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Detection of *Vibrio vulnificus* by direct colony immunoblot

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DETECTION OF *VIBRIO VULNIFICUS* BY DIRECT COLONY IMMUNOBLOT

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
The Department of Food Science

by
Reshani Nisansala Senevirathne
B.S., Wayamba University of Sri Lanka, 2004
May 2007

DEDICATION

- ◆ To love of my life Mr. **Indrajith C Senevirathne**, for all your encouragement, love and care.
- ◆ To my Grand Mother Mrs. **Monica Margret Amarasinghe** who bought me my first book to read from her hard earned pension.
- ◆ To my Father Mr. **Sunil P De Alwis** who worked hard day and night in a far away country so we could eat and dress and to my Mother Mrs. **Ranjani C Amarasinghe** who gave love unconditionally everyday.

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ABSTRACT

Vibrio vulnificus (Vv) is a natural occurring bacterium of world wide estuarine environments, which concentrate on filter feeding shellfish. Under cooked seafood contaminated with this pathogen is a leading cause of 95% of seafood-related, foodborne deaths. The development of a rapid, reliable and user-friendly method for Vv enumeration would help to reduce mortality rate. A direct colony immunoblot (DCI) method was developed and used as a rapid enumeration with high sensitivity and the specificity. This method was optimized using Vv agar plates incubated for 16 h at 35 °C. Colonies were transferred from incubated plates to polyvinylidene fluoride (PVDF) membranes and treated with rabbit anti-flagellar Vv antibodies for 1 h, then washed 3 times. The membranes were then incubated with peroxidase-conjugated goat anti-rabbit IgG for 1 h, and washed 3 times. Finally, the color development mixture was added (Tris buffer, 3, 3'-diaminobenzidine, NiCl₂ and H₂O₂) for 5 min. Positive colonies produced a purple color. Total time duration for enumeration of Vv by the DCI was 3.5 h. The DCI method was compared with the FDA recommended DNA probe hybridization (DNAH) method (6-10 h) and most probable number MPN method (50 h) for enumeration of naturally occurring Vv in oysters. There was no significant difference between the DCI and DNAH methods at 0h, 4h, 8h, 12h and 24h, with both methods having Vv counts of about 2.90 Log CFU/g. By day 7 there was a significance difference between the two methods, with the DNAH exhibiting higher Vv counts (2.62 Log CFU/g) compared to the DCI (2.22 Log CFU/g). By 14 days the counts for both methods were not significantly different from each other (1 Log CFU/g). The DCI method exhibited comparable Vv counts in raw oysters compared with those of the DNAH method except for day 7, which may be due to false positive colonies detected by the DNAH method. The DCI could be a more reliable, inexpensive, rapid and user-friendly method for enumeration of Vv in raw

oysters. This could possibly be used as a rapid enumeration method by regulatory agencies or the seafood industry.

CHAPTER 1. INTRODUCTION

Vibrio vulnificus can be identified as an indole positive motile straight or curved bacterium, which is responsible for the majority of the sea food related fatalities in the United States (Hlady and others 1993). *Vibrio. vulnificus* infections are correlated with the distribution of this organism in estuarine environments which is influenced by several factors such as pH, temperature, salinity, competitors, exposure to light, and nutrient deprivation (Kasper and Tamplin 1993). Most of the seafood related *V. vulnificus* infections in the United States is results from the consumption of raw oysters harvested from the Gulf of Mexico during warmer months (California Department of Health Services). These infections are very aggressive among immuno-compromised individuals and most of the diagnoses are usually made postmortem. Most *V. vulnificus* infections are unrecognized and underreported, which is also a reason for high mortality rates (Todar 2005). To reduce *V. vulnificus* related health hazards quick solutions are needed. One way to reduce the *V. vulnificus* health hazard is by implementing new technologies such as rapid enumeration methods.

We cannot rely on some *V. vulnificus* identification methods such as biochemical tests because it is not always correct (Nishibuchit and Seidler 1985).This methods required tedious subculture of several isolates for verification. Some biochemical tests are varying with the strains and that make identification questionable unless sufficient number of tests are properly done (Wright and others 1993). To overcome that problem several DNA based rapid identification methods such as DNA probe hybridization (DNAH) and Polymerase Chain Reaction (PCR) have been recently developed by several investigators (Cerde-Cuellar and others 2000, Wright and others 1993, Lee and others 1998). Most of these methods are expensive and time consuming, hence a reliable and versatile rapid, enumeration method is needed.

State of California has released emergency restriction on Gulf of Mexico oysters during April to October unless they undergo postharvest treatment to reduce *V. vulnificus* to a nondetectable level (<30 CFU/g). This will cause financial losses to Gulf Coast sea food industry. The ISSC recommends routine monitoring of the summer oysters harvest and undergoing post-harvest treatment until oysters contain < 30 CFU/g (Interstate Shellfish Sanitation Conference 2003; Panicker and Bej 2005). A rapid enumeration method would alleviate any financial losses to Gulf Coast seafood industry by real-time monitoring of *V. vulnificus* levels. There by the treatment can be targeted to specific batches of oysters.

Vibrios have been shown to express flagellar (H) antigens unique to each species (Tassin and others 1983). Predicated on this knowledge, anti-H antibodies were produced that are specifically reacted with *V. vulnificus* (Simonson and Siebeling, 1986). A direct colony immunoblot for *V. vulnificus* utilizing the specific anti-H antibodies would not only be less expensive than the DNA probe method but would also reduce assay time.

The major objectives of this research project were to develop direct colony immunoblot (DCI) for *V. vulnificus* using anti-flagellar core (H) antibodies, optimize and identify the specificity of the DCI, adapt the DCI for detection of *V. vulnificus* in mixed *Vibrio* cultures on selective plates, identify detection limit for the DCI procedure, compare the DCI with the FDA approved DNAH method and compare DCI with DNAH & MPN methods using postharvest Gulf Coast oysters.

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CHAPTER 2. LITERATURE REVIEW

2.1 History and the General Information of *Vibrio* Species

Genus *Vibrio* can be defined as Gram-negative motile rods that are mesophilic and chemoorganotrophic. They have a facultative fermentative metabolism, and found in aquatic habitats (Bergey's Manual of Determinative Bacteriology 1994; Thompson and others 2004). The first *Vibrio* species to be discovered was *Vibrio cholerae* and it was first discovered by an Italian physician named Filippo Pacini in 1854. He examined internal mucosal fluid of fatal patients through a microscope and identified *V. cholerae* cells among all of them (Thompson and others 2004). Currently seventy six species of *Vibrio* members has been identified (McGarey 1984; Janda and others 1988). Out of those at least twelve of them are pathogenic to human. These are *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, *V. alginolyticus*, *V. damsela*, *V. fluvialis*, *V. furnissii*, *V. hillisae*, *V. metschnikovii* and *V. mimicus* (Pruzzo and others 2005). Mainly five species from the above list have considerable effect on causing illnesses in humans. The three major human pathogenic species are *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Thompson and others 2004; CDC 1999). The other two species are *V. mimicus*, and *V. hollisae* (Brelvi and others 1999).

Vibrio cholerae is the major pathogen in developing countries as a result of poor water supplies and sanitation. The World Health Organization (WHO) reported that around 11,399 world wide deaths are caused by Cholera during 2000 to 2002 (Thompson and others 2004).

Center for Disease Control and Prevention (CDC) estimates that approximately 76 million illnesses, 325,000 hospitalizations, 5200 deaths of foodborne infection, and 8000 *Vibrio* infections occur annually in the United States (Ho and others 2007). According to the CDC reports 27% of *Vibrio* illness were recorded from Gulf Coast states, 44% from Pacific Coast states, 21% from Atlantic Coast states (excluding Florida), and 8% from inland states in 2004 .

The recorded *V. vulnificus* infections in 2004 Gulf Coast states were 47%, *V. parahaemolyticus* (21%), and non-toxicogenic *V. cholerae* (9%). In the Non-Gulf Coast States were *V. parahaemolyticus* (61%), *V. alginolyticus* (11%), *V. vulnificus* (9%), and non-toxicogenic *V. cholerae* (7%) (CDC 2004).

2.2 General Information of *V. vulnificus*

Vibrio vulnificus can be identified as lactose positive curved or straight *Vibrio* species. Out of the *Vibrio* genus, *V. vulnificus* is the major cause of sea food deaths in United States (Hlady and others 1993). *V. vulnificus* infections are not limited to US and it is common among other countries such as Israel, Denmark, Germany, Spain, Sweden, Japan, South Korea, Singapore, Thailand, Indonesia, and Taiwan (Bisharat 2005). Although it was first reported as a foodborne pathogen associated with eating raw oysters in 1979, Hippocrates reported similar infections in fifth century B.C. He was reporting the death of the king of the island named Taos. The day before he died, he had septicemia with swollen foot with red and black lesions (Blake and others 1979; Bisharat 2002). *Vibrio vulnificus* has been responsible for 95 % of all seafood related deaths in the US (Oliver 1995; McCann 2006; Mitra and others 2004).

Vibrio vulnificus bacteria prefer warm temperature environments such as 86°F to 95°F for with lower salinity levels ranging from 7-16‰ for their reproduction. Generally Gulf of Mexico, North Atlantic, Pacific, and Mid-Atlantic have typical conditions during summer months. Although highest concentration of *V. vulnificus* can be seen in summer months, small number of bacterial concentration can be seen in winter months as well as relatively cooler environments such as New England Coast. The *V. vulnificus* in Gulf Coast oyster meats has shown direct relation ship with temperature (Figure 1). The highest number of *V. vulnificus* growth can be seen in 26 - 30 °C (Motes and others 1998; Kelly 1982; Calero 2003).

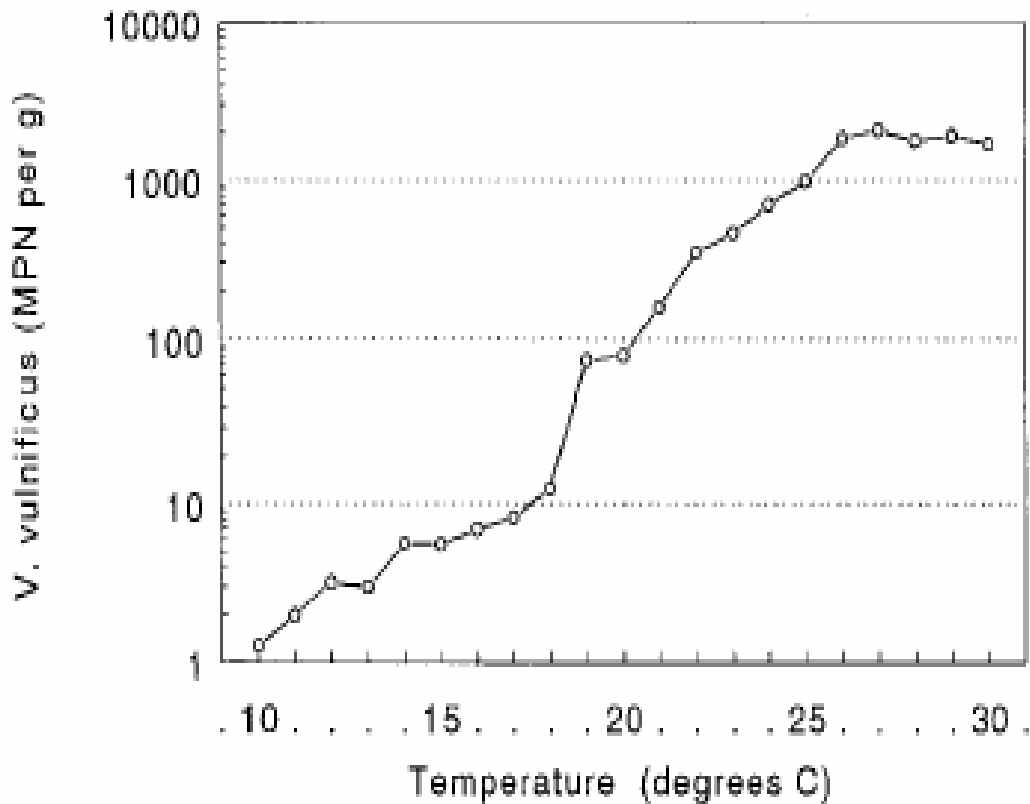


Figure 1. Influence of water temperature on the concentration of *V. vulnificus* in Gulf Coast oyster meats (Motes and others 1998).

Filter feeding mollusks, especially oysters, concentrate *V. vulnificus* in their gut and tissues, and can have about 10^3 to 10^4 *V. vulnificus* cells/g during the warmer months of May through October. In cold months, *V. vulnificus* counts are reduced to 10 cells /g (Figure 2). The *V. vulnificus* growth depend on some other factors such as salinity and their biological factors (Depaola and others 1998; Cook and others 2002; McCann 2006).

2.3 Taxonomy

There are three biotypes of the *V. vulnificus* which have been identified. The biotype 1 is associated with human diseases. It is first identified by CDC in 1976 and named as lactose positive *Vibrio* species.

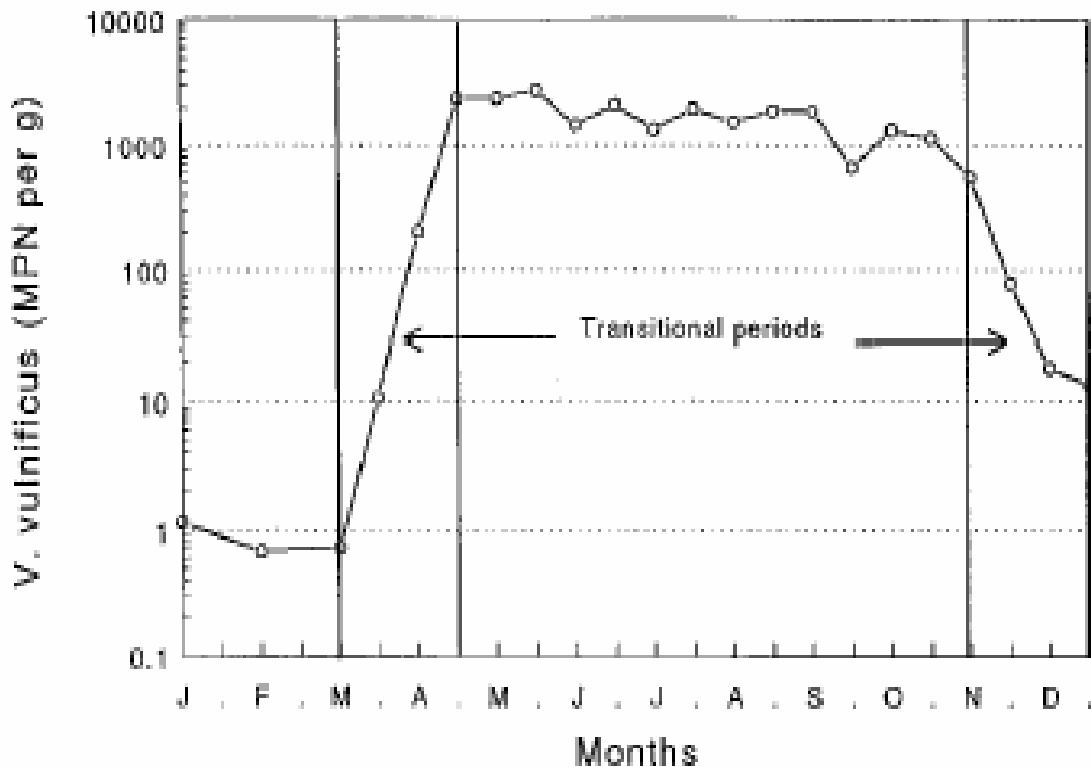


Figure 2. Seasonal distribution of *V. vulnificus* in Gulf Coast oysters (Motes and others 1998).

This is the most common bio type and r-RNA type B only found in Bio type 1. Biotype 2 mainly cause Eel Vibriosis and it was first reported in 1982 and the largest out break reported in Europe in 1991 (Tison 1982). It is possible to differentiate this biotype from biotype 1 strain by negative indole reaction and ornithine decarboxylase reaction. Generally the biotype 2 not known as human pathogen but it can be known as sporadic and opportunistic human pathogen (Bisharat 2002). The r-RNA A can be seen in both biotype1 and the biotype 2. Biotype 3 was reported in 1999 on Israel associated with wound infection in contact with Tilapia fish. Hence it is known as “Israeli biotype of *V. vulnificus*” (Bisharat 2002; Amaro and others 1996; Colodner and others 2004). This bio type shows phenotypic relationship to biotype 1.

2.4 Virulence Factors

The major four virulence factors are lipopolysaccharide (LPS), a capsular polysaccharide (CPS) and toxins such as metalloprotease and cytolysin. The LPS cause septic shock with pyrogenic reaction, which is in charge of potentially fatal hypertension. CPS makes macrophages against phagocytosis to innate immunity and serum bacterial activity. *Vibrio vulnificus* produce metalloprotease as an extra cellular enzyme it shows elastolytic and collagenic activity. The cytolysin enzyme increase intracellular cyclic GMP which leads to damage endothelial cells in the body (McCann 2006).

2.5 Infectious Dose

The office of Public Health, Louisiana Dept of Health and Hospitals reported the infectious dosage of *V. vulnificus* is in the range of 2000 CFU (Infectious Disease Epidemiology Section, 2006). However, less than 100 total organisms can cause illness in high-risk consumers. In April 2003, California banned the import of raw oysters from the Gulf of Mexico between April and October unless the oysters were treated to reduce *V. vulnificus* to undetectable levels. FDA recommended Gulf Coast oysters must be treated after harvest to reduce *V. vulnificus* to <30 CFU per gram of oyster meat During Summer months. (McCann 2006).

2.6 Target Population

Generally healthy individuals do not get infections, ingestion of the organism by individuals with immune-compromising conditions such as diabetes, cirrhosis, leukemia, lung carcinoma, acquired immune deficiency syndrome (AIDS), AIDS- related complex (ARC), or asthma requiring the use of steroids may cause "primary septicemia" (FDA 2006).

There have been no recorded major outbreaks associated with *V. vulnificus* but sporadic cases reported during warmer summer months. Out of all *V. vulnificus* victims 85% are males and 77% of them are white population. The mean and median age limits for the victims are 53. Generally we can say that it has major effect on males who older than 53 years (Leonard 2003).

2.7 Illnesses Caused by *V. vulnificus*

The major three human clinical illnesses are wound infections, gastroenteritis, or a syndrome known as "primary septicemia." The percentage of *V. vulnificus* infections are shown in figure 3.

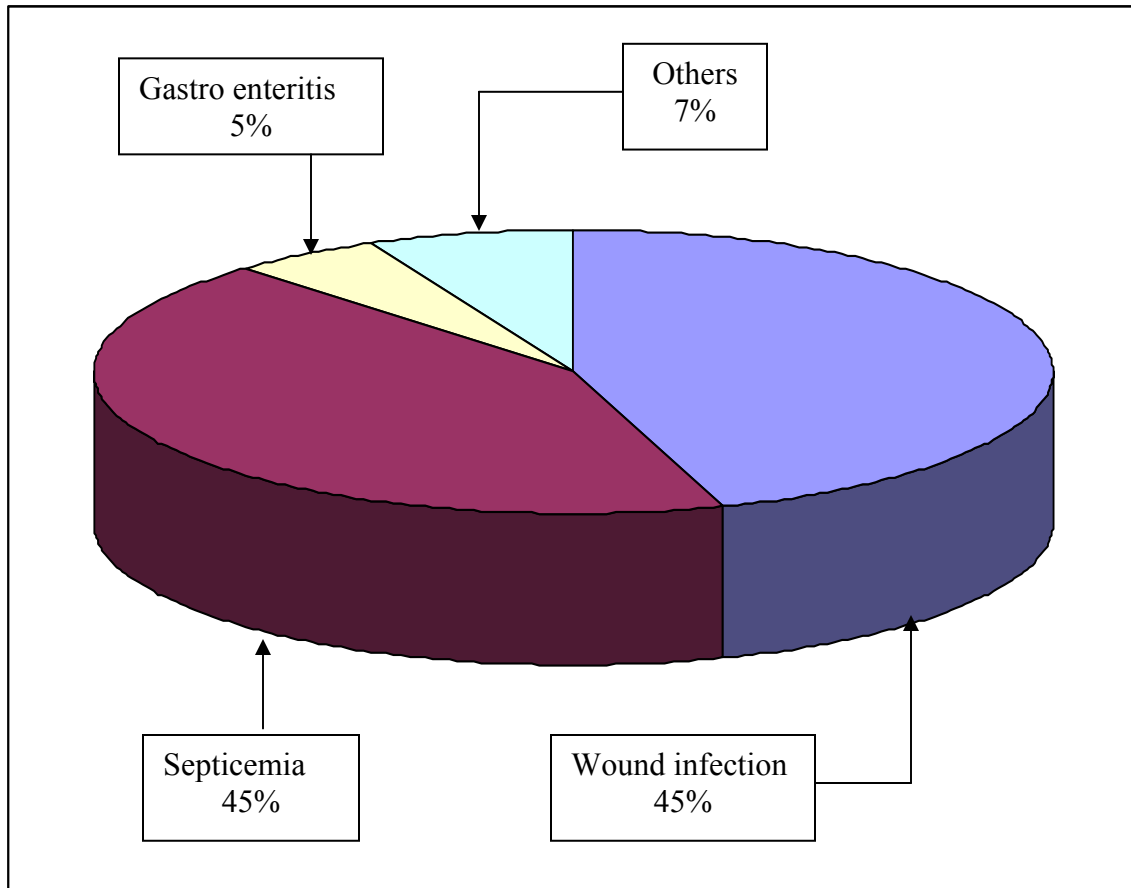


Figure 3. The percentages of *V. vulnificus* infection in Louisiana (Louisiana Dept of Health & Hospitals 2005)

2.7.1 Primary Septicemia

Primary septicemia is the most momentous illness of *V. vulnificus* and the fatality rates of infections are 50- 60% mainly due to under cooked oysters. The symptoms occurred in immunocompromised patients within 36 hours of ingestion. LPS endotoxin in *V. vulnificus* is the major virulence factor for the disease. The immense numbers of cases occur in males over the age 53. It has been discovered the major female hormone estrogen has an effect on LPS endotoxin and help to prevent action of the toxin (Merkel and others 2001). The most commonly reported symptoms are fever, nausea and hypertension. The primary septicemia leads to secondary lesions filled with fluid (Ching and Chuannng 2003).

2.7.2 Wound Infections

Vibrio vulnificus has ability to enter to the body through a pre-existing wound. It is usually started as redness and swelling with the pain at the site of the wound. The illness will progressively affect the whole body and it is fatal about 20% of the time, to prevent death destructive surgical treatments are needed. The symptoms begin within 24 hours of the incubation (CDC 2006).

2.7.3 Gastroenteritis

This is the mildest human syndrome caused by *V. vulnificus*. Healthy individuals usually do not suffer from the other diseases but may suffer from light gastroenteritis. If they do get the illness, the symptoms occur within 16 hours of ingesting the organism. Many cases of *Vibrio*-associated gastroenteritis are under recognized in clinical laboratories due to lack of the proper identification methods (Daniels and others 2000). Therefore rapid identification and enumeration of *V. vulnificus* from oysters are very desirable. A history of alcohol abuse and routine usage of antacid may also act as factor for gastroenteritis (Jonston and others 1986).

Symptoms of *V. vulnificus* infection for the people with immunocompromising conditions may occur within two days. In addition to these three major diseases, *V. vulnificus* has been associated with other clinical syndromes, such as pneumonia, osteomyelitis, spontaneous bacterial peritonitis, eye infections, and meningitis (Daniels and others 2000).

2.8 Identification Methods of *V. vulnificus*

Current FDA recommended identification methods are Most Probable Number (MPN), DNA probed colony hybridization (DNAH/ VVAP) and Polymerase Chain Reactions (PCR). The MPN method is a more labor and time consuming, with a variance of 70%. The DNA probed colony hybridization method based on the specific genes named cytolysin gene which is unique to the species of *V. vulnificus* (DePaola and others 1997). FDA recommended PCR methods also based on targeting the *V. vulnificus* cytotoxin/hemolysin (Hill and others 1991). However, PCR method has several constrains. The first one is lack of reliability of the results at 10^2 CFU/g unless the spiked oyster homogenate had been incubated for 24 hours. The second one detects dead and viable but non-culturable *V. vulnificus* cells (Brasher and others 1998). It is highly advantages to have a rapid identification method to over come these difficulties.

Species in the genus *Vibrio* exhibit flagellar (H) antigens unique to the species. This was first reported by Gardner and Venkatraman (Tassin and others 1983). The anti- *V. vulnificus* H antibodies exhibited species specificity and *V. vulnificus* specific polyclonal antibodies have been developed for the flagellar core protein (Simonson and others 1986). These anti-H antibodies were used to construct coagglutination reagents which agglutinated *V. vulnificus* cells within 1-2 minutes. Among 19 *Vibrio* species examined including 723 *V. cholerae*, *V. mimicus*, *V. parahaemolyticus*, and *V. fluvialis* isolates recovered from seafood and the marine

environment and none of them were agglutinated. Of 435 *V. vulnificus* isolates identified bacteriologically, 432 (99.3%) coagulated with the anti-*V. vulnificus* H reagent.

Several studies have used a anti-H reagent direct colony immunoblot assay to identify several bacteria including *Mycoplasma*, *Salmonella*, *Listeria* and *Streptococcus* (Bhunia and others 1992; Bhunia and others 1991; DeSoet and others 1990; Petter and others, 1993; Rosengarten and others 1996).

2.9 The Direct Colony Immunoassay Methods for *Escherichia coli*

A direct screening immunoblot assay was developed by Hull and others in 1993 for the detection of Shiga-like toxin (SLT)-producing *E. coli* organisms in stool samples using monoclonal antibody. This method was able to distinguish non-toxin-producing *E. coli* from normal stool flora in ratios of 1:1,000 to 1:5,000 with high level of sensitivity and specificity (Hull and others 1993).

Another method was developed by Szakai and others to detect *E. coli* and *Shigella* in water samples using a colony blot immunoassay. In this method samples were filtered through nitrocellulose membranes and filter was tested with a monoclonal antibody. This method also had high sensitivity and the specificity (Szakai and others 2001).

In 2005 the tissue printing on apple was developed by Janes and others. In here infected apple tissue was removed and washed with methanol. It was blocked with Bovine serum albumin reacted with H7 polyclonal goat conjugated horseradish peroxidase and color development solution was added. The colored *E. coli* was clearly visible through the electron microscope (Janes and others 2005).

There is a possibility to develop rapid identification method using direct colony immunoblot method. The objective of this research project was to develop a DCI using the anti-

V. vulnificus H regent. The time for identification of *V. vulnificus* could be shortened by direct colony immunoblot assays.

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**CHAPTER 3. DEVELOPMENT OF DIRECT COLONY IMMUNOBLOT FOR
ENUMERATION OF *V. VULNIFICUS* USING ANTI-FLAGELLAR CORE (H)
ANTIBODIES**

3.1 Introduction

Vibrio vulnificus is omnipresent in the marine environment where it can be isolated from water, sediment and shellfish (Kelly 1982; Oliver and others 1983). It is postulated that less than 100 virulent cells can cause disease in high-risk individuals and the pathogen is responsible for 95% of all seafood related deaths (Oliver 1995; McCann 2006; Mitra and others 2004). During warmer months in the Gulf of Mexico, levels of *V. vulnificus* can often reach 10^3 to 10^4 most probable number per gram (MPN/g) of oyster meat (Motes and others 1998; Cook and others 2002). Due to the rapid progression and high mortality rates of *V. vulnificus* infection in humans, especially those with underlying chronic disease (liver disease, alcoholism, diabetes, gastric disorders, cancer, AIDS/HIV, hemochromatosis/hemolytic anemia and chronic renal failure), oysters are being examined for the presence and level of this pathogen. Conventional detection and enumeration of *V. vulnificus* in oysters by the MPN method (Kaysner and DePaola 2004) can be overwhelming in logistics, material and time, while the expeditious and specific identification of *V. vulnificus* in the laboratory is desirable.

To save assay time and materials, the Food and Drug Administration (FDA) has adopted a direct plating/DNA hybridization method using a non-radioactive labeled probe which targets the cytotoxin gene in *V. vulnificus* (Kaysner and DePaola 2004). This enumeration method is specific and has been shown to be equivalent to the MPN procedure (Wright and others 1993; DePaola and others 1997). However, the method requires expensive reagents, 24-34 hours to perform and often it can be difficult to differentiate between positive and negative colonies at lower hybridization temperatures (50 °C) (Wright and others 1992).

Many vibrios have been shown to express flagellar (H) antigens unique to the species (Miwatani and Shinoda 1971; Bhattacharyya 1975; Tassin and others 1983). Based on this

knowledge, anti-*V. vulnificus* H antibodies were developed and used to construct a coagglutination reagent that agglutinated *V. vulnificus* cells within 1-2 min (Simonson and Siebeling 1986). The anti-H antibodies exhibited species specificity in that, other than *V. pelagius*, only *V. vulnificus* cells were coagglutinated from among 19 *Vibrio* spp. examined including 723 isolate of *V. cholerae*, *V. mimicus*, *V. parahaemolyticus* and *V. fluvialis* recovered from seafood and the marine environment. However, since colonies were transferred from selective plates and grown overnight on slants, the total *V. vulnificus* isolation and identification time was about 2-3 days depending on whether an enrichment or direct plating was employed.

To further shorten the identification time and facilitate *V. vulnificus* detection, a direct colony immunoblot (DCI) using anti-H antibodies could be employed. Direct colony immunoblots have been successfully used to identify a variety of bacteria including *Salmonella*, *Listeria*, *E. coli*, *shigella* and *Streptococcus* (Petter 1993; Bhunia and Johnson 1992; Bhunia and others 1991; DeSoet and others 1990; Szakai and Pal 2001). For these assays, colonies were transferred directly from an agar plate to a membrane where they were detected using specific antibodies and antibody-enzyme conjugates. Like the direct plating/DNA hybridization method, this result in the direct enumeration of specific bacteria with a single, simple and rapid assay based on anti H antibodies. A DCI for *V. vulnificus* would further reduce assay time and would be inexpensive, since the only equipment required would be a shaker. The method would also be simple in that it would require no cell lysis, less time and manipulations, and no stringent temperature requirements.

The objective of this investigation was to examine the possibility of using a DCI to establish a rapid, less expensive yet user-friendly method equivalent to the direct plating/DNA hybridization procedure for the detection and enumeration of *V. vulnificus* in oysters.

3.2 Materials and Methods

3.2.1 Media

Phosphate Buffer Solution (PBS) consisted of 2.4 g of sodium phosphate monobasic anhydrous, 2.84 g of sodium phosphate dibasic and 8.5 g (0.85%) of NaCl and 1L of distilled water. The pH was adjusted to 7.2-7.4 and the broth was autoclaved for 15 min at 121°C. Tween twenty (0.5% T-20) contained 0.5 ml of Tween twenty dissolved in 1L of PBS. Triton X-100 contained 0.5 ml Triton X-100 dissolved in 1L of PBS. Tris buffer solution was prepared by adding 0.788g of Tris in hydrochloric acid to 90 ml of distilled water, the pH was adjusted to 7.6 with sodium hydroxide solution and water was added up to 100 ml. Bovine serum Albumin (1% BSA) 1g was dissolved in to 100 ml of PBS. Alkaline Peptone water (APW) was prepared by dissolving 10g of peptone and 10g of NaCl in 1L of distilled water and the pH was adjusted to 8.2. All solutions were autoclaved for 15 min at 121°C. Thiosulfate-Citrate-Bile-Sucrose (TCBS) agar consisted of 89g of TCBS powder (Troy Biologicals Inc. Troy, MI) and 1L of distilled water. Modified Cellobiose- Polymyxin B-Colistin (mCPC) Agar, *Vibrio vulnificus* agar (VVA) was prepared according to online U.S. Food & Drug Administration Bacteriological Analytical Manual (BAM 2001).

3.2.2 Bacterial Cultures

The following clinical isolates tested were obtained from Louisiana Department of Health and Hospitals (LDHH) and the American Type Culture Collection (ATCC) and environmental strains were isolated from either oysters or sea water at Louisiana State University (LSU) and North Carolina State University (NC state).

Stock cultures were stored at -70°C and subcultures were maintained at room temperature (27 °C) on agar deeps.

Table 1. Different *Vibrio* species used

Species	Culture Number	Type of strain	Source ^a
<i>V. alginolyticus</i>	ATCC [®] 33787	Clinical	ATCC
<i>V. cholera</i>	ATCC [®] 14035	Clinical	ATCC
<i>V. damsela</i>	ATCC [®] 35083	Clinical	ATCC
<i>V. fluvialis</i>	ATCC [®] 33809	Clinical	ATCC
<i>V. mimicus</i>	ATCC [®] 33653	Clinical	ATCC
<i>Vibrio parahaemolyticus</i>	ATCC [®] 17802	Clinical	ATCC
<i>Vibrio vulnificus</i>	Vv 1001	Clinical	LDHH
<i>Vibrio vulnificus</i>	Vv 1002	Clinical	LDHH
<i>Vibrio vulnificus</i>	Vv 1004	Clinical	LDHH
<i>Vibrio vulnificus</i>	Vv 1005	Clinical	LDHH
<i>Vibrio vulnificus</i>	Vv 1006	Clinical	LDHH
<i>Vibrio vulnificus</i>	Vv 1007	Clinical	LDHH
<i>Vibrio vulnificus</i>	Vv 1009	Clinical	LDHH
<i>Vibrio vulnificus</i>	ATCC [®] 27562	Clinical	ATCC
<i>Vibrio vulnificus</i>	C7184	Clinical	ATCC
<i>Vibrio vulnificus</i>	LSU0106VV12 (EN1)	Environmental	LSU
<i>Vibrio vulnificus</i>	LSU0106VV14 (EN2)	Environmental	LSU
<i>Vibrio vulnificus</i>	LSU0541VV49C (EN3)	Environmental	LSU
<i>Vibrio vulnificus</i>	515 4C2 (EN4)	Environmental	LSU
<i>Vibrio vulnificus</i>	WR1 (EN5)	Environmental	NC state

^a ATCC - American Type Culture Collection; LDHH - Louisiana Department of Health and Hospitals; LSU- Louisiana State University ; NC state -North Carolina State University.

Agar deeps contained 8g tryptone, 22.5 g NaCl, 4g nutrient broth, 4g agar, 4g KCl and 4g MgCl₂.6H₂O per liter of distilled water. Cultures were transferred monthly to maintain viability.

3.2.3 Specificity

Vibrio alginolyticus, *V. cholera*, *V. parahaemolyticus*, *V. damsela*, *V. mimicus*, *V. fluvialis* and several clinical and environmental strains of *V. vulnificus* were spotted onto *Vibrio vulnificus* agar (VVA), thiosulfate citrate bile salts sucrose (TCBS) agar, and modified cellobiose polymyxin colistin (mCPC) agar. The plates were incubated for 16 h at 35 ± 2 °C for VVA and 37 ± 2 °C for both TCBS and m-CPC and direct colony immunoblots were then performed .

3.2.4 Flagellar Core Purification

Flagellar cores were isolated from a motile strain of *V. vulnificus* by methods described previously (Simonson and Siebeling 1986). Briefly, the bacterial cells were propagated on Brain heart infusion agar supplemented with 1.5% NaCl. The cells were harvested from the agar surface in 0.15M NaCl and were homogenized for 90 sec in a Waring blender at a medium speed. The bacterial cells were sedimented by centrifugation at 10,000 X g for 10 min and the sheared flagella were obtained by centrifugation of the remaining supernatant fluid at 30,000 X g for 2 hr. The flagella were suspended in 0.1 M Tris buffer, pH 7.8, containing 0.1 mM EDTA, 1 % Triton X-100 and 0.001% thimerosal and the differential centrifugation cycle was repeated 3 times. The H cores were further isolated from residual cellular debris by cesium chloride density ultracentrifugation at 64,000 X g for 18 h. The flagella were examined by electron microscopy to verify that flagellar cores, free of sheath material, were present. The purified cores were stored at 4°C in Tris buffer containing EDTA and thimerosal. Total protein was determined by the bicinchoninic acid (BCA) method (Sigma Chemical Co; BCA Application Note #12 1990).

3.2.5 Production of Anti-H Serum

A New Zealand White rabbit was immunized by subcutaneous injection of 125 µg of core protein followed by alternating intravenous injections of 60 µg protein and subcutaneous injections of 125 µg protein at 3-day intervals over a 40-day period. The rabbit was exsanguinated 45 days after the initial injection, and the *V. vulnificus* H anti-serum was harvested and stored frozen in 5 ml aliquots. Each of this 5ml aliquots sub divided in to several 100µl, 20µl, and 10µl and stored them until use.

3.2.6 Direct Colony Immunoblot

Colonies were directly lifted from incubated *V. vulnificus* agar (VVA) plates to polyvinylidene fluoride membranes and air dried for 10 min. Using forceps, the membranes were placed in separate Petri dishes and washed with gentle agitation at room temperature (27 °C) on a shaker in 20 ml 0.067 M phosphate buffered saline, pH 7.25 containing 0.05% Tween 20 (PBS T-20) for 10 min. The membranes were moved to clean Petri dishes and incubated with 1% bovine serum albumin in PBS for 30 min to block non-specific binding sites. The membranes were washed once and treated with 20 ml rabbit anti-H *V. vulnificus* serum diluted 1:2000 in PBS for 30 min. Unbound antibody was removed by washing four times in PBS T-20 and the membranes were then incubated with 20 ml peroxidase-conjugated goat anti-rabbit IgG diluted 1:2000 (Sigma Chemical Co.) for 30 min. The membranes were again washed four times and a color development solution was added (20 ml 0.05M Tris (pH 7.6), 10 mg 3, 3'-diaminobenzidine tetrahydrochloride, 1 ml 8% NiCl₂ and 0.1 ml 30% H₂O₂) for 5 min. To minimize the effect of the washing chemical and to identify the best washing solution , the washing solution 0.05% Tween 20 (PBS T-20) was replaced by 0.05% Triton X-100.

3.2.7 DNA Probe Hybridization

Colonies were directly transferred from incubated VVA plates to Whatman #541 filter paper disks and *V. vulnificus* was identified by DNA hybridization using an alkaline phosphatase-labeled probe specific for the cytolysin gene (FDA BAM 2001). Briefly, after lifting, colonies on the filter paper were lysed by placing the colony side up in a petri dish containing 1ml of lysis solution (0.5M NaOH and 1.5M NaCl) and using heating on microwave (1000 watts or less) for 30 sec/filter. The filter was neutralized with ammonium acetate solution by swirling on the orbital shaker. Filters were then washed with 1X standard saline citrate (1XSSC) solution and incubated at 42 °C for 30 min with stock Pro K with 10 ml of 1XSSC. Then it was rinsed three times with 10ml of 1XSSC at room temperature (27 °C), and the membranes, along with 10 ml of hybridizing buffer (0.5 g BSA, 1.0g Sodium Dodecyl sulfate (SDS), 0.5 Polyvinylpyrrolidone and 100ml 5XSSC) were placed in to Whirlpack bags and incubated at 55°C for 30 min. The membranes were next placed into 10 ml of pre-warmed hybridizing buffer and 5 pica moles of DNA probe (vvhA probe; 5'Xga gct gtc acg gca gtt gga acc 3') and incubated on 55°C for 1 hr. The membranes were washed with 1XSSC / SDS (10g SDS and 1L 1XSSC) solution at 55°C for 10 min and washed again five times with 1xSSC and color development (NBT/BCIP ready to use tablet and 20 ml d water; Boehinger Mannheim, Cat. No. 1697471) solution was added. Color development was checked with control strips and the reaction was terminated by washing with distilled water.

3.2.8 Enumeration of *V. vulnificus* in Mixed Cultures

Vibrio vulnificus and *V. parahaemolyticus* were grown separately in 10 ml of tryptic soy broth containing 3% NaCl (T₁N₃) overnight at 37°C. Ten µl of the overnight culture was transferred to 10 ml of T1N3 and incubated at 37 °C for 16 hours. The bacterial cells were

collected by centrifugation, washed once with PBS, pH 7.3 and were resuspended in 10 ml PBS. Serial ten fold dilutions of *V. vulnificus* and *V. parahaemolyticus* were made separately in PBS. Ten μ l of each dilution of *V. vulnificus* and *V. parahaemolyticus* were mixed with 80 μ l of PBS and the combined cultures were then spread on VVA plates. The plates were incubated at 35 °C for 16 h and the number of colony forming units (CFU) for *V. vulnificus* and *V. parahaemolyticus* was determined. The direct colony immunoblot was immediately followed by DNA probe hybridization on each mixed culture plate as described above. The concentration of the vibrios in the initial pure cultures was determined by plating aliquots of the *V. vulnificus* and *V. parahaemolyticus* serial dilutions on VVA.

3.2.9 Enumeration of *V. vulnificus* in Spiked Oyster Homoginate

Oysters were collected between Januarys to July 2006 from the Gulf of Mexico. Upon arrival at the laboratory, the oyster shells were scrubbed under running faucet water to remove debris and attached algae and the oysters were kept in a freezer for two days to reduce naturally occurring bacteria. The oysters were opened aseptically using sterilized oyster knives. Serial dilutions of a 16 hr *V. vulnificus* culture were made as described above. Ten ml from each 10^{-1} to 10^{-5} dilution were mixed with 40 ml of alkaline peptone water (APW) and 50 g of oyster meat and the solution was homogenized in a stomacher for 1 to 2 min. One oyster aliquot (50 g) was mixed with 50 ml of APW as a negative control. Serial 10-fold dilutions in PBS were made from each spiked oyster homogenate and 100 μ l aliquots were plated on to VVA plates. The plates were incubated 16 h at 35 °C and colonies were counted. *Vibrio vulnificus* was enumerated directly from each VVA plate by the colony immunoblot followed by DNA probe hybridization as described above.

3.2.10 Statistical Analysis

Both the direct colony immunoblot method and the DNA probe method were analyzed by statistical comparisons of all pairs using one Student's t test following 1-way analysis of variance (ANOVA) (JUMP In version 4.0.3, SAS Institute Inc., Cary, N.C., U.S.A.). Statistical significance occurs at $P < 0.05$. All experiments were repeated 3 times with 2 replications per experiment.

3.3 Results and Discussion

3.3.1 Optimization of the DCI Method

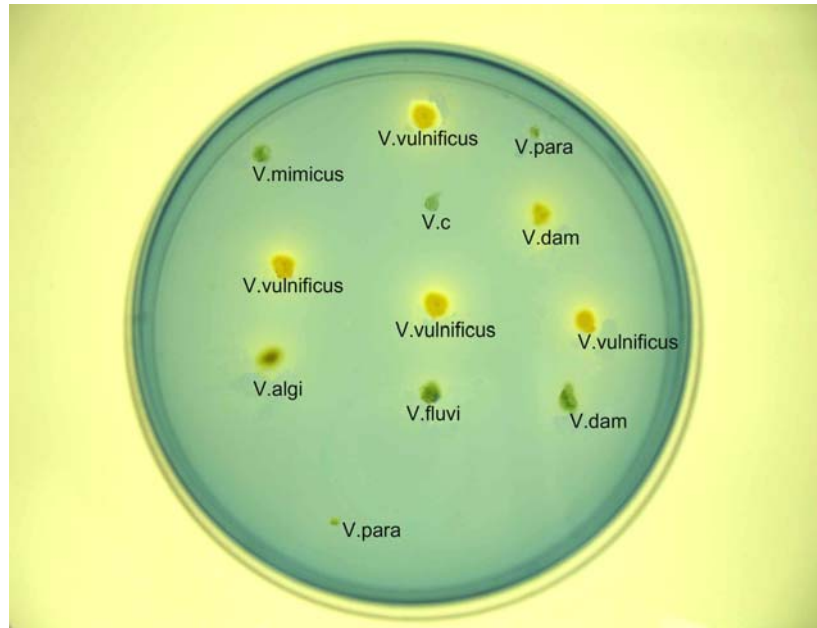
In this study, a DCI rapid enumeration method for detection of *V. vulnificus* was developed that could be completed in 3 ½ hours. To determine which media was the best for detecting *V. vulnificus* by the DCI method the following media was used; VVA, TCBS and m-CPC. It was determined that the most effective media for detection of *V. vulnificus* by the DCI method was VVA, because the colonies on those plate produced less capsule polysaccharide than TCBS and m-CPC plates. The membranes from the TCBS and m-CPC plates, during the washing steps whole clumps of colonies would have washed off of their surfaces due to the over production of capsule polysaccharide. This led to false negative counts. Furthermore, the FDA approved DNA hybridization (DNAH) protocol also recommended using VVA plates prior to hybridization because it helps to distinguished *V. vulnificus* from other bacteria by making bright yellow colonies with the yellow diffusion due to fermentation of D(+) cellobiose (Anonimus, 2002). During enumeration we cannot rely on this color reaction because of several reasons. One reason is some neighboring non *V. vulnificus* also can turn a yellow color; another reason is some other *Vibrio* species such as *V. fluvialis* strains and *V. damsela* can ferment D (+) cellobiose. The last reason is *V. vulnificus* biotype three can not ferment D (+) cellobiose.

The DCI using both washing solutions (T-20 and TX-100), correctly differentiated previously known *V. vulnificus* colonies from six other different *Vibrio* species with a dark purple color (Fig1) and 9 clinical strains and five environmental strains of *V. vulnificus* were positive by the direct colony immunoblot (Fig 2). However, *V. fluvialis* and *V. damsela* colonies exhibited slight purple color reactions, but the intensity of the color was negligible. This weak color reaction is negligible when compared to non-specific color development in DNAH method at lower hybridizing temperatures (Wright and others 1993). Even though good color development was observed by using TX-100 in the wash solution, the chemical damaged the polyvinylidene fluoride membranes. Thus, prevent membrane damage; T-20 instead of TX-100 was incorporated into the washing solution for all subjected studies.

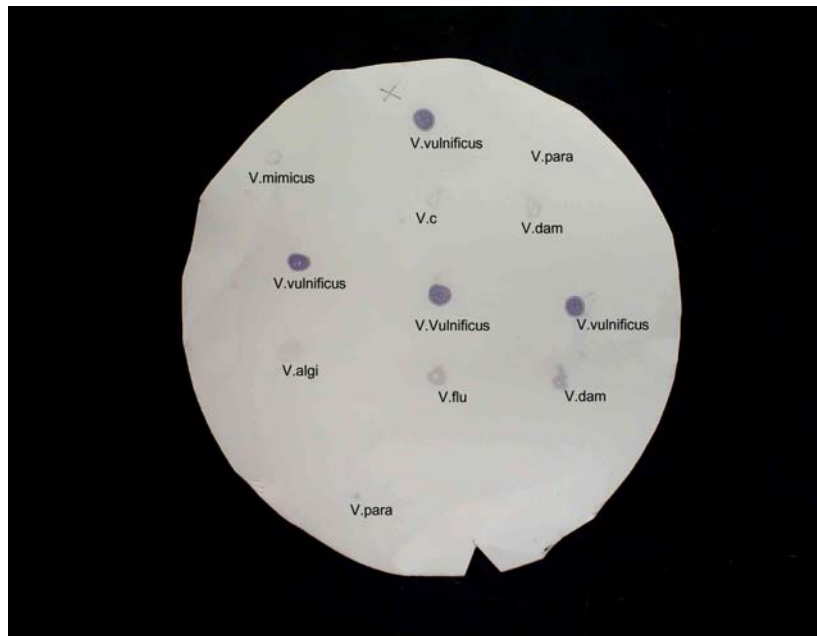
3.3.2 Enumeration of *V. vulnificus* in Mixed Cultures

We were able to enumerate *V. vulnificus* by the DCI and DNAH methods when *V. parahaemolyticus* densities were increased (Table 2). When *V. vulnificus* was mixed with different levels of *V. parahaemolyticus* and plated on to VVA plates, clear color difference could be seen in the colonies. *Vibrio vulnificus* colonies were yellow (cellobiose positive) in color and *V. parahaemolyticus* colonies were green in color (Fig 6 A). *Vibrio vulnificus* had a strong color development with both the DCI and DNAH methods. However, the color was more intense on the DCI than the DNAH method (Fig 6 BC). Furthermore *V. vulnificus* colonies were more closely differentiated from *V. parahaemolyticus* colonies using the DCI as opposed to DNAH. The DNAH has light brown color reaction on *V. parahaemolyticus* colonies (Fig 6 C).

When 4.50 log/ml of *V. parahaemolyticus* was mixed with either 2 log or 1 log of *V. vulnificus* the total number of cells could not be counted on the VVA plates due to over growth by *V. parahaemolyticus*. However *V. vulnificus* colonies could be detected and enumerate by the DCI and DNAH methods.



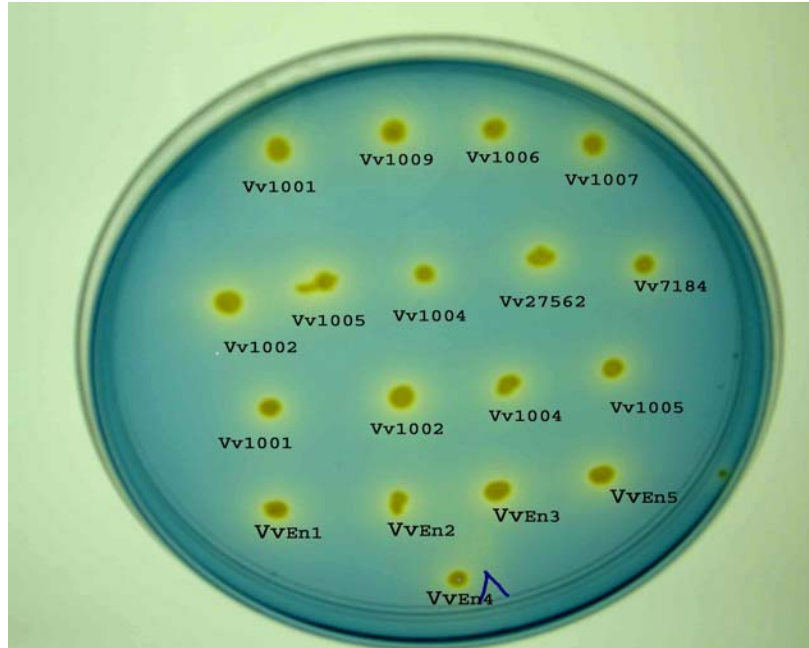
(A) VVA plate with four different *V. vulnificus* strains and other *Vibrio* species



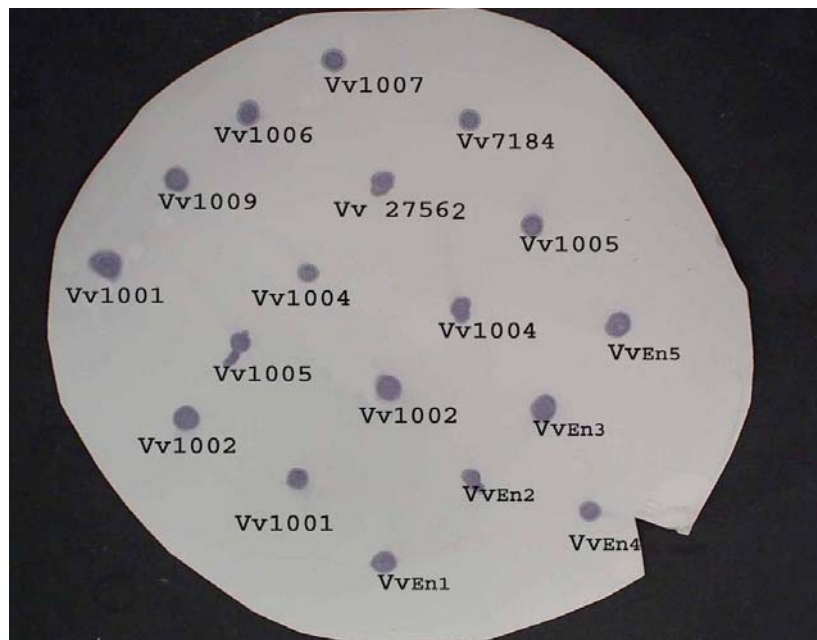
(B) Direct colony Immunoblot with different *Vibrio* species

Figure 4. Detection of *V. vulnificus* from other *Vibrio* species using Direct Colony Immunoblot method

The above bacterial cultures were *V. cholerae* (VC), *V. parahaemolyticus* V. Para), *V. mimicus*, *V. damsela* (V. dam), *V. alginolyticus* (V. algi), *V. fluvialis* (V. flu) and *V. vulnificus* 1002, 1004, 1005, and 1007.



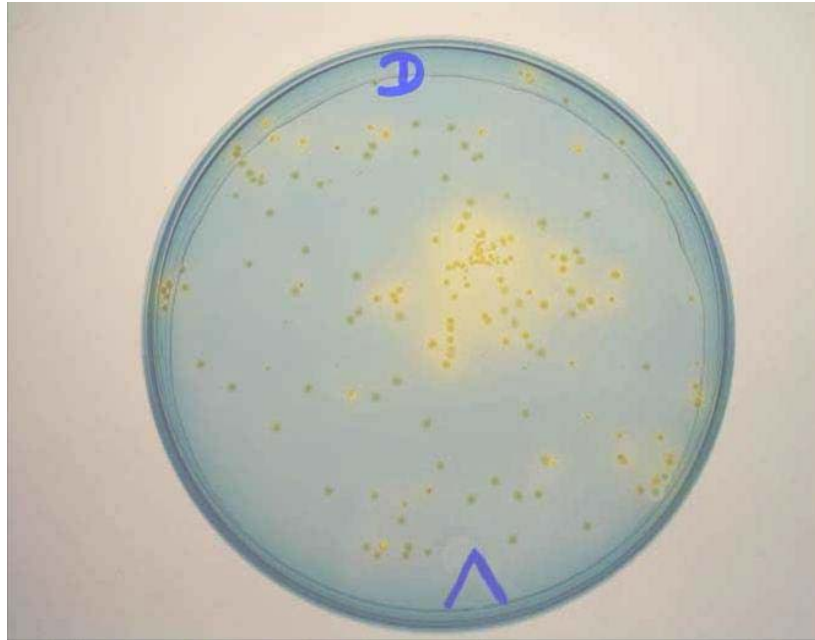
(A) VVA plate with different clinical and environmental strains of *V. vulnificus*



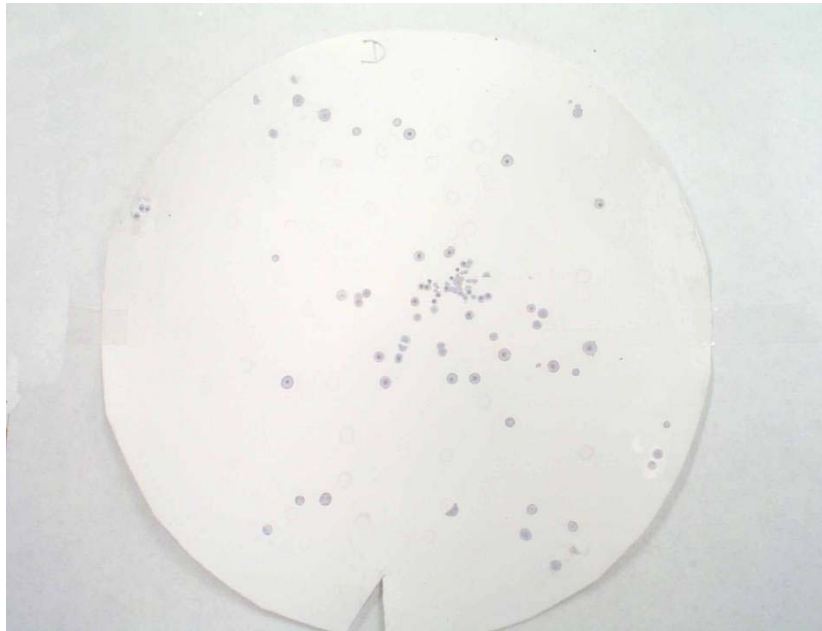
(B) Direct colony Immunoblot with different clinical and environmental strains of *V. vulnificus*

Figure 5. Detection of different strains of *V. vulnificus* using the Direct Colony Immunoblot method.

The Bacterial cultures Vv 1001, 1002,1004,1005,1006,1007,27562 and 7184 are *Vibrio vulnificus* clinical isolates and Vv En 1,2,3,4 and 5 are environmental isolates.

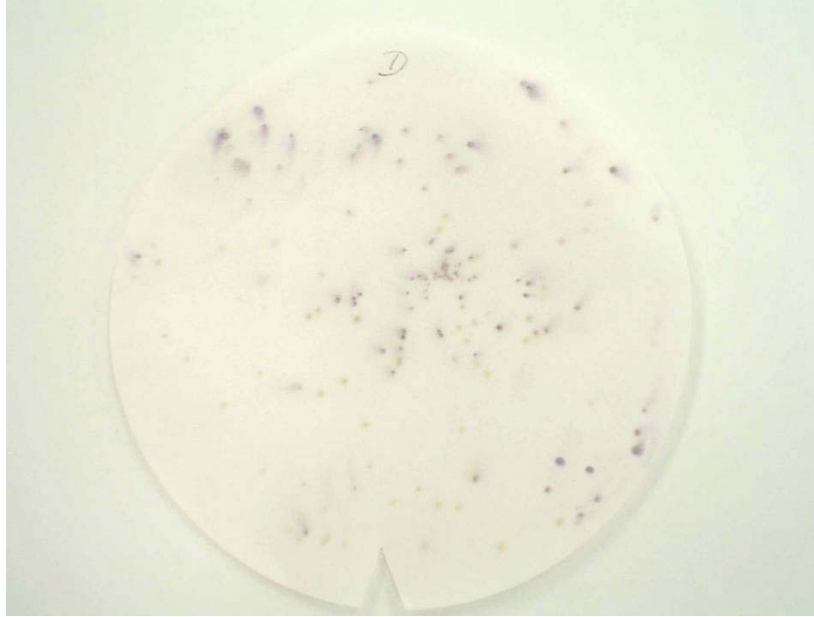


(A) VVA plate with mixed culture of *V. vulnificus* and *V. parahaemolyticus*



(B) DCI with mixed cultures of *V. vulnificus* and *V. parahaemolyticus*

Figure 6. Detection of *V. vulnificus* using Direct Colony Immunoblot and DNAH methods (Fig continued)



(C) DNAH with mixed cultures of *V. vulnificus* and *V. parahaemolyticus*

At the lower *V. parahaemolyticus* (0, 1.49, 2.27, or 3.55 log CFU/ml) the plate counts could be counted and the color difference could be detected on VVA plates. Using both DCI and DNAH, positive *V. vulnificus* colonies could be clearly identified. There were no significance differences among DCI and DNAH methods (Table 2).

3.3.3 Enumeration of *V. vulnificus* from Oysters

To further confirm the accuracy and specificity of the DCI, oyster homogenates were spiked with different levels of *Vibrio vulnificus* (2 to 6 log CFU/ml). The oysters were collected during January to June, and had about 2 log CFU/g of natural *V. vulnificus*. These natural total counts were relatively constant and the standard deviation among different samples was ± 0.15 (Table 3). When the *V. vulnificus* inoculum was low in the oyster homogenate, green and white colonies were observed on VVA plates. Some rapid identification methods, such as PCR, showed that oyster homogenate inhibitory interfered with PCR (Hill and others 1991). The oyster homogenate did not interfere with DCI. One to 200 colonies were accurately detected by DCI

and DNAH methods. Both methods were comparable and had no significant differences (Table 3).

Table 2. Enumeration of *V. vulnificus* by DCI and DNAH methods inoculated into different challenge levels of *V. parahaemolyticus*.

Number of cells in the sample		Enumeration of <i>V. vulnificus</i>		
<i>V. vulnificus</i>	<i>V. parahaemolyticus</i>	DCI ^a	DNAH ^a	
		log CFU/ml		
2 log	Unable to count	4.50 ± 0.41	2.02 ± 0.19 A	2.01 ± 0.17 A
	2.16	3.55 ± 0.40	2.14 ± 0.19 A	2.07 ± 0.28 A
	2.06	2.27 ± 0.16	2.10 ± 0.09 A	2.09 ± 0.09 A
	1.99	1.49 ± 0.40	2.17 ± 0.10 A	2.18 ± 0.16 A
	2.04	0	2.15 ± 0.21 A	2.10 ± 0.19 A
	1 log	Unable to count	4.50 ± 0.41	1.20 ± 0.15 B
	1.32	3.55 ± 0.40	1.29 ± 0.35 B	1.19 ± 0.24 B
	1.22	2.27 ± 0.16	1.16 ± 0.26 B	1.16 ± 0.26 B
	1.13	1.49 ± 0.40	1.17 ± 0.05 B	1.18 ± 0.03 B
	1.12	0	1.00 ± 0.24 B	0.97 ± 0.25 B

^a All analyses were based on three separate experiments with each mean ± standard deviation being average of three determinations. Mean with each vertical column followed by the same letter are not significantly different (P ≥ 0.05) from each other. Statistical comparisons of all pairs were analyzed using one –way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC)

Table 3. Enumeration of *V. vulnificus* by DCI and DNAH in seeded oyster slurry

Number of cells in the sample		Enumeration of <i>V. vulnificus</i>	
Inoculum ^b	Total counts ^c	Direct colony blot ^a	DNA probe ^a
		log CFU/ml	
6.37 ± 0.24	6.66 ± 0.45	6.52 ± 0.34 A	6.42 ± 0.32 A
5.55 ± 0.31	5.67 ± 0.30	5.34 ± 0.22 B	5.35 ± 0.22 B
4.08 ± 0.07	4.84 ± 0.25	4.56 ± 0.35 C	4.54 ± 0.27 C
3.00 ± 0.17	4.78 ± 0.58	3.77 ± 0.20 D	3.76 ± 0.17 D
1.79 ± 0.30	3.31 ± 0.26	2.65 ± 0.35 E	2.63 ± 0.33 E
0	2.08 ± 0.15	1.52 ± 0.29 F	1.58 ± 0.32 F

^a All analyses were based on three separate experiments with each mean ± standard deviation being average of three determinations. Mean with each vertical column followed by the same letter are not significantly different (P ≥ 0.05) from each other. Statistical comparisons of all pairs were analyzed using one –way analysis of variance (ANOVA)(SAS Institute Inc., Cary, NC)

^b Number of *V. vulnificus* added per milliliter of undiluted oyster homogenate

^c Number of total bacteria as determined by growth on VVA

Current identification methods used for enumeration of *V. vulnificus* are more time-consuming than the proposed DCI method. The FDA approved MPN method is labor-intensive and required selective media biochemical test kits for the conformation. This selective media biochemical test kits are expensive and inaccurate (Nishibuchit and Seidler 1985; Arias and others 1998) because it required tedious subculture of several isolates for verification with sufficient number of tests. Some biochemical tests are varying with the strains (Wright and others 1993) where as the DCI method does not vary with the strains.

DNA based rapid identification methods such as DNAH and polymerase chain reaction (PCR) have been recently developed by several investigators (Morris and others 1987, Wright and others 1993, Cerda-Cuellar and others 2000, Lee and others 1998, Wang and others 2003, Panickar and Bej 2005). Wang and others 2003 claimed that their newly developed real time quantitative taqman PCR was less time consuming than other conventional PCR related *V. vulnificus* estimating methods. This method takes 5 hours and 40 minutes (3hours and 40min for the serum separation and 2 hours for the PCR amplification) with additional 48 hours for incubation period of the plates.

The methods such as phosphatase-labeled probe hybridization and PCR methods required enrichment in alkaline peptone water. Enrichment involves competition with other, possibly faster growing organisms in a mixed population, and may result in overgrowth of unwanted bacteria (Wright 1993); where as the DCI method does not need an enrichment step.

Enumeration of *V. vulnificus* with the alkaline phosphatase-labeled oligonucleotide probe, and cytotoxin-hemolysin gene labeled probe showed exact correlation with identification based on the cytolsin genes (Wright and others 1993, Morris and others 1987). Both of above methods proved as species specific markers, but they are very expensive and complex methods.

Wright and others 1993 claimed that alkaline phosphatase- labeled oligonucleotide probe take less than 24 hours for the probing with 16 to 72 hours incubation of the pure cultures and the FDA recommended DNAH protocol also required minimum a 6-10 hours with 16 to 24 hours incubation of the cultures (Anonymous 2002).

The DCI method was completed in only 3 and ½ hours with 16 hours of incubation. So it is very unlikely that they could be used by the DCI method which is inexpensive, more accurate, less time consuming and oyster companies could frequently test their oysters.

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**CHAPTER 4. COMPARISON OF THE DIRECT COLONY IMMUNOBLOT TO DNA
HYBRIDIZATION FOR ENUMERATION OF *VIBRIO VULNIFICUS* IN OYSTERS**

4.1 Introduction

Vibrio vulnificus was first reported as a foodborne pathogen linked with eating raw oysters in 1979 and it is responsible for 95% of all seafood related deaths (Oliver 1995; McCann 2006; Mitra and others 2004) causing three major diseases in human specifically gastroenteritis, wound infections and primary septicemia (Blake and others 1979; Hlady and others 1996; Klontz and others 1988; Strom and others 2000). The Infectious Disease Epidemiology Section in Louisiana reported infectious dosage of *V. vulnificus* in a range of 2000 CFU (Infectious Disease Epidemiology Section, 2006) but there are indications that less than 100 virulent cells can cause illness in high-risk persons (Anon 2006).

The main reason for *V. vulnificus* infections in the United States are consumption of raw or undercooked oysters mainly from the Gulf of Mexico. It has been shown the *V. vulnificus* densities in post harvest oysters increased during warm temperatures (California Department of Health Services 2003; DePaola 1997). Low temperature storages on the boats might help to reduce this number down.

Because of an increased number of incidents, the California state released emergency restrictions on the sale of all oysters harvested from the Gulf of Mexico during April to October unless they undergoes post harvest treatment to reduce *V. vulnificus* to a non detectable level (<30 CFU/g of oyster) (Panicker and Bej 2005). Hence regular *V. vulnificus* detecting surveillance system is needed for the summer months.

The objective of this investigation was to compare newly developed DCI with DNAH & MPN methods using Gulf Coast oysters during summer months and to examine the effect of *V. vulnificus* densities in Gulf Coast oysters by chilling them on ice.

4.2 Materials and Methods

4.2.1 Oyster Collection and Handling

Mature oysters (*Crassostrea virginica*) were harvested from the Gulf of Mexico off the coast of Louisiana from April 2006 through May 2006 and September 2006 through November 2006. We were unable to collect oysters during June, July, August and October 2006. Adult oyster samples from the first dredge of the oyster beds (Beginning of oyster dredging) in the Gulf of Mexico were collected and divided into 5 individual mesh bags containing 15 oysters each. These mesh bags were tagged as “Beginning” oyster samples. One such mesh bag was chilled immediately on ice and the remaining mesh bags were stored in a shaded area on the boat. This sampling operation was repeated towards the middle and the end of the oyster dredging operation and the mesh bags tagged respectively as “Middle” and “End”.

A commercial oyster harvesting trip represents an eight hour period hence, the “Beginning” oyster samples correspond to time zero, the “Middle” oyster samples correspond to 4 hours and the “End” oyster samples correspond to 8 hours of the commercial oyster trip. One bag each from the beginning, middle and end of the dredging operation was chilled on ice at the dock (10 -12 hours after harvesting). The remaining nine bags (Three each from beginning, middle and end of the dredging operation) were transported to the oyster warehouse on a refrigerated truck (5-10 °C). These bags were then collected from the oyster warehouse on the next day and transported in coolers to the Louisiana State University Food Microbiology Laboratory, chilled on ice. One bag each from the “Beginning”, “Middle” and “End” time periods was analyzed for growth of *V. vulnificus* at the end of 24 hours. The remaining 6 bags were stored in a walk in cooler (< 10 °C) at the Food Microbiology Laboratory for analysis of *V.*

vulnificus levels at day 7 and day 14 of storage. The oysters showed no mortality upon storage at $< 10^{\circ}\text{C}$ for a period of 14 days.

4.2.2 Plating Oyster Homogenates

The oysters from 0h, 4h, 8h, 12h and 24h were cleaned and opened aseptically using sterilized oyster knives on the same day it arrived. Oyster aliquots (150 g – 200g) were mixed with equal amounts of Alkaline Peptone Water. Serial 10-fold dilutions in PBS were made from each oyster homogenate and 100 μl aliquots were plated onto VVA plates. The plates were incubated 16h at 35°C and colonies were counted. *Vibrio vulnificus* was enumerated directly from each VVA plate. Same procedure was followed for 7 and 14 days as well.

4.2.3 Enumeration by DCI and DNAH Methods

Vibrio vulnificus was enumerated directly from each VVA plate by direct colony immunoblot (DCI) and DNA probe hybridization (DNAH) using previously described methods in chapter 3.2.

4.2.4 Enumeration by the MPN- PCR Method

The MPN method described in the FDA BAM (2004) was used to estimate amount of *V. vulnificus* in oyster homogenates, except that a species-specific DNA probe targeting the real time Polymerase chain Reaction (PCR) was used for identification (MPN-PCR) (Hara-Kudo and others 2005) instead of biochemical utilization assays. Briefly, oyster homogenate was serially diluted, inoculated into a series of marked MPN tubes containing alkaline peptone water (FDA BAM) (three tubes/dilution), and incubated for 24 h at $37^{\circ}\text{C} \pm 2$, and then a each MPN tube showing growth was streaked onto a VVA plate. Yellow colored cellobiose positive conies were transferred into marked APW tubes using sterile loop and incubated for 24 h at $37^{\circ}\text{C} \pm 2$. All tubes were denatured at 95°C for 5 min and stored at -20°C for PCR conformation.

PCR conformation was done by following method. All stored APW tubes were thawed and properly mixed using vortexer and placed 25 µl of taq PCR master mix 3 µl of diluted primer, 16 µl of deionized water and 3 µl of template DNA to marked eppendorf tubes. The PCR protocol consisted of holding samples 2 min at 94°C, followed by denaturation, 30 sec at 94°C 1 min at 72°C and then 10 min at 72°C. Amplified PCR were verified by electrophoresis with a 4% low-melting-temperature agarose (NuSieve GTG; BioWhittaker Molecular Applications, Rockland, Maine) and stained with SYBR green 1 nucleic acid gel staining at 15 min. Each gel was viewed under UV light and MPN were calculated using MPN tables.

4.2.5 Statistical Analysis

Both the direct colony immunoblot method and the DNA probe method were analyzed by statistical comparisons of all pairs using the Student's t test following 1-way analysis of variance (ANOVA) (JUMP In version 4.0.3, SAS Institute Inc., Cary, N.C., U.S.A.) and by regression analysis. Statistical significance occurs for $P < 0.05$. All experiments were repeated 3 times with 2 replications per experiment.

4.3 Results and Discussion

The main purpose of this study was to compare our newly developed DCI method with FDA approved DNAH method and modified MPN-PCR method using Gulf Coast oysters. Several investigators have reported that *V. vulnificus* can be multiplied on the post harvest shellfish (Cook and Ruple 1989; Hood and other 1983). Hence as a sub objective of the present research; one part was undertaken to determine the timely icing effect on the reduction of *V. vulnificus* densities in oysters during post harvest storage. The oysters were put on ice at different time as explained in the materials and the methods. The DCI and DNAH were used to test enumerate effects of reduction *V. vulnificus* densities on different time of the icing (0 hrs, 4 hrs, 8

hrs, 12 hrs and 24 hrs) and during the cold storage on 5 °C (168 and 336 hrs). The warehouse oyster samples (24 hr) were tested using the MPN-PCR method besides DCI and DNAH methods. Densities of the *V. vulnificus* during summer months in the Gulf of Mexico can reach 3 to 6 log CFU/g of oyster meat (Tamplin and others 1982, Depaola and others 1998; Cook and others 2002). We collected oysters from April to November to coincide with the same time period as transitional period of the *V. vulnificus* (Motes and others 1998). Our results showed about 3 log CFU/g of *V. vulnificus* counts in the oyster meat during the research (Table 4).

We were able to enumerate *V. vulnificus* densities (log CFU/g) during different time intervals when oysters were placed on ice by both DCI and DNAH methods. There was close agreement between the two methods for enumerating *V. Vulnificus* in oysters under a variety of icing and storage conditions (Table 4, Figure 7). No significant differences were observed between the DCI and DNAH methods for the oyster samples collected from the boat (0 hrs, 4 hrs, 8 hrs samples), dock (12 hrs), and the warehouse (24 hrs). On the 7th day of storage (168 hrs) the DNAH exhibited significantly higher *V. vulnificus* counts compared to the DCI. This difference may be due to nonspecific binding of the DNA probe (Wright and others 1993). During the 7th day of storage of oysters, the number of non-*V. vulnificus* species, which cause non specific bindings are increased Wright and others (1993) showed alkaline phosphatase – labeled oligonucleotide probe have cross reactivity with *V. fluvialus* at lower temperature (50 °C). Although they mentioned that these signals were visibly lower, it is dependant on personal visual conditions of the individual membrane count.

There was no statistically significant difference between the DCI or DNAH methods results of *V. vulnificus* densities of oyster samples which placed on ice at beginning (t=0), middle (t=4) and end (t=8), which were dredged at all collection locations (Table 4). Non existence of

the significance difference of the *V. vulnificus* densities pointed to no effect in oysters chilling on ice for the reduction of *V. vulnificus* densities. It pointed out that the *V. vulnificus* were able to survive even if the oysters were placed on ice.

Table 4. Comparisons of DCI and DNAH on *V. vulnificus* densities of environmental oyster samples, when placed on the ice at different time.

Collected Location	Time		Enumeration of <i>V. vulnificus</i>	
	Icing	Sampling ^a	Direct colony ^b blot	DNA probe ^b
	Hours		log CFU/g	
Boat	0	B	2.83 ± 0.71 A	2.89 ± 0.79 A
		M	2.77 ± 0.55 A	2.89 ± 0.71 A
		E	2.88 ± 0.46 A	3.04 ± 0.63 A
Dock	12	B	2.82 ± 0.60 A	3.06 ± 0.26 A
		M	2.62 ± 0.48 A	2.63 ± 0.26 A
		E	2.85 ± 0.37 A	3.12 ± 0.56 A
Warehouse	24	B	2.62 ± 0.20 A	2.97 ± 0.29 A
		M	2.67 ± 0.25 A	2.88 ± 0.53 A
		E	2.81 ± 0.32 A	2.78 ± 0.89 A
Food micro lab cooler 7 th Day	168 ^c	B	2.29 ± 0.19 B	2.60 ± 0.11 A
		M	2.09 ± 0.62 B	2.67 ± 0.20 A
		E	2.27 ± 0.78 B	2.57 ± 0.32 A
Food micro lab cooler 14 th Day	336 ^d	B	0.92 ± 1.59 C	1.16 ± 1.54 C
		M	1.54 ± 1.43 C	1.30 ± 1.30 C
		E	0.43 ± 0.75 C	0.79 ± 1.37 C

^a B-Sample harvested at beginning of the harvesting trip and directly placed on to ice (t= 0), M-Sample harvested at the beginning of the trip then placed on to ice after 4 hours (t= 4), E- Sample harvested at beginning of the trip then placed on to ice after 8 hours (t= 8)

^b All analyses were based on three separate experiments with each mean ± standard deviation being average of three determinations. Mean with each vertical column followed by the same letter are not significantly different ($P \geq 0.05$) from each other. Statistical comparisons of all pairs were analyzed using one –way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

168^c- Samples were harvested at the beginning of the trip collected after 24 hours and tested after 168 hours (7th Day).

336^d- Samples were harvested at the beginning of the trip collected after 24 hours and tested after 336hours (14th Day).

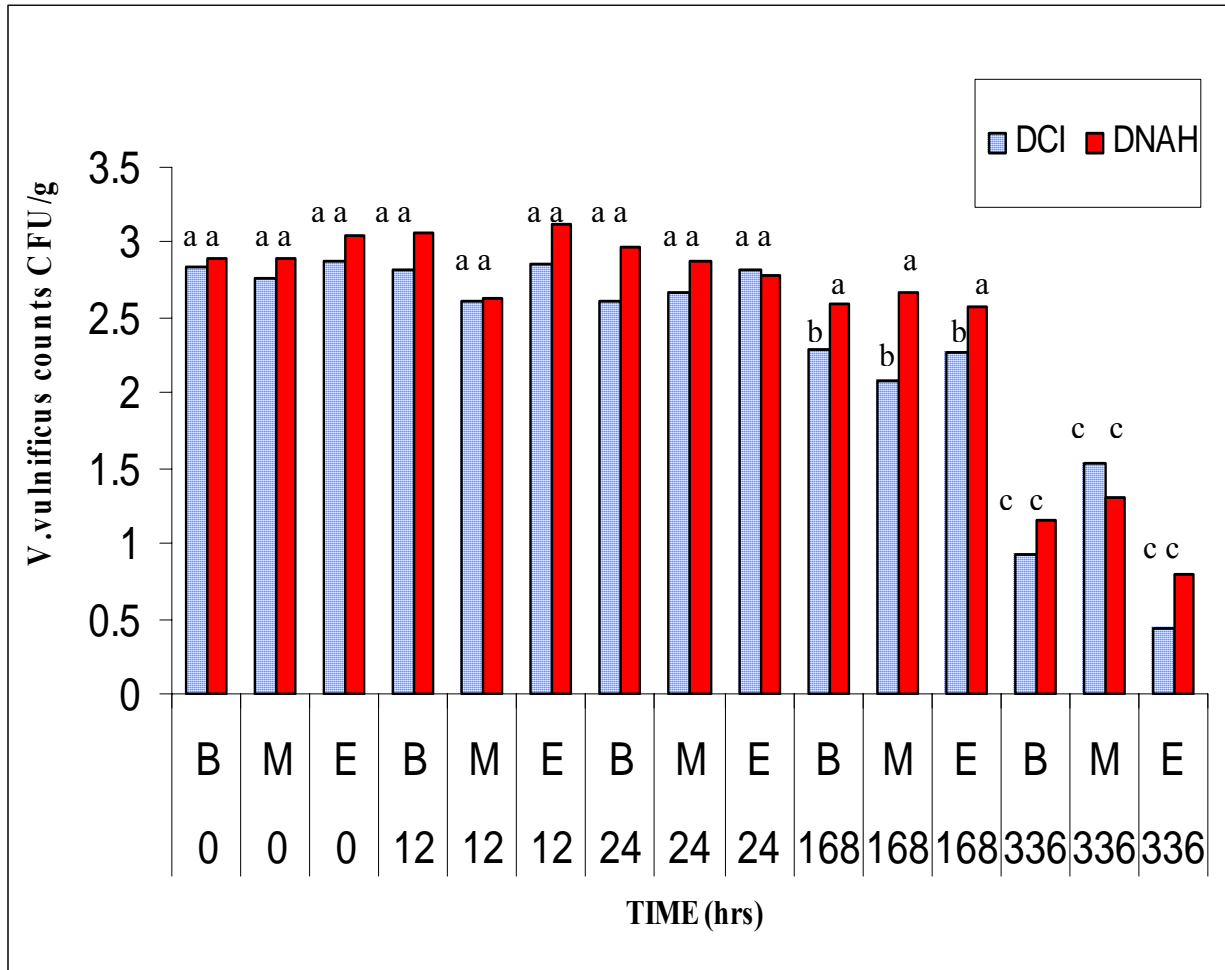


Figure 7. *V. vulnificus* densities (log CFU/g) in Gulf coast oysters analyzed by the DCI and DNAH methods.

B-Sample collected at beginning of trip time (t= 0), M-Sample collected at middle of the trip after 4 hours (t= 4), E-Sample collected at end of the trip after 4 hours (t= 4)

4.4 Comparison of DCI Enumeration Method with DNAH and MPN-PCR Methods

Vibrio vulnificus densities in warehouse oyster samples were enumerated by the DCI, DNAH and MPN-PCR methods. The bacterial counts of the three enumeration methods were not significantly different (Table 5) as observed. In the present studies, PCR was used as a conformation for enumeration of *V. vulnificus* by MPN. Three similar methods were used by Calero (2003), Randa and others (2004) and Hara-Kudo and others (2005) to detect *V. vulnificus* in seafood. The alkaline peptone water (APW) enrichment, that FDA BAM MPN procedure uses

not a selective broth for *V. vulnificus*. In this media other competitive *Vibrio* species also grow fast. Although the present studies MPN-PCR showed similar results, sensitivity may vary due to over growth of other competitive organisms. We used general PCR to confirm MPN positive tubes, which also effect for the sensitivity by counting dead *V. vulnificus* cells. Other negative impact of the MPN-PCR method is the time and labor required during this method. The method required streaking of positive turbid MPN tubes for isolation and 3-6 similar positive colonies were tested for PCR conformation more of the bacteria used a lot media. Calero (2003) has showed in her study that the DNA probe (DNAH) methods were less time consuming than the FDA BAM recommended MPN assay. For this study MPN-PCR method required at least 3 to 4 days. Our DCI method required only 3 ½ with 16 hours of incubation. It is inexpensive and less labor consuming as well.

Table 5. Comparisons of DCI, DNAH and MPN on *Vibrio vulnificus* densities of environmental oyster samples, when placed on the ice at 24 hours.

Time hours	Enumeration of <i>V. vulnificus</i>		
	Direct colony blot ^a	DNA probe ^a	MPN-PCR ^a
	log CFU/ml		
0	2.82 ± 0.02 A	3.13 ± 0.09 A	2.81 ± 0.72 A
4	2.86 ± 0.44 A	2.79 ± 1.25 A	3.18 ± 0.02 A
8	2.80 ± 0.15 A	3.05 ± 0.63 A	2.81 ± 0.89 A

^a All analyses were based on three separate experiments with each mean ± standard deviation being average of three determinations. Mean with each vertical column followed by the same letter are not significantly different (P ≥ 0.05) from each other. Statistical comparisons of all pairs were analyzed using one –way analysis of variance (ANOVA)(SAS Institute Inc., Cary, NC)

4.5 *Vibrio vulnificus* Counts during Cold Storage

In order to identify the survival capability of *V. vulnificus* in oysters during refrigeration the oysters were tested at 0, 12, 24, 168 and 336 hours. *Vibrio vulnificus* counts on the oyster samples that were tested at 0, 12 and 24 hours were not significantly different from each other (Table 1). *V. vulnificus* was capable of maintaining survival status during cold storage 5 °C until 14th day (336 hours), but the counts showed about a 0.5 log CFU/g reduction on the 7th day (168 hours) and around 1.5 log CFU/g reduction on the 14th day (336 hours) (Figure 2). The total plate counts showed positive growth in the oysters during storage. Furthermore, the total plate counts had increased 2 log CFU/g by 336 hours in oysters (Figure 3.).

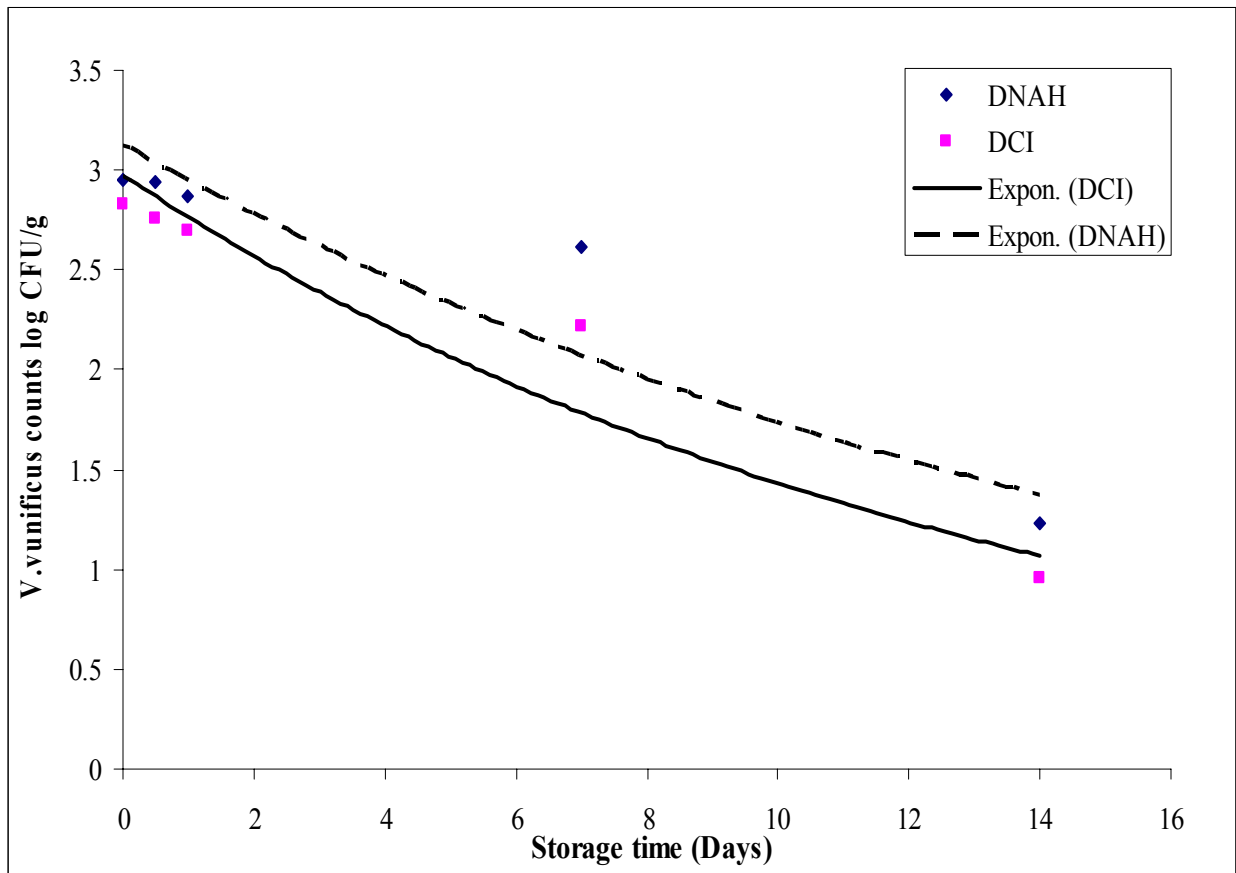


Figure 9. *V. vulnificus* densities (log₁₀ CFU/g) in Gulf coast oysters analyzed by the DCI and DNAH methods during the storage.

Monthly distribution of *V. vulnificus* densities in fresh water oysters were compared with DCI and DNAH methods (Fig 12). The seasonal distributions of mean *V. vulnificus* counts were comparable with the results of the other investigators (Figure 4) (Motes and others 1998, DePaola and others 1997). Previous studies had 2 to 4 log CFU/g range of *V. vulnificus* densities in fresh water oysters during summer months. We had similar results with 2.5 to 3.5 log CFU/g of *V. vulnificus* counts in fresh water oysters (Figure 4) during April to November. No significant difference between DCI method and the DNAH method was observed.

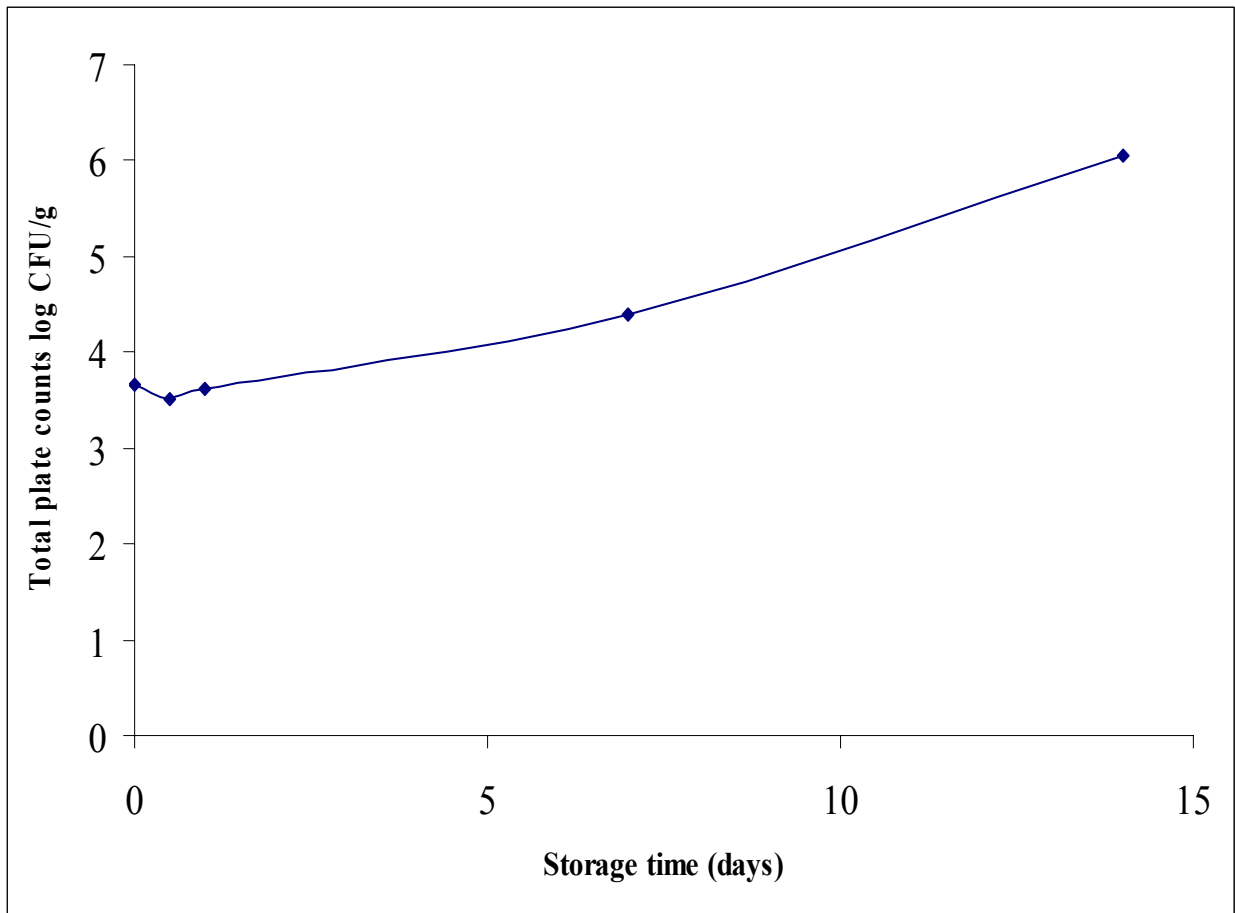


Figure 10. Direct Total plate count densities (log₁₀ CFU/g) in Gulf coast oysters on VVA plates during the storage

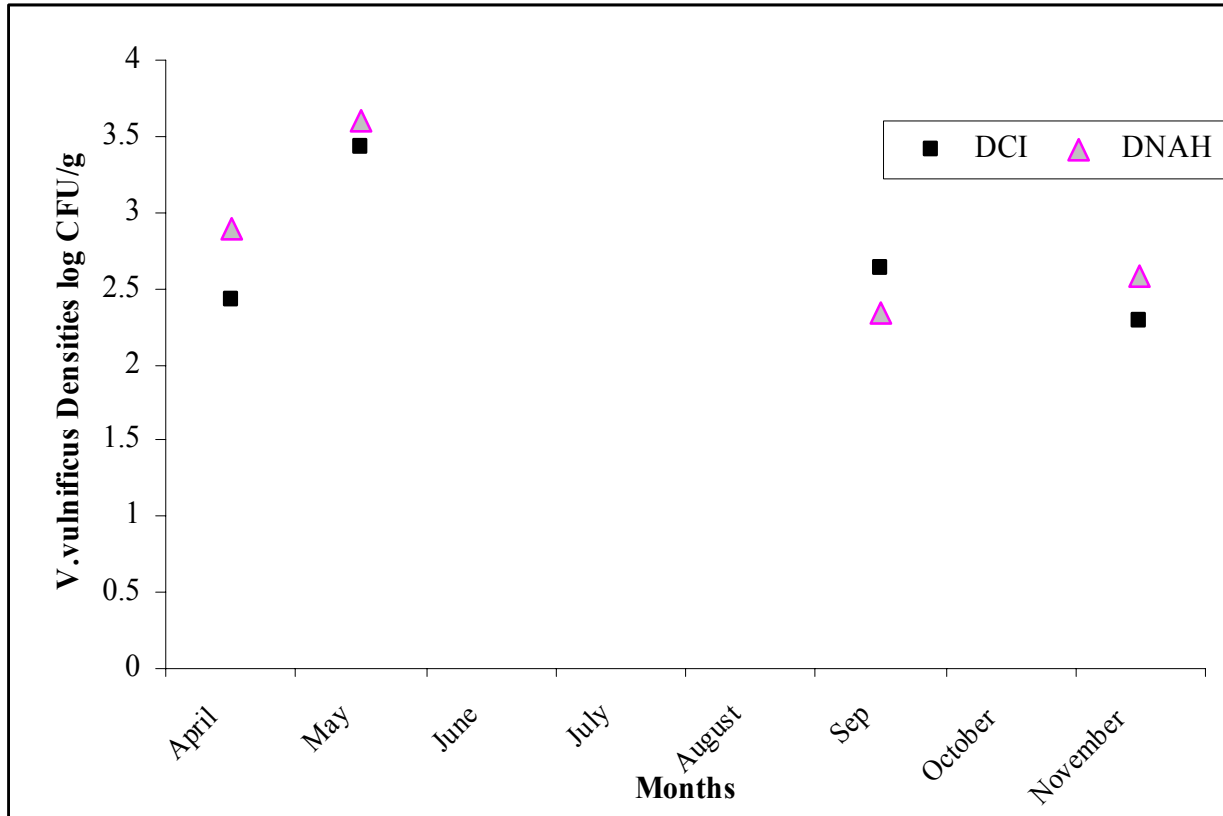


Figure 11. Monthly distribution of *V. vulnificus* densities in freshly harvested oysters

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CHAPTER 5. CONCLUSION

This study demonstrated that, incubation temperature does not seem to affect the DCI method whereas the DNAH method was influenced by fluctuations in incubation temperatures. The newly developed DCI method for rapid enumeration of *V. vulnificus* is compatible, more sensitive, and inexpensive and less time consuming than FDA approved DNAH method and MPN-PCR method.

The virulence strains of the *V. vulnificus* species are not well defined from avirulent strains of the species (Campbell and Wright 2003). Hence none of the available methods could discriminate virulent from avirulent strains of the species. The DCI method targeted polar flagellar also can not differentiate virulence strains like other methods.

This research indicates that the DCI probe provides a very sensitive and specific means of enumerating environmental *V. vulnificus* isolates, giving results comparable to those obtained by using much more complex DNAH and MPN-PCR methods.

Using DCI, our study has refined previous findings on the densities of *V. vulnificus* in gulf coast oysters during summer month (3 log CFU/g). In addition, we have shown time of oyster chilling on ice has no significant effect on densities of *V. vulnificus* levels in post harvest oysters. In addition, we have shown that during the storage population of *V. vulnificus* decline. This DCI could be improved as the standard protocol for the environmental monitoring of *V. vulnificus* in shellfish and estuarine waters and it could possibly be used as a rapid enumeration method by regulatory agencies or the seafood industry.

VITA

Reshani Nisansala Senevirathne was born in Western province of Sri Lanka. She received her primary education from Holy Family Convent Kaluthara and St Joseph Balika Girls School in Colombo 14, Sri Lanka. She received her secondary education from St. Paul's girls school Milagiriya in Colombo 4, entered Wayamba University of Sri Lanka in 1999. She received her Bachelor of Science Degree in agricultural science majoring in food technology in 2004, and she earned another Bachelor of Science Degree majoring in chemistry in 2000 from Institute of Chemistry, Ceylon. She entered Louisiana State University in 2005 for her Graduate studies.

During her graduate career, she was an active member of the Food Science Club and elected Vice President of Food Science Club of Louisiana State University in 2006-2007. She was awarded the Barkate Scholarship for Outstanding Graduate Student in 2006 by the Department of Food Science. Reshani has received IFT – Gulf Coast Section 2006 Tom Quinn Student Scholarship, based on her abstract, “Direct Colony Immunoblot for Enumeration of *Vibrio vulnificus*”. She was a team member of a multi-state project titled “Ecology and control of pathogenic strains of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in U.S. Gulf Coast oysters” and Food Product Development competition team of LSU in IFT 2007.

After completing her master's program, Reshani intends to pursue a doctorate in food microbiology at the Louisiana State University.