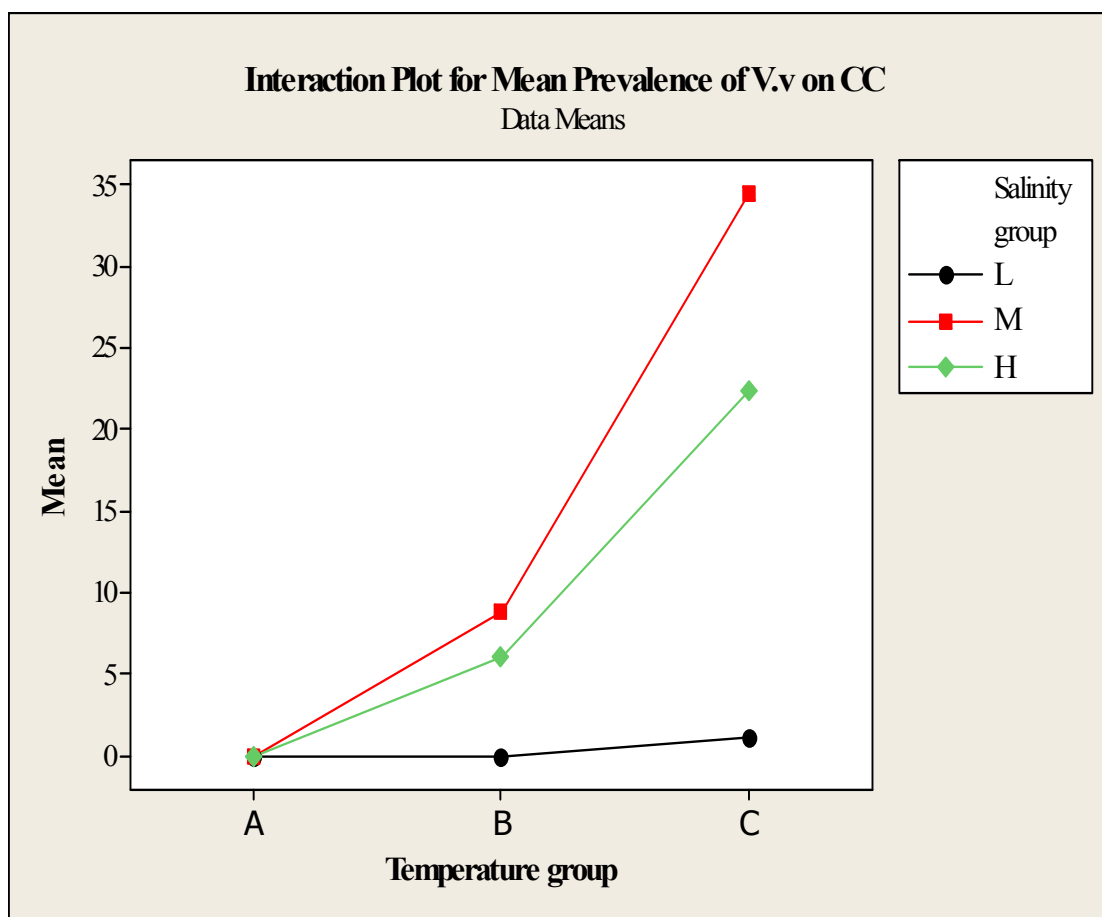


Figure 12b: Interaction Plot for Mean Prevalence of *V.vulnificus* (V.v) on the CC agar.

Overall, the salinity group M ranging 2 – 14 ppt has highest prevalence of *V.vulnificus* than other groups at warmer temperatures. In this study an optimal salinity range (2-14 ppt) is playing a pivotal role in their abundance as reported in previous research, 5 - 10 ppt (Randa *et al.* 2004), 17 ppt (Motes *et al.*, 1998) and 7- 16 ppt (Kelly, 1982).

The GLM model leads us to accept the alternative hypothesis that there is a significant difference in response between the levels of temperature and salinity on both TCBS and CC agar. It is evident that the effect of temperature and salinity from TCBS (R-sq 57.02%) and CC agar (R-sq 60.73%), play an essential role (R-sq ~ 60%) influencing the occurrence of *V.vulnificus* population. The remaining 40% may be influenced by other environmental parameters (Kaneko & Colwell, 1973; Kaneko & Colwell, 1975; Baross *et al.*, 1978; Sochard, 1979; Davis, 1982; Williams, 1985; DePaola *et al.*, 1997; DePaola *et al.*, 1998; Pelon *et al.*, 1995).

4.3.13 Main Effects and Interaction Plot for Prevalence of *V. parahaemolyticus* on TCBS agar

The main effects of salinity ($p=0.016$, $\alpha=0.05$), on the mean prevalence of *V. parahaemolyticus* (Figure 13a) are more positively correlated than that of warmer temperature ($p= 0.019$, $\alpha= 0.05$), which is similar to DePaola *et al.* (2003) studies. However, there is a significant effect of both the factors on the mean prevalence of *V. parahaemolyticus*. Interaction plots (Figure 13b) indicates that the effect of temperature on the mean prevalence of *V. parahaemolyticus* at each salinity levels is statistically evident ($p= 0.015$, $\alpha= 0.05$) and there is interaction between the factors. Similar to *V. vulnificus*, the lowest salinity group L (< 2 ppt) does not support prevalence of *V. parahaemolyticus* at any temperature levels. It was observed in main effects plot that a rise in salinity increases the mean prevalence of *V. parahaemolyticus* (Figure 13a), these results were comparable to previous studies (DePaola *et al.*, 2003; Zimmerman *et al.*, 2007). However due to lesser number of higher saline stations sampled in 2011, the interaction plot is not very clear for salinity group M and H (Figure 13b).

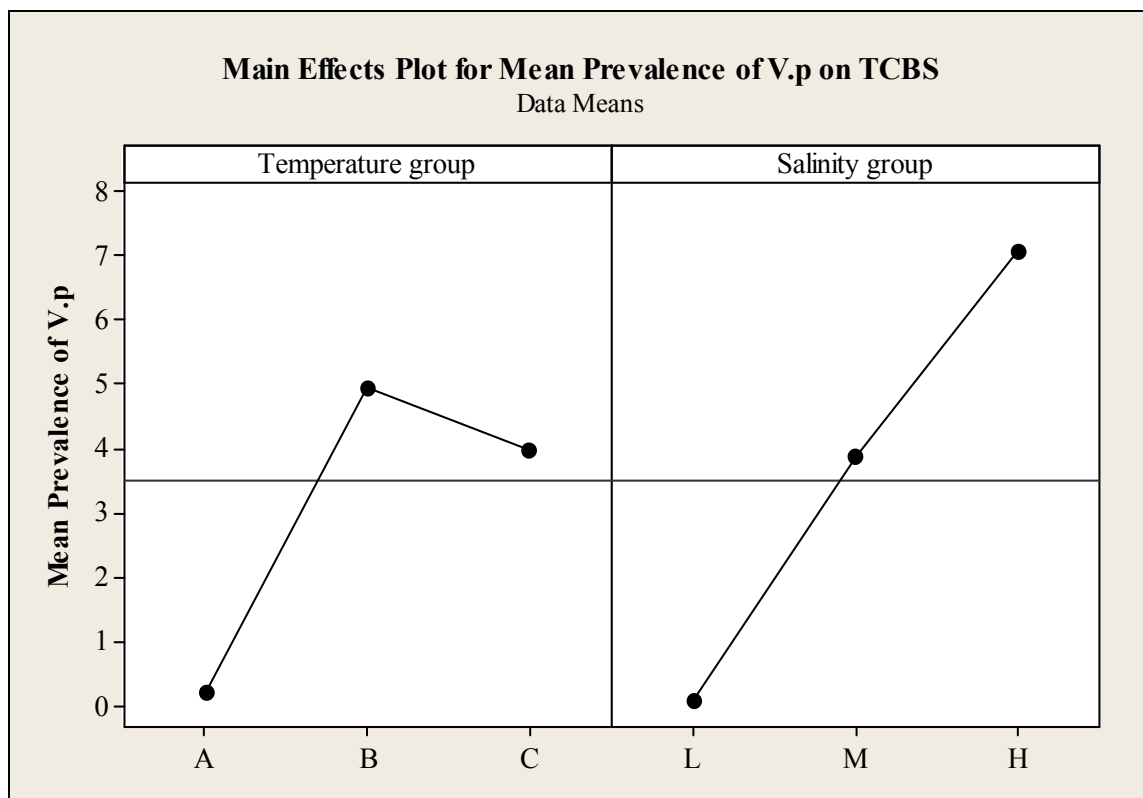


Figure 13a: Main Effects Plot for Mean Prevalence of *V. parahaemolyticus* (V.p) on TCBS agar.

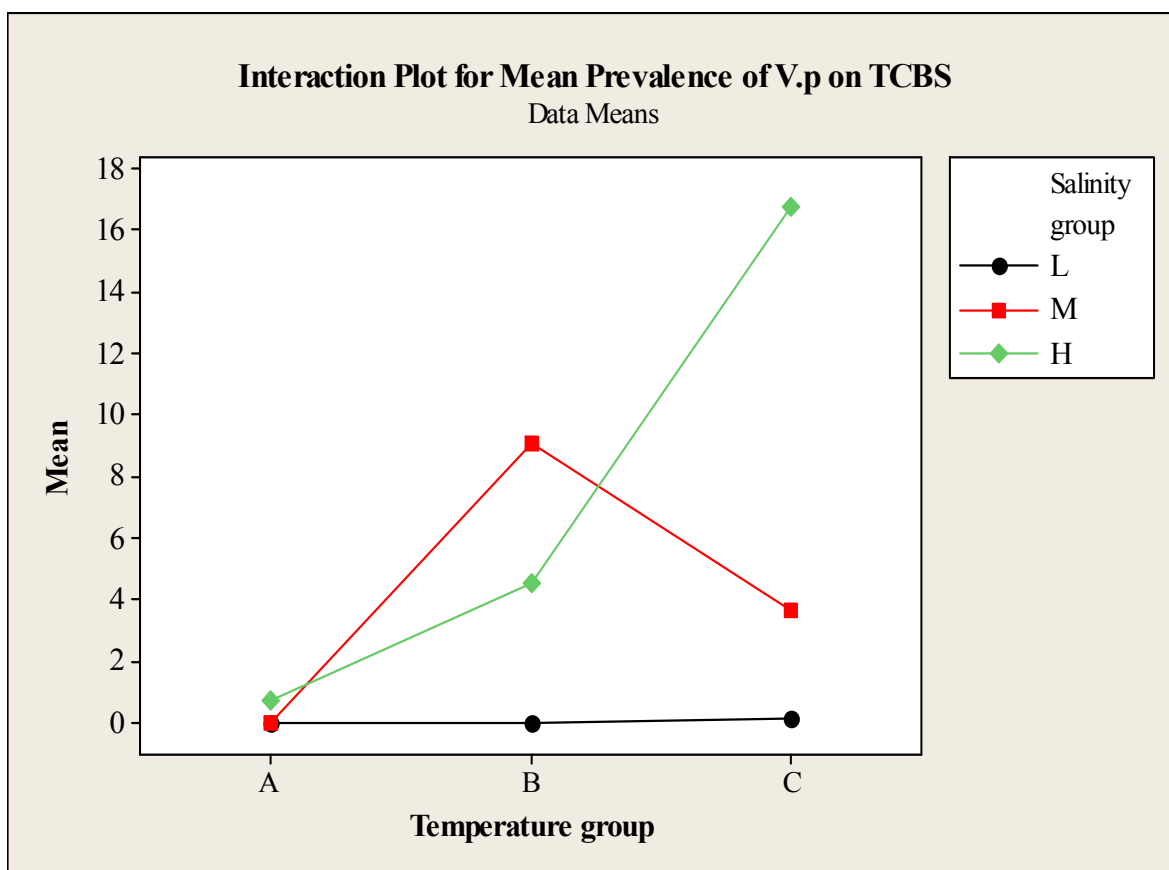


Figure 13b: Interaction Plot for Mean Prevalence of *V. parahaemolyticus* (V.p) on TCBS agar.

The R-sq value (39.06%), suggests that temperature and salinity in this model play a minor role in variability of the mean prevalence of *V. parahaemolyticus*. Salinity has been previously reported to play a minor role in *V. parahaemolyticus* abundance (Deepanjali *et al.*, 2005; Martinez-Urtaza *et al.*, 2008; Sobrinho *et al.* 2010). Their prevalence might be influenced by other abiotic factors such as turbidity and chlorophyll (Johnson *et al.*, 2010; Rehnstam-Holm *et al.*, 2010) or release of non potable water from ship ballast and bilge (McCarthy and Khambaty, 1994). Biotic factors such as phytoplankton (Rehnstam-Holm *et al.*, 2010), bacteriocins (Joerger, 2003; Riley & Wertz, 2002) and bacteriophages (Baross *et al.*, 1978; DePaola *et al.*, 1997; DePaola *et al.*, 1998) may cause reduction in the levels of *V. parahaemolyticus* at salinity group M at higher temperatures. These microbes may not occur in higher saline group H, which heightens the mean prevalence of *V. parahaemolyticus*, apart from the fact that warmer climate also enables proliferation of *V. parahaemolyticus*.

CHAPTER 5

CONCLUSIONS AND FUTURE RESEARCH

This was the first known effort to develop a fast, cost-effective multiplex PCR (a pentaplex PCR termed pPCR) assay for simultaneous detection of bacterial isolates for 2 species-specific genes (*vvh* in *V. vulnificus* and *tlh* in *V. parahaemolyticus*) and 3 pathogenic genes (*viuB* in *V. vulnificus*, *tdh* and *trh* in *V. parahaemolyticus*). The pPCR assay with an annealing temperature of 57°C was able to detect the 5 target genes with 100% efficiency when 7 reference strains were tested and could differentiate bacterial isolates for *V. vulnificus* or *V. parahaemolyticus* from *V. cholera* and other *Vibrio* species. An efficiency of 96.7- 98.7 % was achieved with 293 environmental isolates collected from the Northern Gulf of Mexico water samples on TCBS and CC agar. The efficiency of pPCR is comparable to previously published protocols (Bej *et al.* 1999; Panicker *et al.* 2004a), which targeted the genes in *V. vulnificus* and *V. parahaemolyticus* separately.

Furthermore, the validity of the pPCR assay was confirmed by the 16S rDNA sequencing results. Four isolates identified as *V. vulnificus* or *V. parahaemolyticus* by the pPCR assay showed a 16S rDNA sequence similarity of 98.9 – 99.9 % to known strains of *V. vulnificus* or *V. parahaemolyticus*, and the 41 environmental isolates determined as non- *V.v./V.p* species by the pPCR assay had highest similarities to known non- *V.v./V.p* strains.

The assay for detection of the 5 target genes could be completed in a laboratory setting within 5-6 hours after collection of isolates. The study suggests that the pentaplex PCR assay has great advantages that can be applied by various food industries, environmental and water quality monitoring laboratories and also, be adopted into the current FDA's- Bacteriological Analytical Manual, for rapid screening of bacterial isolates for *V. vulnificus* and *V. parahaemolyticus* and their virulent strains from *V. cholera* and other bacterial pathogens.

Furthermore, the pPCR could provide additional time saving solutions to detect both *V. vulnificus* and *V. parahaemolyticus* cells in a mixture such as MPN broths, however in this case, further research for differentiating the two amplicon products of *viuB* (504 bp in *V. vulnificus*) and *trh* (500bp in *V. parahaemolyticus*) in close proximity needs to be resolved. In this study, the

lowest detection limit of the pPCR for pathogenic strains of *V. vulnificus* and *V. parahaemolyticus* was 30 cells/ ml. As the protocol uses a crude DNA template for pPCR assay, further purification and quantification of the DNA template would enhance the detection limit.

The pPCR assay was applied for screening 782 isolates, collected from the Breton Sound and Barataria Bay water samples for *V. vulnificus* and *V. parahaemolyticus* and their virulent strains, with a purpose of determining their prevalence in the environment as impacted by water temperature and salinity. The assay showed higher occurrences of *V. vulnificus* (~49%) than that of *V. parahaemolyticus* (~12%) on TCBS agar. Occurrences of *V. vulnificus* (~49%) in bacterial isolates tested from both TCBS and CC agar were comparable. Overall, the occurrences of pathogenic *V. vulnificus* and *V. parahaemolyticus* strains were less than 1%.

Salinity showed uneven impacts on both the *Vibrio* prevalence. The prevalence of *V. vulnificus* seemed to be higher with a salinity range of 2- 14 ppt relative to higher salinity (>14 ppt), while *V. parahaemolyticus* occurrence increased with increasing salinity (> 14 ppt). The occurrence of *V. parahaemolyticus* showed an increase in salinity range 2- 14 ppt at temperatures 15-25°C, but declined as the temperature increased >25°C. However, the prevalence of *V. parahaemolyticus* in higher saline conditions (> 20 ppt) at higher temperature (25-32°C) regime necessitates further research, as the environmental conditions prevailing in 2011 limited the number of higher saline sampling stations found during warmer months. The freshwater stations, where salinity was below 1.8 ppt, did not support any growth of both the *Vibrio* species, at all seasons.

Water temperature had a positive effect on the abundance of both *V. vulnificus* and *V. parahaemolyticus*, where they peaked in June and August of 2011. The lower prevalence of *V. parahaemolyticus* (~1.3%) in May and July 2011 was attributed to the Mississippi River flooding and precipitation that largely lowered the salinity; however, this condition did not affect the prevalence of *V. vulnificus* during warmer months (May through August).

Both species could not be cultivated at cooler weather conditions (below 15°C) in all salinity ranges. The occurrence of pathogenic strains was higher in November (~20°C) than in June - August (30-32°C), which has also been reported by other studies. This indicates the

capability of the pPCR assay to detect pathogenic genes giving an insight to their seasonal occurrence. However, isolates from a larger number of cooler months need to be tested to draw a sound conclusion whether the prevalence of the pathogenic strains is seasonally dependent.

One interesting observation in this study was that one of the environmental bacterial isolates was identified as *Vibrio mediterranei* through 16S rDNA sequencing, which, leads us to do further research as to how untreated waters in ship ballast/bilge from Mediterranean Sea and other coastal waters apart from tidal wave action could disseminate microbes with antagonistic factors that probably could change the prevalence of *V. vulnificus* and *V. parahaemolyticus* in the coastal environment. The GLM analysis of the main effects and interaction plots suggests that temperature and salinity in our model are playing a major role that influences the prevalence of *V. vulnificus* (~60 %) in a greater degree than that of *V. parahaemolyticus* (~39%). The results also implies that other biotic (such as bacteriophages, antagonistic microbes) and abiotic factors (such as nutrients, turbidity, chlorophyll, attachment to copepods, and exoskeleton of crabs) may also impact the prevalence of *V. vulnificus* and *V. parahaemolyticus* and should be studied to develop better risk management strategies for *V. vulnificus* and *V. parahaemolyticus*.

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VITA

Nabanita Bhattacharyya was born and raised in Madras (Chennai) which lies on the Southeastern coastal plains of India. She had majored in Plant Science and Biotechnology and earned a Master's degree from the University of Madras in 2004. After graduating, she worked for Central Leather Research Institute, India as a Biotech laboratory assistant, gaining experience in production of eco-friendly enzymes that would help leather industries and the environment, by reducing the use of hazardous chemicals used for leather processing. Later in 2005, Nabanita was accepted as a Lecturer and taught courses in Biotechnology at two prestigious schools affiliated to the University of Madras. After four years of her service as a tutor, teaching graduates students in India, she wanted to continue her pursuit of higher studies and research in environmental science. In the fall of 2009, Nabanita accepted a graduate research assistantship position at Louisiana State University, where she spend the following three years gaining knowledge and doing research in environmental science, toxicology and microbial ecology. She will graduate with a Master of Science in August 2012 and is looking forward to continue her studies as a Doctoral student in Louisiana State University that will help her build a future career as an academician and a researcher.