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# Production and application of antibodies produced against the flagella and hemolysin of *Vibrio parahaemolyticus*

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**PRODUCTION AND APPLICATION OF ANTIBODIES PRODUCED AGAINST  
THE FLAGELLA AND HEMOLYSIN OF *VIBRIO PARAHAEMOLYTICUS***

**A Dissertation**

**Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agriculture and Mechanical College  
in partial fulfillment of the  
Requirements for the degree of  
Doctor of Philosophy  
in**

**The Department of Food Science**

**by**

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**May, 2009**

**This work is dedicated to  
my better half Proyag Datta  
and  
my 1 year old son, Sohumi Datta.**

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## ABSTRACT

*Vibrio parahaemolyticus* is a marine bacterium which is the leading cause of bacterial gastroenteritis due to consumption of raw or undercooked bivalve molluscan shellfish in United States and is considered an important seafood-borne pathogen throughout the world. The conventional methods for enumerating this bacteria from seafood is laborious and time consuming, hence the objective of our study was to develop antibody based method which would be sensitive, user-friendly and less time consuming to detect and enumerate *Vibrio parahaemolyticus* from oysters. In order to condense time and material two rapid and specific antibody based test methods were developed. In the first method, monoclonal antibodies were produced against the polar flagella of *Vibrio parahaemolyticus*. The monoclonal antibody was employed in Immunomagnetic separation (IMS) protocol and was then coupled with Real-time PCR (q-PCR). IMS coupled to q-PCR was able to detect bacterial numbers in less than 3 hrs with a sensitivity of 1,000 CFU/g of oyster homogenate. In the second method anti-hemolysin serum was produced and employed in Direct colony immunoblot (DCI) protocol to detect pathogenic *Vibrio parahaemolyticus*. DCI when compared with the currently used conventional method, DNA hybridization (DNAH) showed good correlation and was a faster and simpler method requiring less equipment and stringent laboratory conditions than DNAH. From our results we have found that IMS+q-PCR or DCI could be an useful addition to the methods available for enumerating pathogenic *V. parahaemolyticus* in seafood.

**CHAPTER 1**  
**INTRODUCTION**

In the United States, seafood accounts for over 26% of foodborne disease outbreaks. A majority of these outbreaks are a result of consumption of raw or undercooked bivalve molluscan shellfish contaminated with *Vibrios* that are naturally present in the marine environment (Joseph et al., 1983; Rippey, 1994, Hlady and Klontz, 1995).

*Vibrio parahaemolyticus* is one such naturally occurring marine bacterium responsible for the majority of seafood-associated human gastroenteritis cases here in United States and considered an important seafood-borne pathogen throughout the world (Su and Liu, 2007; Kaysner and DePaola, 2001). It has been well documented that most of the strains of *V. parahaemolyticus* isolated from the environment are not pathogenic (Nishibuchi and Kaper, 1995). Virulent strains of this bacterium genome contain two principal genes that code for a thermally stable direct hemolysin (*tdh*) and a thermally stable direct acting-related hemolysin (*trh*). Nearly all clinical isolates contains one or both of the genes whereas less than 0.3% of food and environmental isolates contain either *tdh* or *trh* (DePaola et al., 1990, DePaola et al., 2000; DePaola et al., 2003). Hence there is a need for an approved and standardized method which is sensitive, reliable and is capable of accurate identification and quantification of total and pathogenic *V. parahaemolyticus* that could be used to monitor seafood products. Ideally, a method is needed which can easily detect and enumerate *V. parahaemolyticus* cells by the direct examination of shellfish, seafood or water without involving lengthy enrichment steps or overnight incubation.

Oysters harvested from the Gulf of Mexico are laden with *V. parahaemolyticus* along with other *Vibrio* species. A survey from four regions (Gulf of Mexico, North

Atlantic, Pacific and Mid-Atlantic) of retail oyster showed that summer months had the highest bacterial counts in oysters (Cook et al., 2002). During these summer months *V. parahaemolyticus* in freshly harvested Gulf coast oysters can exceed  $10^4$  MPN/g (Ellison et al., 2001; Cook et al., 2002). Conventional bacteriological methods for the detection of total *V. parahaemolyticus* in raw oysters and waters can be overwhelming in logistics, materials and time. Three analytical schemes for enumerating *V. parahaemolyticus* are suggested by current FDA Bacteriological Analytical Manual (BAM). The first is the most probable number (MPN) procedure which is most commonly used by many laboratories. The second method is a membrane filtration procedure using hydrophobic grid membrane filter (HGMF). The third method is a direct plating method using DNA probes for identification of the total *V. parahaemolyticus* population and pathogenic TDH containing strains. The entire procedure takes 3-5 days to confirm the presence of total or pathogenic *V. parahaemolyticus*. Thus there is a need to develop a more reliable and rapid method to detect the presence of pathogenic strain.

Species within the genus *Vibrio* can be identified serologically through detection of species-specific H antigens expressed in the core protein of the polar flagellum (Bhattacharyya, 1975; Simonson and Siebeling, 1986). In order to condense time and material, a rapid and specific antibody based test method could be developed which could detect species-specific antigens. This antibody based method could be coupled with Real-time PCR (qPCR) to increase the sensitivity and also reduce the detection time to 24 h.

In order to develop a more reliable and sensitive method our objectives were to:

- 1) Isolate polar flagella of *V. parahaemolyticus* using differential centrifugation methods and to produce anti H-monoclonal antibodies against *V. parahaemolyticus* polar flagellar cores.

- 2) Develop and optimize an immunomagnetic separation (IMS) protocol using the anti H-monoclonal antibody to isolate and concentrate *V. parahaemolyticus* from oyster tissue homogenates and mantle fluid.
- 3) Develop IMS/q-PCR based assay protocol to detect *V. parahaemolyticus* directly from spiked oyster homogenates and mantle fluids.
- 4) Develop direct colony immunoblot (DCI) using anti-hemolysin serum, and examine the possibility to use DCI to detect and enumerate pathogenic *V. parahaemolyticus*.

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**CHAPTER 2**  
**LITERATURE REVIEW**



## **2.1 General Information on *Vibrio parahaemolyticus***

*Vibrio parahaemolyticus* is a Gram negative, non-sporeforming, curved rod-shaped facultative halophilic bacterium which was encountered for the first time in Japan as a cause of acute gastroenteritis associated with consumption of shellfish and fish products (Sakazaki et al., 1968). It is widely distributed and natural inhabitant of estuarine marine environment. Since its discovery 50 years ago, *V. parahaemolyticus* has been indicated as a major cause of foodborne illness around the globe (Yeung and Boor, 2004).

Microscopically *V. parahaemolyticus* appears as a straight, often curved rods with round ends. The bacteria usually occur as single cells but occasionally can form chains. *V. parahaemolyticus* possesses a single polar flagella with a sheath when grown in liquid media whereas it has unsheathed, peritrichous flagella when grown on solid media (Baumann et al., 1973).

## **2.2 Cultural and Biochemical Characters of *Vibrio parahaemolyticus***

*Vibrio parahaemolyticus* is a mesophile with optimum growth temperatures between 35°C and 39°C and hence they are most prevalent in the summer season (Yeung and Boor, 2004). Furthermore, during the winter season *V. parahaemolyticus* can reach a viable but non-culturable (VBNC) state, and as the summer season approaches they start to multiply fast. For multiplication *V. parahaemolyticus* needs salt. Optimum salt concentration ranges from 0.5% to 10% with optimum levels between 1% and 3%. *Vibrio parahaemolyticus* is also able to utilize and tolerate various concentrations of metal ions which help the organism to out-compete other marine flora (Yeung and Boor, 2004). *Vibrio. parahaemolyticus* is included under terrestrial halophile type of bacteria which

require magnesium for oxidation of substrate whereas sodium plays a role in preventing lysis of cells and accelerates cytochrome oxidase , electron transport chain and ATP formation of oxidative phosphorylation (Joseph et al., 1982). *Vibrio parahaemolyticus* is a facultative anaerobe which posses both respiratory and fermentative metabolism.

### **2.3 Ecology of *Vibrio parahaemolyticus***

*V. parahaemolyticus* is widely spread throughout the world in marine environment. It has been isolated from waters in Japan, Korea, Thailand, Indonesia, India to European countries like Denmark, Germany , Italy and Spain. It has also been isolated from waters of the Western Hemisphere, from Canada, Panama and US and from Madagascar in Africa (Joseph et al., 1982). It is found to be present in water sediments, plankton, fish and shellfish samples.

*V. parahaemolyticus* illness is mainly associated with consumption of contaminated raw or undercooked shellfish. Oysters, clams and mussels and cooked crustaceans such as shrimp, crab have also been implicated with *V. parahaemolyticus* infections (Blake et al., 1980, Yeung and Boor, 2004). *V. parahaemolyticus* can increase to high levels in oysters because of their ability to enhanced concentration and survival in oysters. Oysters are filter feeders and *V. parahaemolyticus* are associated with zooplanktons which results in enhancing the bioconcentration of the bacteria in oysters (Depaola et al., 1990).

In the Unites States, *V. parahaemolyticus* was first noted in the Chesapeake Bay in association with dead and dying blue crabs in 1969. Whereas the first fully documented case occurred in Maryland in 1971 which was also associated with steamed crab (Johnson, 1984). The ecology of Chesapeake Bay was examined and it was found that the incidence of *V. parahaemolyticus* was correlated with water temperatures. The

organism was not found in sea waters during the winter months but it could be isolated from the sediments in small numbers. It was later established that *V. parahaemolyticus* could survive the winter months by attaching to the planktons and then proliferate inside of the planktons (Kaneko and Colwell, 1973). With the increase in water temperatures *V. parahaemolyticus* is released from the planktons and can be easily detected in the waters.

Oysters which were harvested particularly from Gulf of Mexico showed high *Vibrio* counts. A survey of retail oysters in the Gulf of Mexico, North Atlantic, Pacific and Mid-Atlantic regions found high *Vibrio parahaemolyticus* and *Vibrio vulnificus* counts during summer months (Cook et al., 2002). During the summer months *Vibrio parahaemolyticus* counts in freshly harvested oysters could exceed  $10^4$  MPN/gm (Cook et al., 2002).

#### **2.4 Epidemiology of *Vibrio. parahaemolyticus* Infection**

*V. parahaemolyticus* is naturally found in oysters, but not all of them are pathogenic. It is noted that more than 90% of clinical *V. parahaemolyticus* isolates are pathogenic whereas less than 1% of food or environmental isolates are pathogenic (DePaola et al., 1990, DePaola et al., 2000; DePaola et al., 2003). The pathogenic *V. parahaemolyticus* produces a thermostable direct hemolysin (TDH), which is the product of the *tdh* gene (Honda et al., 1993; Depaola et al., 2003). The occurrence of *tdh* positive *V. parahaemolyticus* in environmental samples and seafoods ranges from 0% to 6% (Cook et al., 2002; Thompson and Venderzant, 1976).

There are three clinical manifestations of pathogenic *V. parahaemolyticus* infection; wound infection, primary septicemia and occurrence of gastroenteritis. Hladly and Klontz (1960) reported diseases caused by pathogenic *V. parahaemolyticus* are as follows; 51% gastroenteritis, 24% wound infection and 17% septicemia respectively. Out

of this, fatality rates were only 1% for gastroenteritis, 5% for wound infection 44% for septic disease. Raw oyster consumption was the main cause for 68% gastroenteritis cases and 83% of primary septicemia cases. (Hladly and Klontz , 1960).

Patients with wound infections have a wound that has been exposed to seawater or seafood drippings. The majority of wound infections occur in fishermen and seafood processors (Hladly and Klontz, 1960). A study by Strom and Paranjpye (2000) showed that 69% of wound infection is related to occupational exposures amongst oyster shuckers and commercial fishermen.

*V. parahaemolyticus* can cause septic disease but these infections are relatively rare (Hladly and Klontz, 1960). In a case control study by Hladly and Klontz it was seen that 91% of the primary septicemia cases and 86% of the wound infections occurred in April through October months, the summer months. Out of the primary septicemia cases, 48% of these patients had pre-existing liver disease (Hladly and Klontz, 1996).

Amongst all the *Vibrios*, *V. parahaemolyticus* is the one mainly associated with gastroenteritis. Since 1988, US Centers for Disease Control and Prevention (CDC) has collected epidemiological and clinical data about the sporadic *V. parahaemolyticus* infections in states which participate in the Gulf Coast *Vibrio* Surveillance System. The states involved with the surveillance were Florida, Alabama, Louisiana and Texas (Daniels et al., 2000). State and local health departments report all infections associated with *V. parahaemolyticus* to the CDC. Over 10 years data from 1988-1997 on *V. parahaemolyticus* infections were gathered that has provided clinical and epidemiological information. A total of 345 cases of *V. parahaemolyticus* infections were reported in the Gulf Coast *Vibrio* Surveillance system, 202 (59%) of the patient had gastroenteritis, 118

(34%) had wound infection and 17 (5%) has septicemia. Among the 202 patients with *V. parahaemolyticus* gastroenteritis, 77 (88%) were caused by eating raw oysters in the week before the illness occurred. Among the 17 patients with septicemia and with known food history, 10 (91%) had eaten raw oysters (Daniels et al., 2000).

## **2.5 Virulence Factors**

The pathogenicity of *Vibrio* is attributed to various virulence factors including enterotoxin, hemolysin, cytotoxin, protease, lipase, siderophores and/or haemagglutinins (Zhang and Austin, 1995). Hemolysin is an exotoxin, which causes lysis of erythrocyte membranes, causing the cell to rupture with the liberation of iron-binding protein such as haemoglobin, transferrin and lactoferrin. In most cases the pore-forming activity of hemolysin is not restricted to erythrocytes, but extends to a wide range of cells including mast cells, neutrophils and polymorphonuclear cells which leads to tissue damage (Iida and Honda, 1997).

The disease caused by *V. parahaemolyticus* is associated with thermostable-direct hemolysin (TDH) or Kanagawa hemolysin found in the pathogenic strains (Chun and Seol, 1975). *V. parahaemolyticus* strain's ability to cause beta-hemolysin on Wagatsuma blood agar is referred to as the Kanagawa phenomenon (KP). It is related to the ability to cause gastroenteritis. TDH has been associated with clinical isolates and only less than 5% of environmental isolates produces TDH (Nishibuchi and Kaper, 1995). *Vibrio parahaemolyticus*-TDH is a protein toxin that has no lipid or carbohydrate composition with a molecular weight of 42 KDa as shown by gel filtration. TDH is a dimer structure made of two identical subunits with a molecular mass of 21 KDa that is heat-stable at 100°C (Honda and Ida, 1993). TDH appears to act on the cellular membrane as a pore-forming toxin which changes the ion influx in intestinal cells, inducing colloidal osmotic

lysis in erythrocytes, thereby leading to diarrhea (Honda et al., 1992; Honha and Ida, 1993; Fabbri et al., 1999 and Yeung and Boor, 2004). Purified TDH has haemolytic, cytotoxic, mouse lethality and cardiotoxic activities (Iida and Honda, 1997, Zhang and Austin, 2005).

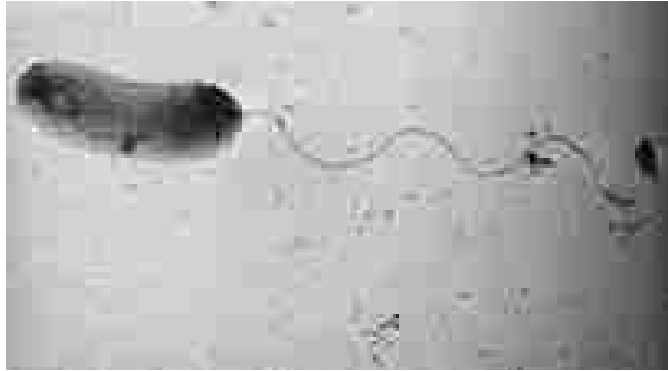
TDH causes haemolysis by three steps, first it binds to the erythrocyte membrane, secondly it causes formation of a transmembrane pore and lastly it causes the disruption of cell membrane (Honda et al., 1992). Clinical isolates of KP-negative *V. parahaemolyticus* isolated from Maldives travelers produced a new identified toxin named Vp-TDH related hemolysin (TRH) (Honda et al., 1990). In polyacrylamide gel electrophoresis, Vp-TRH migrates as a single band and at a lower mobility than Vp-TDH with a molecular weight of 48 Kda with each subunit having a molecular weight of 23 KDa each. Unlike Vp-TDH, Vp-TRH is heat inactivated at 60°C for 10 mins (Honda and Ida, 1993). *V. parahaemolyticus* strains that carry the *tdh* and *trh* (TDH-related hemolysin) genes are considered virulent strains have been associated with clinical cases (Kanaguchi et al., 2004). Comparative analysis have found that *tdh* and *trh* genes have 68.6% nucleotide sequence homology suggesting that they might be derived from a common ancestor (Shirai et al., 1990). As only 1-2 % of environmental samples are TDH positive, determining the total number of *V. parahaemolyticus* present in the environmental samples would not reflect the total number of pathogenic strains present, hence there is a need for an alternative assay for determining pathogenic strains *V. parahaemolyticus* numbers by using a reliable rapid method. A method is needed which would accurate enough to determine the pathogenic *V. parahaemolyticus* in presence of other vibrio spp. which are naturally present in the samples.

## 2.6 Serological Identification of *Vibrio*

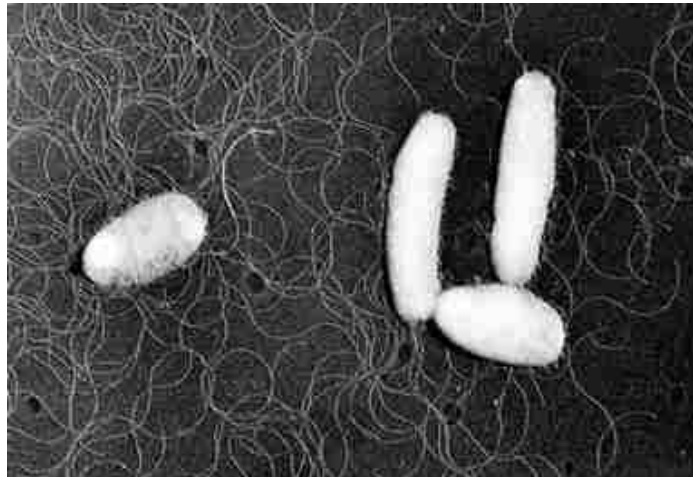
Species within the genus *Vibrio* can be identified serologically through the detection of unique H antigens expressed in the core protein of the polar flagellum (Bhattacharyya et al., 1975; Tassin et al., 1983). *Vibrio parahaemolyticus* has polar monotrichous flagella apart from lateral peritrichous flagella which differ from each other physically, functionally and antigenically. (Yabuchi et al., 1963 ; Shinoda et al., 1970, Mitwani et al., 1970, Shinoda et al., 1974, Kim et al., 2000). Flagella have two subunits which have similar physicochemical properties with molecular weights of approximately 40,000 daltons but not identical as shown by amino acid analysis (Shinoda et al., 1970). The bacteria produces monotrichous polar flagellum when grown in liquid medium but on solid media it produces peritrichous or lateral flagella (Shinoda and Nakahara, 1977). The lateral flagella are unsheathed and more fragile and are functional in viscous environments that enable the bacteria to colonize surfaces. Polar flagella on the other hand has sheath and the sheath looks more like an extension of the cell outer membrane (McCarter, 2001). Gel diffusion tests and electron microscope observations shows that polar and peritrichous flagella differ antigenically (Shinoda et al., 1974).

In order to confirm the presence of *V. parahaemolyticus* serological test that detects the species-specific flagellar (H) antigens could be used. There are two antigenic determinants in the flagellum of *Vibrio*, one of which is the surface antigen which exists on the surface of the flagellar filament and is responsible for H-agglutination when antiserum reacts with flagellar antigen. The other antigen exists inside the flagellum and is exposed only when dissociation of flagellar filament takes place (Shinoda et al., 1982).

Species-specific anti-*V. vulnificus* H polyclonal antibodies were produced and used to construct coagglutination reagents which reacted with 99% of bacterial isolates identified bacteriologically as *V. vulnificus* (Simonson and Siebeling, 1986).



**Figure 2.1: Polar Monotrichous Flagella of *V. parahaemolyticus* (McCarter, 2001)**



**Figure 2.2: Lateral Peritrichous Flagella of *V. parahaemolyticus* (McCarter, 2001)**

This offered a specific, rapid and economical approach for the detection of the pathogen one step beyond primary isolation. *Vibrio cholerae* and *V. parahaemolyticus*, however, exhibit H determinants shared with other *vibrios* which complicates the use of polyclonal anti-H antibodies to detect these pathogens serologically (Simonson and Siebeling, 1986). This was rectified in the case of *V. cholerae* by the production of several anti-H Monoclonal antibody (Mabs) which reacted only with the flagella of *V.*



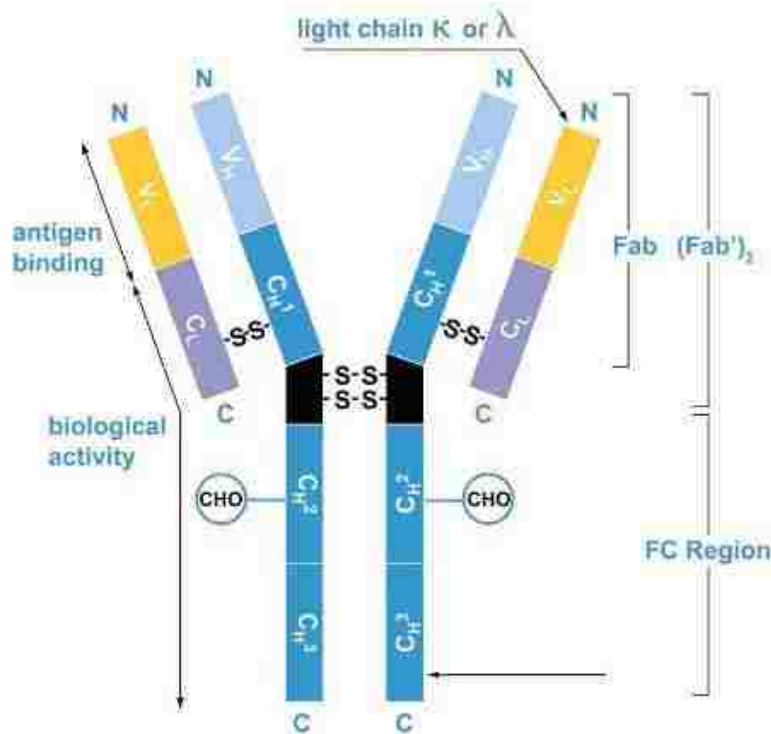
*cholerae* and *V. mimicus* thereby eliminating cross-reaction with *V. fluvialis*, *V. metschnikovii*, *V. anguillarum*, and *V. ordalii* (Simonson and Siebeling, 1988). Hence production of monoclonal antibodies against the polar flagellar core of *V. parahaemolyticus* might also eliminate the cross-reaction observed with other *vibrio* spp. thereby resulting in the desired species specificity for use in an Immunomagnetic bead separation (IMS) protocol. In addition, the production of anti-H secreting hybridomal cell lines would ensure the continuing availability of anti-*V. parahaemolyticus* Mabs for employment in IMS procedures and other analytical purposes.

### **2.6.1 Immunoglobulins**

Immunoglobulins function as antibodies. Antibodies are antigen binding proteins present on the surface of B-cell membranes. Once they are exposed to antigens they differentiate into plasma cells that secrete antibodies (Kuby, 2000). Antibodies have a common structure of four peptide chains, consisting of two identical light chains, of about 25,000 kDa and two identical heavy chains of about 50,000 kDa. Each light chain is bound to the heavy chain by a disulfide bond and a combination of hydrogen bonds, salt linkages and hydrophobic bonds. Similarly noncovalent interactions and disulfide bridges link two identical heavy and light chain combinations to each other to form a basic four chain immunoglobulin structure (Fig 2.3).

The amino terminal ends of polypeptide chains (N) show considerable variation in amino acid composition and is termed as Variable region (V) to distinguish it from the constant (C) regions. Each L chain consists of one variable domain ( $V_L$ ) and one constant domain ( $C_L$ ). The Heavy chains consist of variable domain ( $V_H$ ) and three constant domains  $CH^1$ ,  $CH^2$ ,  $CH^3$  (Figure 3).

The five primary classes of antibodies are IgG, IgM, IgA, IgD and IgE. They vary from each other by the type of heavy chains and functionality. The differences in heavy chains polypeptides allow these antibodies to function in different types of immune responses and at a particular stage of immune response (Kuby, 2000)



**Figure 2.3: Structure of Antibody**

(<http://www.abcam.com/ps/CMS/Images/abstructure.JPG>)

IgG, is the most abundant class present in serum constituting about 80% of the total serum immunoglobulin. Due to its relative abundance and excellent specificity towards antigens, IgG is the principle antibody used in immunological research (Kuby, 2000).

### 2.6.2 Monoclonal Antibodies

Antibodies are produced and purified in two basic forms: Polyclonal and Monoclonal antibodies. An immunological response towards an antigen is heterogeneous

which results in many different cell lines of B-lymphocytes producing antibodies against the same antigen. The resulting serum antibodies are heterogenous which consists of mixture of antibodies, each specific for one epitope. As it is a heterogenous collection of

For research purpose, an antibody molecule which is more specific is needed. Since an individual B-lymphocyte produces and secretes only one specific antibody molecule, clones of B-lymphocytes produce monoclonal antibody. Thus **monoclonal antibodies** are derived from a single clone and thus will be specific for a single epitope.

Fusing an antibody-producing B-cell with a myeloma cell (a cancerous plasma cell), a hybrid cell is produced called a Hybridoma, which has the immortal growth properties of the myeloma cell and antibody secreting property of B cell. The resulting clones of hybridoma cells can be cultured indefinitely.

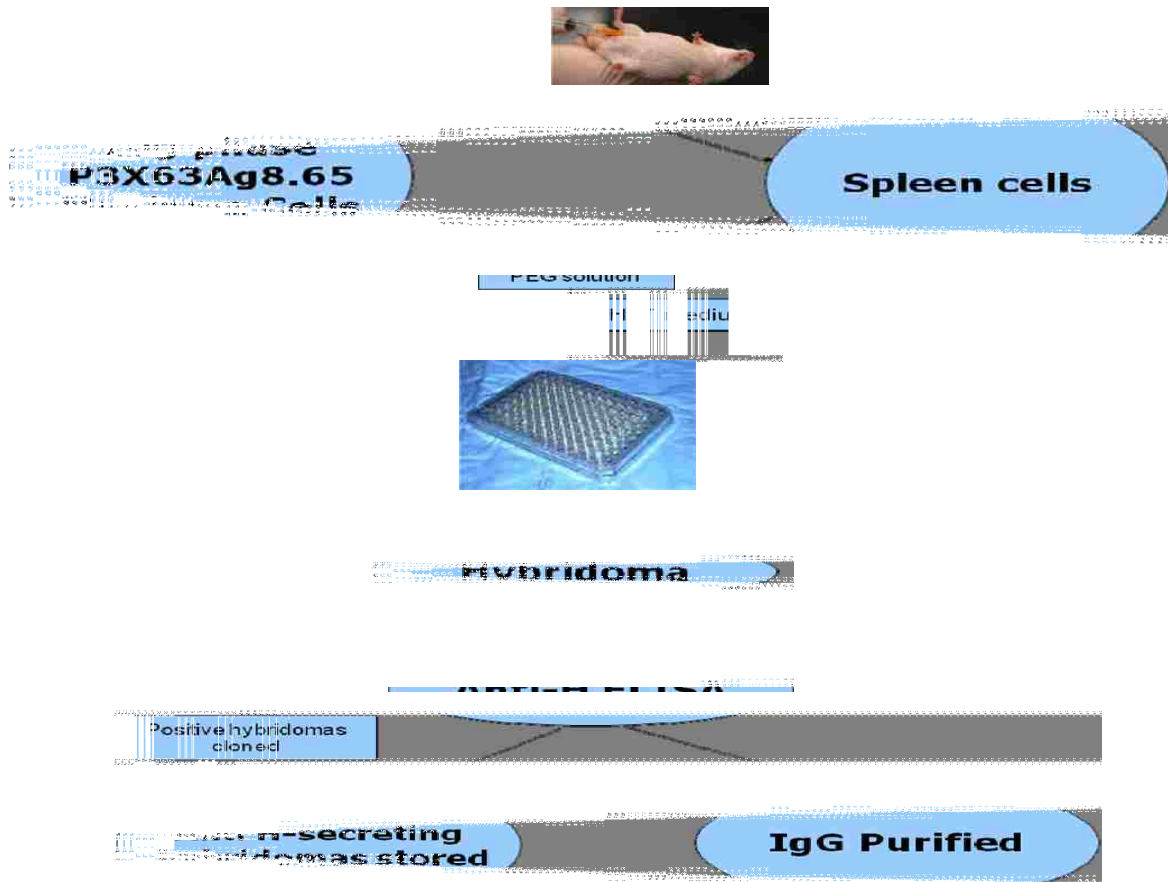
Monoclonal antibodies were initially used *in vitro* diagnostic reagents. They are now proving to be useful as diagnostic, imaging and therapeutic reagents in clinical medicine. Several monoclonal antibody diagnostic tests kits are available commercially and are used to test for pregnancy, pathogenic microorganisms, blood levels of various drugs, detecting antigens shed by certain tumors (Kuby, 2000).

### **2.6.3 Production of Monoclonal Antibodies**

Monoclonal antibodies are produced by fusing B-cells to myeloma cells using polyethylene glycol. The B-cells are derived from the animals that have been immunized with an antigen of interest. The myeloma cells contribute to the immortal properties of the fused cells.

After the fusion, there is a generation of three different combinations of fused cells; unwanted fusion of a B-cell with a B-cell or myeloma cell with a myeloma cell and the desired fusion of B-cell with a myeloma cell. The hybridoma technique uses HAT

medium (Hypoxanthine, Aminopterin and Thymidine) which selectively allows the fused cells to grow. HAT selection depends on the fact that mammalian cells synthesize nucleotides by two different pathways; the De novo and Salvage pathways.



**Figure 2.4: Monoclonal antibody production specific for a given antigen developed by G.Kohler and C. Milstein in 1975.**

When De novo pathway is blocked by the aminopterin (present in the HAT medium), the cell utilize the salvage pathway which bypasses the aminopterin block by converting purines and pyrimidines directly into nucleotides for synthesis of DNA and RNA. The enzyme which catalyze the salvage pathway include hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK). A mutation in either of

these two enzymes blocks the ability of the cell to use the salvage pathway. HAT medium contains aminopterin which blocks the de novo pathway and hypoxanthine and thymidine which allows the growth by the salvage pathway. Hence the cell that lack either HGPRT or TK dies in the HAT medium as they lack the ability to use the salvage pathway. The B-cells and myeloma cells survive because the B-cell contributes the missing enzyme for the salvage pathway. In the hybridoma technique, the myeloma cells used are double mutants and they lack HGPRTase and therefore are not selected in HAT. The reason for using mutant myeloma cells is to make sure that antibodies produced by hybridoma cells are encoded only by spleen cell partner and the myeloma cells only contributes the immortal growth properties to the fused cells (Figure 2.4).

Once monoclonal antibodies are produced, they are screened for antigenic specificity. Enzyme linked Immuno Sorbent Assay (ELISA) is such a screening technique. After monoclonal antibodies are screened and identified, they are recloned to ensure that the culture is monoclonal then can be propagated to produce the desired monoclonal antibody.

## **2.7 Enumeration of *V. parahaemolyticus***

### **2.7.1 Culture Based Methods**

Development of method for detecting *V. parahaemolyticus* has interested researchers for the past 50 years. Since then various different methods have been developed for isolation, enrichment and enumeration of *V. parahaemolyticus*. For raw molluscan shellfish, FDA Bacteriological Analytical Manual (BAM) has stated standard methods which could be used to enumerate this pathogen (Kayser and DePaola, 2001). The most common method for enumeration of this organism in food and water sample is most probable number (MPN) method. The MPN concept is based on estimation of

organisms based on probability (Luan et al., 2008). The method utilizes enrichment broths. Replication of three or five tubes can be used, with inoculation of at least three different samples (Joseph et al., 2007). The MPN method is very time consuming and laborious. In this method, 10 –fold dilution of shellfish samples is carried out in PBS, followed by inoculations of dilutions in APW, in triplicates. APW is incubated for 18 to 24 hrs and positive tubes for growth are streaked onto TCBS (Thiosulfate-citrate-bile salts-sucrose) agar for isolation. The plates are incubated followed by examination of typical colonies. Biochemical identification is used to further identify *V. parahaemolyticus*. As an alternative BAM manual suggests the use of species-specific alkaline phosphatase –labeled DNA probes. The probes target the *tdh* gene which identifies the pathogenic *V. parahaemolyticus* (Stephenie et al., 2007).

Membrane filtration technique is another technique that can be used to enumerate *V. parahemolyticus*. Arabinose, ammonium sulfate, sodium cholate (ACC) media at pH 8.6 is used but it is noted that enumeration of *V. parahaemolyticus* is affected ACC media. Hence there is a tendency for underestimation of the true incidence of *V. parahaemolyticus* (Joseph et al., 2007).

## **2.7.2 Molecular-Based Detection Methods**

### **2.7.2.1 DNA Hybridization**

Currently the FDA recommends the use of DNA probe colony hybridization for detection of pathogenic *Vibrio parahaemolyticus*. This method relies on detection of pathogenic *tdh*<sup>+</sup> gene of *V. parahaemolyticus* for enumerating the bacterial numbers from raw shellfish. Nishisibuchi and others (1985) were the first to report specific DNA probe for detecting the *tdh* gene of pathogenic *V. parahaemolyticus*. Lee and others (1992) developed a different oligonucleotide probe which targets *tdh* gene while

MaCarthy and others (1999) reported that an alkaline phosphate –labelled *tlh* gene correctly identified all the *V. parahaemolyticus* strains they tested. Gooch and others (2001) used alkaline phosphatase (AP)-labelled *tlh* probes for DNA probe colony hybridization for enumeration of *V. parahaemolyticus* after direct plating on T1 N3 (1% tryptone, 3% NaCl, 2% agar) medium. It is reported that spread plating on T1 N3 after APW enrichment followed by colony hybridization using AP-labelled *tdh* probe is superior in recovery of pathogenic *V. parahaemolyticus* compared to a more conventional streak plate method (Nordstrom and DePaola, 2003). But this method is very laborious and time consuming. It takes approximately 2 days to obtain the result.

#### **2.7.2.2 Polymerase Chain Reaction (PCR) Method**

Detection of pathogens by PCR has been shown to be a highly specific, sensitive and less time consuming method than the conventional methods available. A lot of work has been carried out using numerous PCR techniques such as multiplex PCR, nested PCR and real- time PCR (q-PCR) to rapidly screen and detect the presence of pathogenic or spoilage bacteria (Wang and Levin, 2007). There has been subsequent work done with PCR for detecting the total and pathogenic *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* strains (Bej et al., 1999; Panicker et al., 2003; Campbell and Wright, 2004; Panicker et al., 2004; Wang and Levin, 2007). The challenge of using PCR based methods with oyster products is the natural presence of PCR inhibitors. In addition post processing procedures such as gel electrophoresis are required for PCR based methods. These methods are not inherently quantitative and dead cells may be counted that could give a false positive result (Blackstone et al., 2003; Campbell and Wright, 2003).

Real-time PCR (q-PCR) has an advantage as it eliminates the requirement from post PCR processing, by measuring the accumulation of PCR amplicons during each

cycle of PCR in real-time and thereby reducing the analytical time and labor (Blackstone et al., 2003). Fluorogenic probes used in q-PCR targets gene-specific sequences internal to the primer site, which gives q-PCR an added degree of specificity compared to the conventional methods (Blackstone et al., 2003). q-PCR method is based on the amplification of the *tdh* gene for the detection of pathogenic *V. parahaemolyticus* (Blackstone et al., 2003). No cross reactions with *tdh* negative *V. parahaemolyticus* or other *Vibrio* spp. was observed. The studies have shown that the detection rate of *tdh* positive cells by q-PCR (47%) is higher than with the streak /probe methods (11%) (DePaola et al., 1990; Cook et al., 2001) which may be due to testing more number of potential *tdh* positive cells by real-time PCR than by testing of colonies with DNA probing methods (Blackstone et al., 2003).

Real-time PCR studies carried out for the detecting of *V. vulnificus* in oyster and sea water have shown that the entire method starting from sample processing, enrichment and real-time amplification took less than 8 h making it a single day assay method compared to the other conventional methods which takes about 3-4 days to complete (Panicker et al., 2003). Kaufman *et al.* (2004) used qPCR for enumeration of total *V. parahaemolyticus* directly from oyster mantle fluid. Analysis of non-seeded mantle fluids using DNA hybridization and qPCR demonstrated significant correlation between the two methods. However, a loss of efficiency was observed when numbers of *V. parahaemolyticus* were low and/or PCR inhibitors were present in the mantle fluids of certain oysters. Limited number of PCR inhibitors has been identified so far in general, and complex nature of oyster homogenate matrix makes it difficult to identify these inhibitors in oysters. Collagen may be one of the possible inhibitors present in oysters. One approach to eliminate these problems and improve the recovery and detection of *V.*



*parahaemolyticus* in seafood and shellfish samples is through the use of immunomagnetic separation (IMS).

#### **2.7.2.2.1 Immunomagnetic Separation (IMS)**

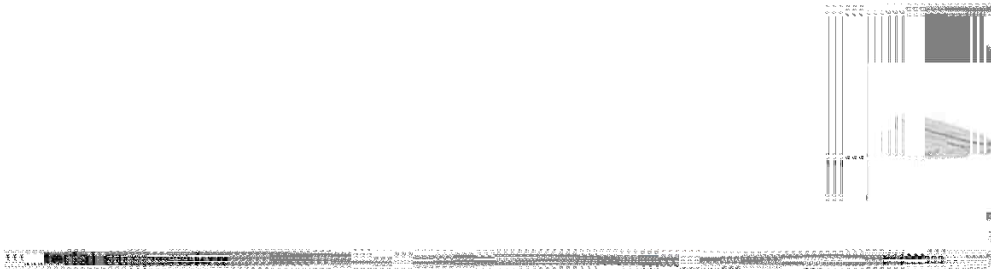
Immunoassays are widely used for the rapid detection of foodborne pathogens. These assays are easy to use, inexpensive, specific as they target the host and can reduce the detection time as compared to the conventional methods that are used in industries and laboratories (Brovko et al., 2004). There are many immunoassays based on Enzyme-linked immunosorbent assay (ELISA) which are commercially available but require minimum  $10^5$  - $10^6$  cells for detection.

Immunomagnetic separation method is an assay which is widely used to extract the target bacteria from other microorganisms and also to remove the bacteria from food components which may act as inhibitors to PCR analysis (Benoi and Donahue, 2003). Employment of IMS as a pre-PCR step would also concentrate the pathogen to a suitable volume and separate *V. parahaemolyticus* from inhibitor elements in shellfish or seafood homogenates, thereby eliminating the need for DNA purification or enrichment cultures.

Immunomagnetic separation method has been shown to be efficient in recovering eukaryotic cells and prokaryotic pathogens from fluids media such as blood, food and faecal samples (Enroth and Engstrand, 1995). It has been proved to be a powerful tool for extraction of a desired organism from heterogeneous bacterial suspension such as from food matrices (Grant et al., 1998).

Immunomagnetic separation uses small super-paramagnetic particles or beads coated with antibodies against surface antigens of cells. It relies on the interaction between cell surface antigens and antibodies attached to paramagnetic beads. The desired cells are separated by placing a bead suspension in a strong magnetic field.

Paramagnetic beads can be recoated with anti-mouse or anti-rabbit antibodies that can be polyclonal or monoclonal antibodies that are linked to the beads either directly or indirectly (Skjerve et al., 1990, Tomoyasu, 1992, Olsvik et al., 1994, Grant et al., 1998). The antibodies are bound to a secondary anti-rabbit antibody, which is coupled covalently to the magnetic bead and then the immunocaptured bacteria are further used (Stark et al., 1996). In the direct method of IMS immunocapture, the beads are coated with the desired antibody and then the antigen is added. After immunocapture the beads are collected and plated to count the number of bacteria attached to the beads. Studies using fluorescent microscopy revealed that large clumps and not just single cells attached to the same IMB or group of beads (Grant et al., 1988). Hence in the indirect method, the supernatant fluid is plated to enumerate the cells which do not bind to the beads but are lost in the supernatant after the magnetic separation step as shown in the figure.



**Figure 2.5: Schematic diagram of the indirect IMS procedure.**

There are several advantages of IMS technique, firstly all the target bacteria are separated from the sample and concentrated from a large volume which could then be taken to a volume suitable where it could be detected. Secondly the process also removes growth-inhibitory reagents which might be present in the sample (Olsvik et al., 1994). Immunomagnetic separation method could also be used as a sample preparation system

where the target bacteria are captured from the crude sample by antibody-antigen binding through a magnetic concentrator reducing the risk of false-positive results due to contamination. And also intact bacterial cells could be used as the sample and the beads with immunocomplex could be transferred directly to the PCR tube without the need of any phenol chloroform extraction method (Stark et al., 1996).

#### **2.7.2.2.2 Real- time PCR (q-PCR)**

The use of DNA technology offers an alternative way to detect microorganism. Amplification of DNA sequences by PCR using primers specific for a given microorganism are used and this method has proven to take less time and is more sensitive than the conventional methods (Widjoatmodjo et al., 1991).

q-PCR offers many advantages. Here the amplification and detection occurs in an integrated system where fluorescent dyes/probes allow constant reaction monitoring. It also has a rapid cycling time (20-40 mins for 35 cycles) with high sample throughput (approx 200-500 samples/day). There is low chance of contamination due to sealed reactions. q-PCR has increased sensitivity, with a broad dynamic range of detection. It allows for quantification of results and now it is no more expensive than 'in-house' PCR. Conventional PCR is directed towards positive recognition of the amplicons. The post-PCR analysis depends on either the size or sequence of the amplicon. Gel-electrophoresis is used to measure the size of the amplicon, but size analysis has limited specificity since different molecules of approximately the same molecular weight cannot be distinguished. q-PCR on the other hand simplify amplicon recognition by providing a means to monitor the accumulation of specific products continuously during cycling. Some of the disadvantages of q-PCR is that development of protocols and analysis requires a high

level of technical skills. It also has a limited capacity for multiplexing using all chemistries (Edwads et al., 2004)

The SmartCycler System from Cepheid is ideally suited for quantitative q-PCR and it has automatic, rapid heating and cooling repeated cycles for testing the samples (SmartCycler II, operation manual, 1997-2006, Cepheid). The sample in the reaction tube is inserted into the thermal cycling chamber module for amplification. The chamber consists of two heated plates made of ceramic material which enables high thermal conductivity and at the same time ensures temperature uniformity and rapid heat transfer. The SmartCycler optical system uses a high intensity light –emitting diodes (LEDs), silicon photodetectors and appropriate filters for excitation and detection of four different spectral bands. SmartCycler polypropylene reaction tubes are unique because they have been designed very thin which allows rapid rates of heating and cooling of the reaction mixture thereby allowing rapid amplification (SmartCycler II, operation manual, 1997-2006, Cepheid).

Real-time PCR allows the analysis of data during the log-linear phase rather than the end point of a reaction, as in conventional PCR, which is not quantitative as the final yield of product is not primarily dependent upon the concentration of the target sequence in the sample. q- PCR overcomes this limitation by incorporating a fluorescent dye labeled sequence-specific probe to see and monitor the amplified product in real-time.

A plot of fluorescence versus the cycle threshold ( $C_t$ ) for each sample can be plotted to get a log-linear phase of the reaction. The cycle threshold ( $C_t$ ) is the first cycle at which there is a significant increase in fluorescence above the background fluorescence (SmartCycler II, operation manual, 1997-2006, Cepheid).

**TaqMan Sequence Detection Chemistry** (<http://www3.appliedbiosystems.com>).

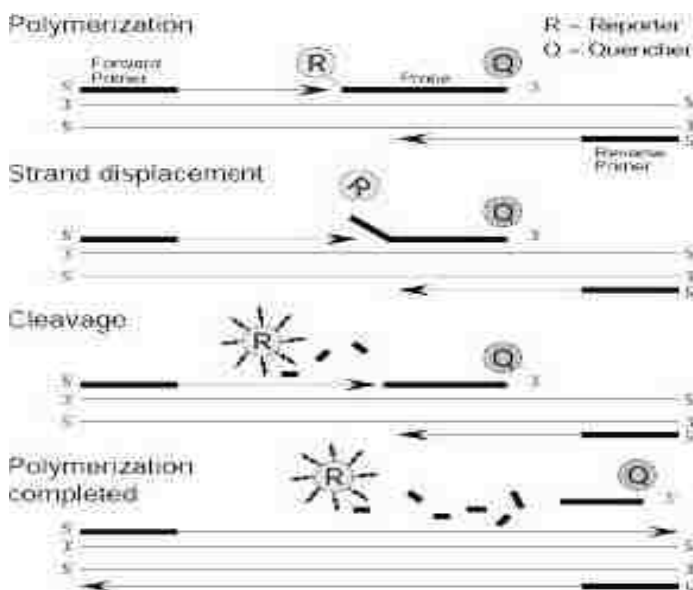
The TaqMan chemistry uses a fluorogenic probe which enables the detection of a specific PCR product as it accumulates during PCR process. The entire process of Taqman could be divided into four steps:



**Figure 2.6: Cephid SmartCycler system**  
(<http://www.cephid.com/systems-and-software/smartcycler-system>)

1. An oligonucleotide probe is constructed containing a reporter fluorescent dye on the 5' end and a quencher dye on the 3' end. When the probe is intact, the proximity of the quencher dye reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET).
2. When the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of Taq DNA polymerase as this primer is extended.

3. The cleavage of the probe separates the reporter dye from the quencher dye and thereby increasing the reporter dye signal. The cleavage removes the probe from the target strand, allowing primer extension to continue till the end of the template strand.
4. Additional reporter dye molecules are cleaved from their respective probes with each cycle run, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced.



**Figure 2.7: Taqman Sequence Detection**

Applied Bio sciences, <http://www3.appliedbiosystems.com>

Blackstone and others (2003) developed a method to detect pathogenic *V. parahaemolyticus* by targeting *tdh* gene using a Taqman probe. The q-PCR method was more sensitive when compared to a streak plate/probe method. The assay was less time and resource consuming. When TaqMan q-PCR assay was carried out targeting the *tlh* gene to detect total *V. parahaemolyticus* in shellfish and seawater, they found a strong correlation between cycle threshold and log concentration of *V. parahaemolyticus* (Kaufman and others, 2004). Takahashi and others (2005) developed a q-PCR assay

targeting *toxR* gene which quantifies total *V. parahaemolyticus* in shellfish and seawater. Another q-PCR method was developed targeting the *gyrB* gene, which is a well conserved gene in *V. parahaemolyticus*. The method has a detection sensitivity of 1CFU per PCR reaction when the method was applied to pure culture and 8 CFU per PCR reaction in spiked raw oyster (Cai and others 2006). Ward and Bhej (2006) developed a TaqMan multiplex q-PCR method for identification of *V. parahaemolyticus* which targets *tlh*, ORF8, *tdh*, and *trh* genes of *V. parahaemolyticus*. This method identifies total and pathogenic *V. parahaemolyticus* with detection limits of 1 CFU/g of oyster after overnight enrichment. Another multiplex q-PCR was developed which was optimized to amplify individually or in different combinations, two virulence-associated genes, *tdh* and *trh*, along with a species-specific *tl* gene from various *V. parahaemolyticus* strains (Bej et al., 1999). In this method the minimum level of detection was found to be less than 100 cells per 10 g of enriched oyster tissue homogenate for *tdh* gene and less than 10 cells for *tlh* and *trh* genes (Bej et al., 1999). A micro q-PCR detection system has been designed for detecting *V. parahaemolyticus* in raw oysters which targets *toxR* gene. This assay was able to detect *V. parahaemolyticus* at 1.5 CFU /g of oyster and the method was found to be rapid and more specific than standard culture methods (Kim et al., 2008).

#### **2.7.2.2.3 IMS Coupled with Real-time PCR (IMS + qPCR)**

The immunomagnetic separation and PCR assays combined will selectively extract and identify bacteria by specific antibodies with primer-specific PCR amplification (Jenikova et al., 2000). IMS represents a novel approach for the isolation of particular bacteria of interest and q-PCR is the final step of specific detection. The disadvantage of immunochemical detection is that it is not able to discriminate between related microorganisms by antisera and it has lower sensitivity. On the other hand PCR

method has its own disadvantages. It requires enrichment of sample and also amplification of target DNA that could be inhibited by large amounts of contaminating DNA or inhibitors present in the sample (Widjoatmodjo et al., 1991). Combination of IMS +PCR overcomes the disadvantages of both these methods. This method combines selective separation of desired bacteria using magnetic beads coated with monoclonal antibodies from the sample, followed by amplification of the DNA in PCR method (Widjoatmodjo et al., 1991).

Detection of *Coxiella* directly from milk by PCR amplification alone was unreliable but the combination of IMS followed by direct PCR clearly detected *C. burnnetti* with adequate sensitivity (Muramatsu, et al., 1996). Direct IMS method has proved to be the most rapid method for sample extraction and preparation. In another study IMS-PCR proved to be rapid and a specific method for detection of *Salmonella* in foods with lower fat content with the combination of filtration bags (Jenikova et al., 2000). In addition, *Salmonella* in chicken meat was rapidly detected by using magnetic immune-polymerase reaction assay. *Salmonella* serogroup B-specific monoclonal antibody improved the sensitivity but still enrichment was required for the detection of low levels of bacteria. Even though enrichment increased the assay time, they were able to detect 0.1 CFU of *Salmonella enteritidis* per g of chicken meat (Fluit et al., 1993). Hudson and others studied the rapid detection of *Listeria monocytogenes* in ham samples using IMS followed by PCR method. They showed in their IMS + PCR study that although recovery of cells by IMS was not optimal, inclusion of IMS was essential to derive DNA suitable for amplification (Hudson et al., 2001). From these studies we come to a conclusion that IMS could be used as a sample preparation step before performing real-time PCR analysis.



## 2.8 Direct Colony Immunoblot (DCI)

Direct Colony Immunoblot (DCI) is an antibody based detection method. It was developed by Hull and others in 1993 to detect Shiga-like toxin (SLT)-producing *E. coli* organisms in stool samples. DCI was able to detect non-toxin-producing *E. coli* from normal stool flora with high level of sensitivity and specificity (Hull et al., 1993). In another method *E. coli* and *Shigella* was detected from water samples using DCI method. The use of nitrocellulose membranes were used to filter out the sample and then the filter was tested using a monoclonal antibody (Szakai and others 2001). DCI method was used in tissue printing of apple slices to show the movement of *E. coli* 0157:H7 into the apple tissue from wounds on the surface of the apples (Janes et al., 2005).

Recently, DCI method was developed to detect *V. vulnificus* using anti-flagellar antibodies (Senevirathne et al., 2009). This method could detect all the clinical and environmental *V. vulnificus* strains tested and did not cross-react with other *Vibrio* species. DCI method was also found comparable to standard DNA hybridization method demonstrating no significant statistical differences while enumerating *V. vulnificus* from mixed culture or from oyster homogenates. DCI method also demonstrated clear color development and was found to be less time-consuming than DNA hybridization (Senevirathne et al., 2008).

We have developed anti-hemolysin antibodies against the hemolysin of *V. parahaemolyticus* (unpublished data). Hence this antibody in DCI protocol would be able to distinguish a pathogenic (+*tdh*) *V. parahaemolyticus* from a non pathogenic (-*tdh*) *V. parahaemolyticus*. DCI could be used as a rapid, user friendly method to enumerate pathogenic *V. parahaemolyticus* by the regulatory agencies.

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### **CHAPTER 3**

## **IMMUNOMAGNETIC SEPARATION AND COAGGLUTINATION OF *VIBRIO* *PARAHAEMOLYTICUS* WITH ANTI-FLAGELLAR MONOCLONAL ANTIBODY\***

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### 3.1 Introduction

*Vibrio parahaemolyticus* is a naturally occurring marine bacterium responsible for the majority of seafood-associated human gastroenteritis cases in the United States and is considered an important seafood-borne pathogen throughout the world (Levine et al., 1993; Daniels et al., 2000; Su et al., 2007). Conventional bacteriological methods for the detection and enumeration of *V. parahaemolyticus* can be costly in labor, materials and time nevertheless the expeditious identification of *V. parahaemolyticus* in the laboratory is desirable (Kaysner and DePaola, 1998). Ideally, a method is needed which can easily detect and enumerate *V. parahaemolyticus* cells by the direct examination of shellfish, seafood or water that does not involve lengthy enrichment steps or overnight incubation.

After *V. parahaemolyticus* outbreaks in the United States (CDC, 1998; CDC, 1999; DePaola et al., 2003) the Interstate Shellfish Sanitation Conference (ISSC) implemented a plan for monitoring the levels of *V. parahaemolyticus* in freshly harvested oysters. Since the standard most probable number (MPN)/biochemical method for enumerating *V. parahaemolyticus* (Elliot et al., 1992) was so labor-intensive and time-consuming, the procedure the ISSC recommended involved direct plating of oyster homogenates onto agar plates and, after an overnight incubation, transferring resultant colonies to filters that could be hybridized with DNA probes to detect total (*tlh*) and pathogenic (*tdh*) strains of *V. parahaemolyticus* (Cook et al., 2000). The probes were successfully used for the direct examination of total and pathogenic *V. parahaemolyticus* in oysters harvested from Washington, Texas and New York (DePaola et al., 2000). Gooch et al., 2001 compared two direct plating methods to the MPN protocol using probes specific for *tlh* to confirm *V. parahaemolyticus* isolates in Alabama oysters. They



concluded that both direct plating methods were equivalent to, yet faster and less labor intensive than the MPN method for enumeration and confirmation of total *V.*

*parahaemolyticus* in oyster homogenates. Since then, probes for *tlh* and *tdh* have been successfully and routinely employed for the detection of *V. parahaemolyticus* in retail oysters (Cook et al., 2002), shellfish and sediments (DePaola et al., 2003), and freshly harvested oysters (DePaola et al., 2003; Kaufman et al., 2003). However, the direct plating/DNA probe hybridization procedure still requires an overnight incubation and exhibits, at best, a detection sensitivity of 10 CFU/g (Kaufman et al., 2003).

It has also been shown that detection of *V. parahaemolyticus* by PCR is specific and less time-consuming than the conventional bacteriological method (Bej et al., 1999; Basher et al., 1998; Hongprayoon, 1993). However, to achieve the desired detection sensitivity of  $10^1$ - $10^2$  CFU/g, either enrichment cultures were employed or homogenates had to be subjected to DNA purification prior to PCR analysis. A real-time PCR (q-PCR) method based on the amplification of the *tdh* gene was developed and evaluated for the detection of pathogenic *V. parahaemolyticus* (Blackstone et al., 2003). The research demonstrated that q-PCR was a rapid and reliable technique for detecting virulent *V. parahaemolyticus* in pure cultures and oyster homogenate enrichment cultures. Kaufman et al., 2004 used q-PCR for enumeration of total *V. parahaemolyticus* directly from oyster mantle fluid, however, a loss of efficiency was observed when numbers of *V. parahaemolyticus* were low and/or PCR inhibitors were present in the mantle fluids of certain oysters.

One approach to circumvent these problems and improve the recovery and detection of *V. parahaemolyticus* in seafood and shellfish samples is through the use of immunomagnetic separation (IMS). Immunomagnetic separation has been successfully

used to concentrate and isolate numerous pathogens and has often been used as a pre-PCR step to concentrate and separate the organism of interest from polymerase inhibitors in the sample matrix (Corona-barrera et al., 2004; Enroth et al., 1995; Fluit et al., 1993; Roberts et al., 1997; ,Wolfgang et al., 1994). A successful IMS method for concentration of *V. parahaemolyticus* utilizing species-specific antibodies coupled to PCR would improve detection sensitivity and separate *V. parahaemolyticus* from other bacteria, eliminating interference with DNA amplification. Employment of IMS as a pre-PCR step would also concentrate the pathogen to a suitable volume and separate *V. parahaemolyticus* from inhibitory factors in shellfish or seafood homogenates, thereby eliminating the need for DNA purification or enrichment cultures. The first requirement for optimizing an IMS method for the isolation of *V. parahaemolyticus* would be to generate antibodies which will specifically recognize the pathogen.

Species within the genus *Vibrio* can be identified serologically through the detection of unique H antigens expressed in the core protein of the polar flagellum (Bhattacharya et al., 1975; Sakazaki et al., 1968; Shinoda et al., 1970; Tassin et al., 1984; Tassin et al., 1983). Based on this knowledge, species-specific anti-*V. vulnificus* H polyclonal antibodies were produced and used to construct coagglutination reagents which reacted with 99% of those bacterial isolates identified bacteriologically as *V. vulnificus* (Simonson and Siebeling , 1986). Since little cross-reaction with other *Vibrio* species was observed, this offered a specific, rapid and economical approach to the detection of the pathogen one step beyond primary isolation. *Vibrio cholerae* and *V. parahaemolyticus*, however, exhibit H determinants shared with other vibrios which complicates the use of polyclonal anti-H antibodies to detect these pathogens serologically (Bhattacharyya and Mukerjee, 1974; Shinoda et al., 1970; Shinoda et al.,

1970; Simonson and Siebeling, 1986; Tassin et al., 1984). This situation was rectified, in the case of *V. cholerae*, by the production of several anti-H MAbs which reacted only with the flagella of *V. cholerae* and *V. mimicus* thereby eliminating cross-reaction with *V. fluvialis*, *V. metschnikovii*, *V. anguillarum*, and *V. ordalii* (Simonson and Siebeling, 1986). Likewise, production of MAbs reactive with the polar flagellar core of *V. parahaemolyticus* might also eliminate the cross-reaction observed with *V. alginolyticus*, *V. natriegens*, *V. harveyi*, *V. campbellii* and *V. carchariae*, thereby resulting in the desired species specificity for use in an IMS protocol. In addition, the creation and preservation of anti-H secreting hybridomal cell lines would ensure the continuing availability of anti-*V. parahaemolyticus* MAbs for analytical purposes. This report describes the production and analysis of several MAbs reactive with the polar flagellar core of *V. parahaemolyticus* and the use of one of the MAbs in an optimized IMS protocol.

## **3.2 Materials and Methods**

### **3.2.1 Flagellar Core Purification**

Flagellar cores were purified from the polar flagella of *V. parahaemolyticus* ATCC 17802 by methods previously described (Simonson and Siebeling, 1986). *V. parahaemolyticus* cells were grown overnight in alkaline peptone broth on a shaker at 35°C. The overnight culture was then centrifuged at 10,000 rpm for 10 min at 4°C. The cell pellet was then resuspended in 0.15 M NaCl and the cell suspension was chilled on ice for 15- 30 min. This suspension was then spun in a Waring blender at a medium speed for 45 sec to shear the polar flagella. The suspension was then centrifuged in a fixed angle rotor at 10,000 rpm for 10 min to remove the cell debris. The supernatant was then pelleted at 16,000 rpm for 2 h. The pellet was resuspended in 250 ml of TET buffer

(10mM Tris, 2mM EDTA, pH 8.0, 1% Triton X-100). The entire step was repeated three times. The purified cores were then examined by transmission electron microscopy (TEM) to verify that naked cores, free of sheath material and cell debris, were present. Flagellin concentration was determined by BCA protein assay (Pierce, Rockford, IL).

### **3.2.2 Electron Microscopy**

Carbon coated grids were floated on drops of either a suspension of a *V. parahaemolyticus* ATCC 17802 nutrient broth culture containing 3% NaCl (NB+) which had been incubated for 18 hr at 35°C, centrifuged at 5,000 rpm for 15 mins and resuspended in 0.01 M phosphate buffered saline (PBS), pH 7.0 or purified flagellar cores. The flagella were then negatively stained with 2% uranyl acetate.

### **3.2.3 Polyacrylamide Gel Electrophoresis**

The approximate molecular weight of the purified flagellar core protein was determined using sodium dodecylsulfate gel electrophoresis (SDS-PAGE) under reducing conditions using a 10% acrylamide resolving gel. Molecular weight markers (10-250 kDa) were used as standards to estimate the molecular weight of the *V. parahaemolyticus* flagellin (Bio-Rad, Hercules, CA).

### **3.2.4 Immunization**

BALB/c mice were immunized at 2-week intervals for 4 to 8 weeks by intraperitoneal injection of 50 µg purified *V. parahaemolyticus* polar flagellar core protein. Mice exhibiting elevated anti-H flocculation titers (Tassin et al., 1983) were boosted with 50 µg of flagellin and their spleen cells were collected after 3 days.

### **3.2.5 Hybridoma Production**

The method used to promote cell fusion was modified from that described previously (Simonson and Siebeling, 1998). Spleen cells from immunized mice were

fused at a 4:1 ratio with log-phase P3X63Ag8.653 nonsecreting myeloma cells by the slow addition of a 50% polyethylene glycol (PEG) solution (Hybrimax, Sigma Chemical Co., St. Louis, MO). The cells were sedimented at 800 rpm for 7 mins and resuspended in RPMI 1640 containing 15% fetal bovine serum, 2 mM glutamine, 1% nonessential amino acids, 100 units/ml penicillin-streptomycin, 100  $\mu$ M hypoxanthine,  $4 \times 10^{-7}$  M aminopterin,  $1.6 \times 10^{-5}$  M thymidine and 10% hybridoma cloning factor (BioVeris Corp., Gaithersburg, MD). The cells were then distributed in 96-well, flat bottom tissue culture plates. Cell cultures were fed and maintained at 37° C in a humidified atmosphere of 5 to 7 % CO<sub>2</sub>.

**Anti-H ELISA.** Supernatant fluid from each well exhibiting hybridomal growth was tested by ELISA for the presence of anti-*V. parahaemolyticus* H antibody using *V. parahaemolyticus* polar flagellar cores (2  $\mu$ g protein/well) bound to Costar high binding microtiter plates (Corning Inc., Corning, NY) and alkaline phosphatase-labeled rabbit anti-mouse immunoglobulin G (IgG) (Sigma Chemical Co., St. Louis, MO) diluted 1:500 as described previously (Simonson and Siebeling, 1998). For ELISA, 100  $\mu$ l of purified flagella (2 $\mu$ g/well) added to coupling buffer (1.6g Na<sub>2</sub>CO<sub>3</sub>, 2.9 g NaHCO<sub>3</sub>, 0.9g NaN<sub>3</sub>, 11 dH<sub>2</sub>O) was added to each well of microtiter plate. The plates were incubated at 4° C for overnight. 100 $\mu$ l of BSA was added which served as a positive control. Plates were flicked the next day and 200 $\mu$ l of PBS-BSA was added to each well and kept at 25°C for 1 h to block all the non specific binding sites. The plates were then flicked again and 50 $\mu$ l of rabbit serum was added to each well. The plates were then incubated for 1 h at 25°C. 200 $\mu$ l of washing buffer (1.5mM MgCl<sub>2</sub>.6H<sub>2</sub>O, 2mM Mercapthoethanol (78 $\mu$ l), 500 mg BSA, 500 ml PBS) was then added to each well and plates were washed thrice. 50  $\mu$ l of alkaline phosphatase-labeled rabbit anti-mouse immunoglobulin G (IgG) (Sigma

Chemical Co., St. Louis, MO) diluted 1:500 was added to each well. The plates were again incubated for 1 h followed by washing the plates three times with washing buffer. 200  $\mu$ l of (1 mg/ml) p-nitrophenyl phosphate in diethanolamine buffer (97 ml diethaloamine, 0.2 g NaN<sub>2</sub>, 100mg MgCl<sub>2</sub>.6H<sub>2</sub>O, pH 9.8 adjusted with 1M HCL) was added to each well and plates were incubated for 30 min. The sample supernatant fluids which produced an absorbance of at least 0.5 were initially considered positive. Anti-H secreting hybridomas were cloned at least 2 times by limiting dilution and stored in liquid nitrogen.

### **3.2.6 Monoclonal Antibody Purification**

Three hybridomas secreting *V. parahaemolyticus* anti-H MAbs which exhibited ELISA absorbance > 1.0 were each expanded to 1 liter of cell culture medium not containing aminopterin, thymidine or hypoxanthine and were incubated for 2 weeks. Culture fluids were clarified by centrifugation and IgG was precipitated by the addition of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 50% saturation. The precipitates were collected by centrifugation, dissolved in 50-100 ml 0.067 M PBS, pH 7.2-7.4, and dialyzed against several changes of buffer. After clarification through a 0.45  $\mu$ m filter, IgG was purified by affinity chromatography on proteinA-Sepharose (Sigma Chemical Co., St. Louis, MO) following the manufacturer's instructions. The MAbs were isotyped by ELISA (Southern Biotech, Birmingham, AL) and IgG concentration was determined by BCA protein assay.

### **3.2.7 Coagglutination**

Formalin-fixed *Staphylococcus aureus* Cowan 1 ATCC 12598 cells were prepared by methods reported previously (Simonson and Siebeling, 1998). Anti-H coagglutination reagents were prepared by mixing 75  $\mu$ g of each MAb with 1 ml of *S. aureus* cells. Each of the 3 anti-H coagglutination reagents was tested against the

following *Vibrio* species: *V. parahaemolyticus* ATCC 17802, *V. parahaemolyticus* ATCC 33847, *V. vulnificus* ATCC 27562, *V. cholerae* ATCC 14035, *V. mimicus* ATCC 33653, *V. fluvialis* ATCC 33809, *V. natriegens* ATCC 14048, *V. alginolyticus* ATCC 33787, *V. harveyi* ATCC 14126, *V. harveyi* ATCC 35084 and *V. campbellii* ATCC 25920. The anti-H MAb, designated 3-F-3, which demonstrated the strongest slide coagglutination reaction against the vaccine *V. parahaemolyticus* strain was then tested against 41 additional *V. parahaemolyticus* and 30 *V. vulnificus* clinical and environmental strains. Each *Vibrio* isolate tested by coagglutination was grown on peptone agar slants (1% peptone, 2% NaCl, 0.2% yeast extract, 1.5% agar) for 24 hr at 35° C. The bacterial cells were harvested in 1 ml of 0.1 M Tris buffer (pH 7.8) containing 0.1 mM EDTA, 1% Triton X-100 and 0.001% thimerosal (TET buffer). One drop (30-50 µl) of cell suspension was placed on a clean glass plate and 30-50 µl of coagglutination reagent was placed adjacent to it. The two drops were mixed and observed for 3 min over a light box for evidence of agglutination.

### **3.2.8 Immunomagnetic Separation**

*Vibrio parahaemolyticus* ATCC 17802 was grown in NB+ at 35°C for 18 hr. Serial ten-fold dilutions of the culture were made in 0.1 M PBS, pH 7.2. In separate experiments, 0.1 ml of cell suspension were removed from selected dilutions and placed in sterile eppendorf tubes containing 0.9 ml PBS (9 tubes/dilution). Either 20 µl (about 30 µg) of MAb 3-F-3 or 20 µl of PBS were added to each test or control tube (3 test and 6 control tubes/dilution). The tubes were then placed on a rocker and gently agitated for 30 min at 25° C. Twenty µl ( $10^7$ ) paramagnetic beads coated with sheep anti-mouse IgG (Dynabeads M-280, Dynal Biotech, Oslo, Norway) which were washed once with PBS and suspended in PBS containing 0.1% BSA and 0.02% NaN<sub>3</sub> were added to each test

tube and one set of 3 control tubes and the tubes were agitated an additional 5 min. The beads were then isolated by placement of the tubes in a magnetic concentrator for 5 min and the supernatant fluid was carefully removed. Aliquots of the aspirated fluid were spread on NB+ agar plates and the plates were incubated overnight at 35° C. *Vibrio parahaemolyticus* colonies were counted to determine the number of bacteria which did not bind to the IMB and the number of cells present in control tubes containing no IMB (PBS control tubes). The entire procedure was carried out three times with three replications each.

### **3.3 Results and Discussion**

#### **3.3.1 Production and Characterization of MAbs**

Prior to immunization of mice, purified *V. parahaemolyticus* polar flagellar cores were examined by TEM to ensure that flagellar cores, free from cell debris and flagellar sheath, were obtained (Fig.3. 1 and 3.2). When subjected to SDS-PAGE, the purified flagellar cores exhibited a major protein band corresponding to approximately 45 kDa (Fig.3.3). Two fusion experiments yielded about 20 hybridomas producing MAbs that resulted in an absorbance reading higher than 0.5 by anti-H ELISA. Only hybridomas secreting IgG were selected by intentionally using a second antibody-enzyme conjugate specific for mouse IgG. After positive hybridomas were cloned 2-3 times, 3 stable and rapidly growing hybridomas secreting MAbs specific for *V. parahaemolyticus* polar flagellar cores when tested by anti-H ELISA were selected and expanded in approximately 1 liter of cell culture fluid. After antibody purification and subclass determination of each MAb, anti-H ELISA titers were determined and defined as the reciprocal of the highest dilution of MAb, starting with 250 µg of IgG in well 1 of a



microtiter plate, which gave an absorbance reading of 0.2 or greater in three separate experiments (Table 3.1).

The MAb produced by hybridoma 3-F-3 exhibited a much higher anti-H titer than the other two MAbs, indicating higher affinity for the *V. parahaemolyticus* flagellar core protein.

**Table 3.1: Analysis of antibodies purified from cell culture fluid.**

Hybridoma designation	IgG Concentration	Anti H-ELISA titer <sup>a</sup>	Subclass Designation
3-F-3	1480 µg/ml	512	IgG1
4-E-8	320 µg/ml	4	IgG1
4-B-6	260 µg/ml	8	IgG1

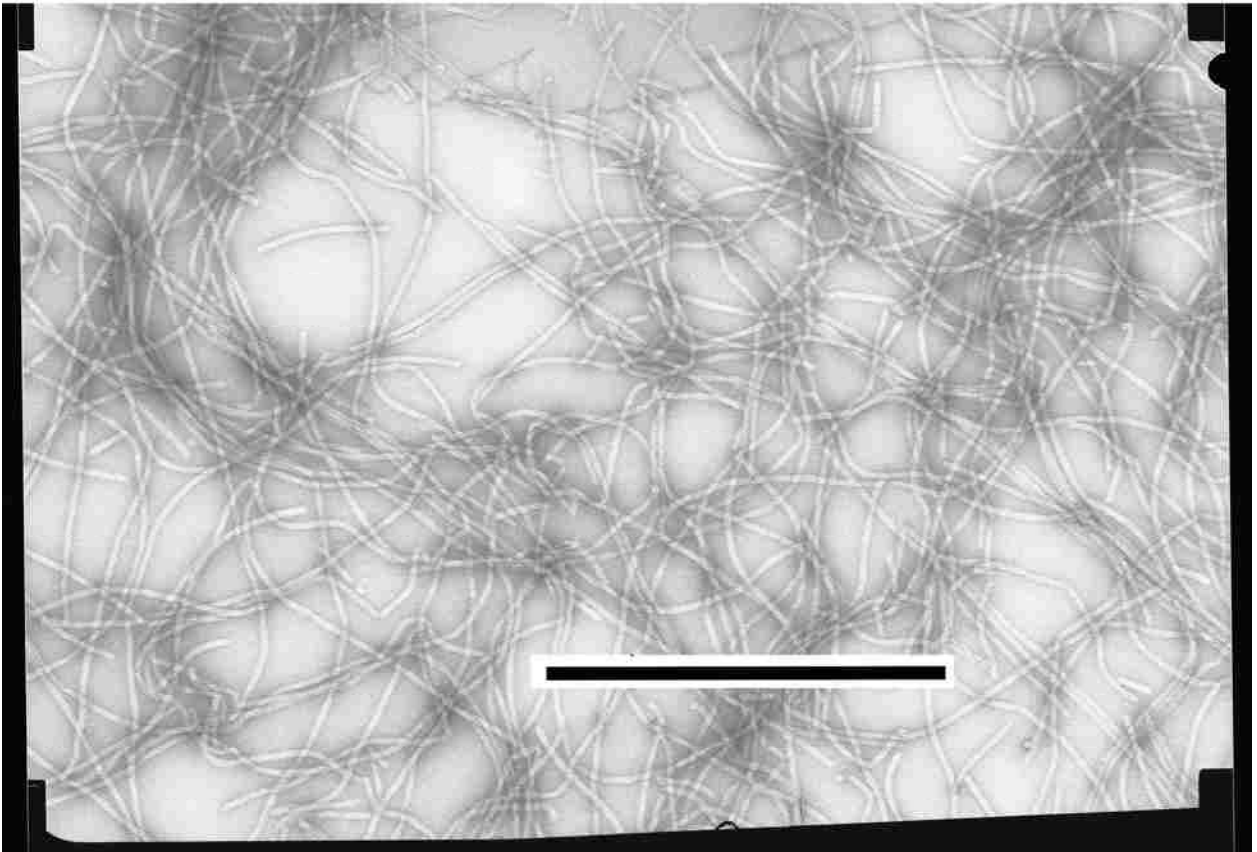
<sup>a</sup>Two-fold dilutions with a beginning protein concentration of 250 µg/well

The serological specificity of the 3 selected MAbs was assessed by slide coagglutination. Each of the 3 selected MAbs was coated onto *S. aureus* cells and the reagents were tested for positive slide coagglutination reactions with several *Vibrio* species which had been harvested and held in TET buffer for 24 h (Table 3.2). Each of the 3 MAbs exhibited positive coagglutination reactions with homologous strains of *V. parahaemolyticus* and heterologous *V. alginolyticus*, *V. harveyi* (which now includes *V. carchariae*) and *V. campbellii* strains.

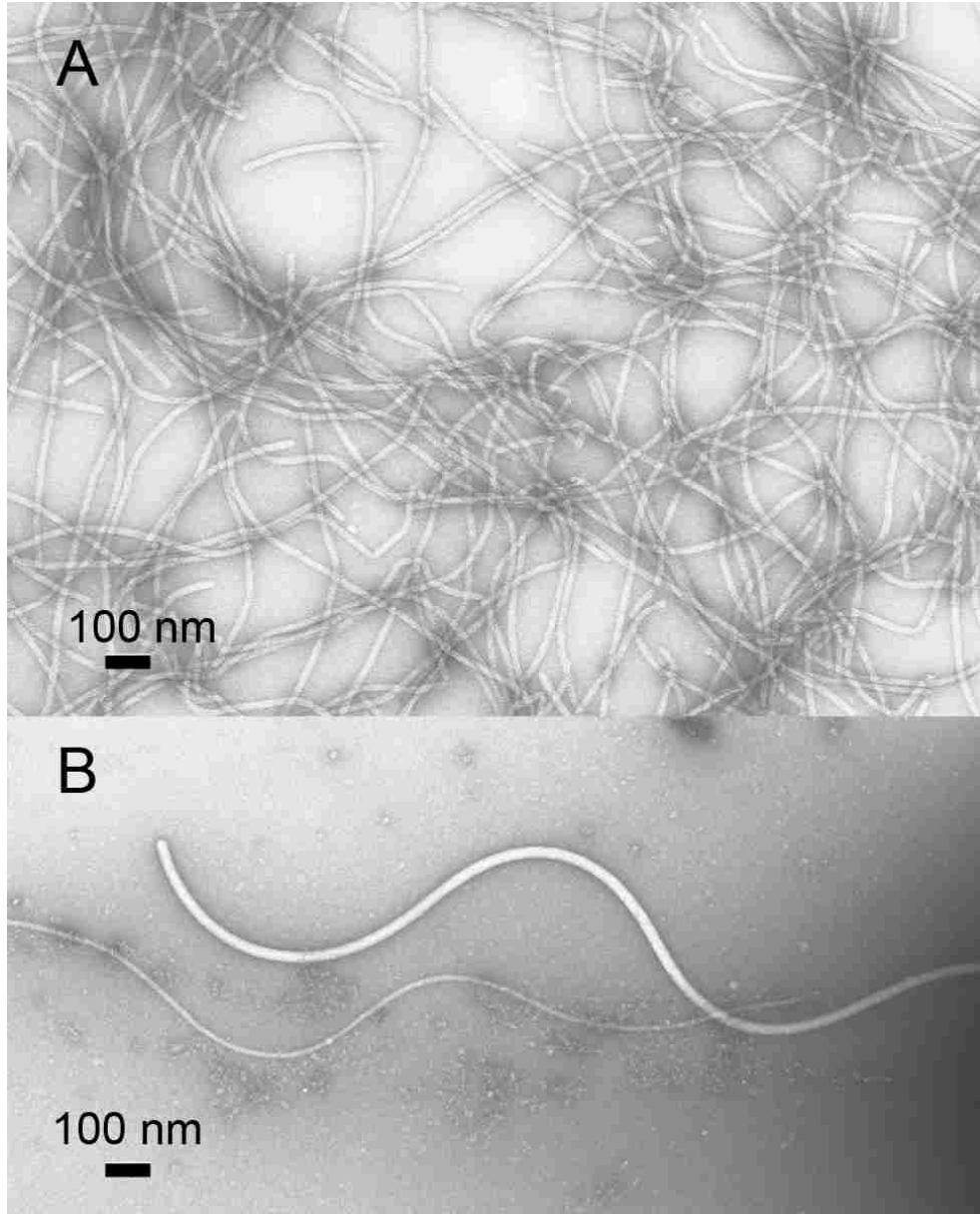
Even when *S. aureus* cells were armed with as little as 15 µg of MAb 3-F-3, positive agglutination reactions were still observed with these *Vibrio* species (data not shown). Further dilution of MAb 3-F-3 resulted in the elimination of a positive agglutination reaction within a min when tested against the *V. parahaemolyticus* vaccine strain. As expected, no reaction was observed between the anti-*V. parahaemolyticus* coagglutination reagents and *V. vulnificus*, *V. cholerae*, *V. mimicus* and *V. fluvialis*.

1  $\mu\text{m}$

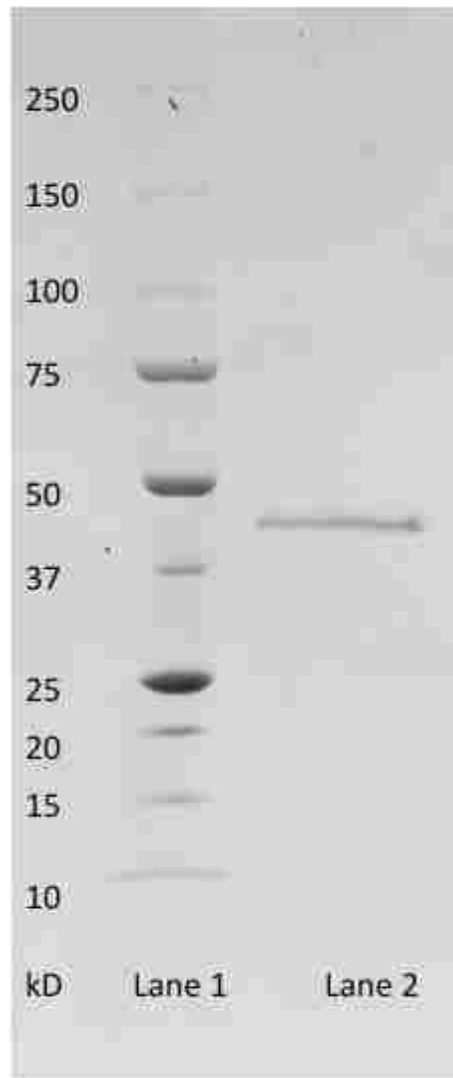
85475



**Figure 3.1: TEM of *V. parahaemolyticus* ATCC 17802 polar flagella (Marker indicates 1 $\mu\text{m}$ )**



**Fig 3.2: Electron micrograph of *V. parahaemolyticus* ATCC 17802 purified flagellar cores (A) and sheathed and unsheathed flagella (B)**



**Fig 3.3: SDS-PAGE of purified *V. parahaemolyticus* flagellar core protein. Lanes : 1, Standard protein markers; 2, 1.5 µg purified flagellin**

### 3.3.2 Coagglutination Reactions

**Table 3.2: H-antigen Relationships Among *Vibrio* Species Examined Serologically by Coagglutination with Anti-*V. parahaemolyticus* H MAbs <sup>a</sup>**

Suspension <sup>b</sup>	MAb designation		
	3-F-3	4-E-8	4-6-B
<i>V. parahaemolyticus</i> ATCC 17802	+++	++	++
<i>V. parahaemolyticus</i> ATCC 33847	+++	++	++
<i>V. vulnificus</i>	0	0	0
<i>V. mimicus</i>	0	0	0
<i>V. fluvialis</i>	0	0	0
<i>V. natriegens</i>	0	0	0
<i>V. alginolyticus</i>	++	++	++
<i>V. harveyi</i> ATCC 14126	++	++	++
<i>V. harveyi</i> <sup>c</sup> ATCC 35084	++	++	++
<i>V. campbellii</i>	+	+	+

<sup>a</sup>Coagglutination reactions were examined and scored to indicate agglutination at less than 1 min (+++), 1-2 min (++) and 2-3 min (+).

<sup>b</sup> ATCC *Vibrio* isolates harvested from 24 h slants in TET buffer (10mM Tris, 2mM EDTA, 1% Triton-X 100, pH 7.8)

<sup>c</sup> Originally deposited as *V. carchariae*

These results substantiate earlier studies with polyclonal antibodies in which *V. parahaemolyticus* anti-H sera cross-reacted with *V. alginolyticus*, *V. campbellii*, *V. harveyi*, *V. carchariae* and *V. natriegens* (Shinoda et al., 1974; Shinoda et al., 1970; Simonson and Siebeling, 1986; Tassin et al., 1983) but not other vibrios tested including *V. cholerae*, *V. mimicus*, *V. fluvialis*, *V. metschnikovii*, *V. anguillarum* and *V. vulnificus* (Sakazaki et al., 1968; Shinoda et al., 1970; Shinoda et al., 1970; Terada et al., 1968). Only the cross-reaction observed between *V. natriegens* and polyclonal anti-*V. parahaemolyticus* H serum (Simonson and Siebeling, 1986) was eliminated by the use of the MAbs selected. It is still possible that a *V. parahaemolyticus* species-specific polar H antigen exists since the coagglutination observed with *V. alginolyticus*, *V. harveyi*, *V.*

*carchariae*, *V. natriegens* and *V. campbellii* was eliminated by absorption of polyclonal anti-*V. parahaemolyticus* H serum with formalin-killed *V. campbellii* cells (Simonson and Siebeling, 1986). In addition, Hongprayoon, 1993 reported the isolation of a hybridoma that produced anti-H MAb specific for *V. parahaemolyticus* that only cross-reacted with *V. alginolyticus* and not 28 other *Vibrio* species tested. Unfortunately the hybridoma and the MAb it produced were lost. The *V. parahaemolyticus* species-specific H antigen may represent a minor flagellar antigen compared to the H antigen shared with the 4 heterologous *Vibrio* species. Thus, many more hybridomas would have to be created and screened before one producing species-specific MAb could be detected.

Shinoda et al., 1977, 1974 reported that the sheathed polar and lateral, nonsheathed flagella produced by *V. parahaemolyticus* differed morphologically and immunologically. To further complicate matters, *V. parahaemolyticus* polar and lateral flagella were shown to possess a surface antigen which was present on intact flagellar cores and an internal antigen which was revealed when the flagellar cores were denatured into flagellin monomers (Shinoda et al., 1980; Shinoda et al., 1979; Shinoda and Okamoto, 1977). It was concluded that the surface antigen-antibody reaction was responsible for H-agglutination whereas the internal antigen-antibody reaction produced the precipitin lines observed in gel diffusion tests. In 1992, Chen et al., 1992 also found that many MAbs raised against *V. alginolyticus* recognized a polar flagellar antigen of 52 kDa common to *V. alginolyticus* and *V. parahaemolyticus*. This common flagellar antigenic determinant represented an internal antigen since it was exhibited by denatured flagella and detected by gel immunoblot. The MAbs that were produced in this study should have been generated in response to immunization with *V. parahaemolyticus* polar flagellar cores only since *V. parahaemolyticus* does not produce lateral flagella when

grown in liquid media (Allen and Baumann, 1971; Belas and Colwell, 1982; Shinoda et al., 1979; Shinoda and Okamoto, 1977). In addition, when analyzed by SDS-PAGE, the lateral flagellar core is composed of a flagellin with a molecular weight of 27 kDa (McCarter, L.L. 1995.) whereas the polar flagellins of *V. parahaemolyticus* and *V. cholerae* exhibit a molecular weight of about 45 kDa (McCarter, L.L. 1995.; Yang et al., 1977). The flagellar core preparation used in this study was composed of a single flagellin with an approximate molecular weight of 45 kDa on SDS-PAGE (Fig.3.3). Thus, the cross reactions observed must have occurred from a shared H antigen displayed on the polar flagellum. In addition, the 3 MAbs selected also must have recognized a surface antigen since intact flagellar cores were used as murine immunogen and also antigen in all anti-H ELISA determinations. Most importantly, MAb specificity was determined by anti-H slide coagglutination which could only occur by a surface antigen-antibody reaction. One might also argue that the MAbs analyzed could be reactive to *V. parahaemolyticus* polar flagellar sheath. This, however, is unlikely since the flagellar sheath was stripped from the core by suspension of the sheared flagella in TET buffer and several rounds of differential centrifugation (Simonson and Siebeling. 1986.). Thomashow and Rittenberg, 1985 and Yang et al., 1977 demonstrated that the flagellar sheaths of *Bdellovibrio bacteriovorus* and *V. cholerae* were readily solubilized by Triton X-100. Also, examination of the *V. parahaemolyticus* purified flagellar cores by TEM prior to immunization revealed no contaminating sheath material or cellular debris (Fig. 3.2A). In addition, the morphology of the purified flagellar cores and intact flagella obtained from an overnight *V. parahaemolyticus* 17802 broth culture were studied by electron microscopy. Both unsheathed and sheathed flagella were evident in the broth culture (Fig. 3.2B). The diameter of the sheathed flagellum was measured and found to

be about 30 nm. The diameters of the unsheathed flagellar cores from the broth culture (Fig 3.2B) and the purified flagellar cores (Fig. 3.2A) were about 15 and 20 nm respectively, indicating that the purified flagellar core preparation that was used as mouse immunogen was free of sheath material. These results demonstrate that *V. parahaemolyticus*, *V. alginolyticus*, *V. harveyi* and *V. campbellii* must share at least one H antigen displayed on the surface of their polar flagellar cores.

One of the goals of this investigation was to assess the efficiency of an anti-H MAb to isolate and concentrate *V. parahaemolyticus* in an IMS protocol. Skjerve et al., 1990 demonstrated that a slide coagglutination reaction was a simple and reliable tool to assess the ability of antibody-coated immunomagnetic beads (IMB) to bind bacterial strains of interest in an IMS protocol. Since MAb produced by hybridoma 3-F-3 exhibited the highest affinity for purified *V. parahaemolyticus* flagellar cores and the strongest coagglutination reaction against the two *V. parahaemolyticus* strains originally tested, it was selected for use in the development of a *V. parahaemolyticus* IMS method. The serological specificity and potential reactivity of MAb 3-F-3 in an IMS protocol was further examined by testing additional *V. parahaemolyticus* and *V. vulnificus* clinical and environmental strains by *S. aureus* slide coagglutination. Of 41 isolates identified genetically as *V. parahaemolyticus*, 41 (100%) were coagglutinated with the anti-H MAb within 30 sec and the MAb did not react with 30 isolates identified as *V. vulnificus* (data not shown).

### **3.3.3 Immunomagnetic Separation of *V. parahaemolyticus***

Several guidelines were followed for the optimization of the IMS method. First of all, there are two ways that antibodies can be used in an IMS protocol. In the direct method, the antigen-specific antibody is first coated onto IMB, which are then mixed



with the test sample. The indirect method differs from the direct approach in that the organisms of interest are first incubated with an excess of antigen-specific antibody and then the IMB are added to the test sample. Roberts and Hirst, 1997 found that indirect IMS produced consistently better results for the detection of *Mycobacterium ulcerans* from heterogeneous samples, increasing sensitivity up to 10-fold. Corona-Barrera et al., 2004 also found that the indirect IMS method was 10- to 100-fold more sensitive for the detection of *Brachyspira* species in porcine feces. Based on this information, we used MAb 3-F-3 in an indirect IMS protocol by incubating an excess of the MAb (30 µg/test sample) with target *V. parahaemolyticus* cells prior to the addition of the IMB. Secondly, the concentration of IMB employed per test sample was predetermined at  $10^7$  since other successful IMS methods recommended bead concentrations in this range (Fluit et al., 1993; Grant et al., 1998). Skjerve et al., 1990 determined that at this concentration of IMB, 5 µg of MAb exhibited near maximum *L. monocytogenes* binding, while Roberts and Hirst, 1997 found that 15µg IgG was required for optimal coating of the IMB. We used 30 µg 3-F-3 IgG / test sample for indirect *V. parahaemolyticus* IMS since that concentration of MAb was in slight excess of the optimal range determined by other researchers. Finally, it has been shown that not just a single bacterial cell but often clumps of cells are bound to each bead during IMS (Grant et al., 1998; Olsvik et al., 1994; Roberts and Hirst, 1997). Since more than one cell can bind to a single bead, then determining the number of bacteria bound to the IMB by plating the IMB and counting resulting CFU would not be accurate. Therefore, the numbers of cells bound by the IMB in this study was determined indirectly by counting the number of unbound *V. parahaemolyticus* cells in the aspirated supernatant fluid.

One objective in developing an IMS method for the isolation of *V. parahaemolyticus* was to increase the detection sensitivity. Thus, the detection sensitivity was explored in this study and not the maximum binding capacity of the anti-H coated IMB. Skjerve et al., 1990 demonstrated a sensitivity of 10-100 *L. monocytogenes*/ml PBS. They found that an increase in the number of bacteria in suspension resulted in an increase in the number of bacteria bound to the IMB from < 10% (100 *L. monocytogenes*/ml) to 50% ( $1.5 \times 10^4$  *L. monocytogenes*/ml). Likewise, when Roberts and Hirst, 1997 mixed 10-fold dilutions of *M. ulcerans* with nontarget bacteria, the IMB were unable to capture less than about 100 *M. ulcerans*. Maximum IMB binding capacity for *L. monocytogenes* and *Mycobacterium paratuberculosis* was about  $10^4$ - $10^5$  CFU/ml when  $10^6$ - $10^7$  antibody-coated IMB were employed for each test sample (Grant et al., 1998, Skjerve et al., 1990). When MAb 3-F-3 was employed in the IMS protocol an average binding of about 35% was exhibited at  $10^2$  *V. parahaemolyticus* CFU/ml and 45% at  $10^3$  CFU/ml (Table 3.3 ).

The binding of *V. parahaemolyticus* at lower concentrations (<100 CFU/ml) could not be determined accurately by plating methods alone since 0.1 ml aliquots were spread on the agar plates and further dilution resulted in less than 10 CFU/plate. About 15% nonspecific binding to the IMB was observed at  $10^3$  *V. parahaemolyticus* CFU/ml. Fluit et al., 1993 developed an IMS coupled to PCR for the detection of low numbers of *L. monocytogenes* in food samples. The procedure involved the isolation of *Listeria* cells by IMS using genus-specific MAbs followed by PCR amplification of a *L. monocytogenes*-specific DNA sequence. The method served to separate *Listeria* cells from PCR-inhibitory factors present in selective food enrichment broths allowing a detection sensitivity of 1 CFU/g.

**Table 3.3. IMS of *V. parahaemolyticus* using Anti-H MAb**

<i>V. parahaemolyticus</i> recovered (CFU/ml) <sup>a</sup>			
Inoculum <sup>b</sup>	IMB	IMB + MAb	% Binding
1810	1633	1167	35.5
1587	1347	973	38.7
1130	953	497	56.1
943	700	483	48.8
343	ND <sup>c</sup>	233	32.0
213	ND	130	39.0

<sup>a</sup> Number of cells remaining in supernatant fluid after IMS. Results are averages of 3 determinations

<sup>b</sup> Number of *V. parahaemolyticus* ATCC 17802 cells in control tubes (PBS only).

<sup>c</sup> Not done

A similar method could be employed using 3-F-3 MAb to capture *V. parahaemolyticus* cells. Even if cross-reacting *Vibrio* species were bound by the MAb to the IMB or attached to the IMB by nonspecific binding, *V. parahaemolyticus* could be specifically detected by coupling the IMS to *tdh* or *tlh* PCR amplification.

In the next chapter, we will concentrate on the detection of *V. parahaemolyticus* by anti-H IMS in oyster homogenates. It is also important to couple the IMS to q-PCR to determine if low levels of *V. parahaemolyticus* captured by IMS can be quantified and to explore the limits of *V. parahaemolyticus* detection sensitivity.

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## **CHAPTER 4**

### **EVALUATION OF IMMUNOMAGNETIC SEPARATION (IMS) COUPLED WITH REAL -TIME PCR (Q-PCR) FOR ENUMERATION OF *V.* *PARAHAEMOLYTICUS* IN SPIKED OYSTER HOMOGENATES AND MANTLE FLUID**



#### 4.1 Introduction

Kanagawa phenomenon can be detected by the thermostable direct hemolysin + *tdh* genes of *V. parahaemolyticus* that causes beta-type hemolysin on a special Wagatsuma agar (Miyamoto et al., 1969). This phenomenon is detected in most of the clinical strains but only 1-2 % of the environmental isolates tested have these genes (Sakazaki et al., 1968). In recent years researchers have developed Polymerase Chain Reaction (PCR) based assays to target +*tdh* genes for the identification of pathogenic *V. parahaemolyticus*. Polymerase Chain Reaction methods have been used in the detection of pathogenic *Vibrio* species from food and environmental enrichment samples (Koch et al., 1993; Depaola and Huang , 1995; Bej et al., 1999). Detection of *V. parahaemolyticus* by PCR has been shown to be rapid, extremely specific and less time consuming than the conventional bacteriological methods. The Thermostable direct hemolysin gene (*tdh* +) can be detected by PCR amplifications and the results are consistent when compared to DNA hybridization assays (Lee and Pan, 1993).

Even though the conventional PCR- based methods are highly specific and sensitive, it requires post-PCR processing such as gel electrophoresis and moreover conventional PCR is not inherently quantitative which eventually increases the analytical time and labor (Blackstone et al., 2003). The post-PCR analysis in conventional PCR depends on either the size or sequence of the amplicon. Gel electrophoresis is used to measure the size of the amplicon, but size analysis has limited specificity since different molecules of approximately the same molecular weight cannot be distinguished. Real time PCR (q-PCR) on the other hand simplify amplicon recognition by providing a means to monitor the accumulation of specific products continuously during cycling thereby eliminating the post-PCR analysis and at the same time it can be used for

quantification (Fortina et al., 2001; Kimura et al., 2000; Edwards et al., 2004 ). Quantification is accomplished by using fluorogenic probes that target the gene-specific sequences which are internal to the primer sites hence q-PCR imparts an added degree of specificity compared to the conventional PCR based methods (Blackstone et al., 2003). Blackstone and others developed a q-PCR method based on the amplification of *tdh* gene for the detection of pathogenic *V. parahaemolyticus* in pure cultures and in oyster enrichments (Blackstone et al., 2003). Kaufman and others used real-time PCR for enumerating total *V. parahaemolyticus* directly from oyster mantle fluid. Non seeded mantle fluids using DNA hybridization and q-PCR showed significant correlation between the two methods, but there was a loss of efficiency when the numbers of *V. parahaemolyticus* numbers were low or when PCR inhibitors were present in the mantle fluids of oysters (Kaufman et al., 2004).

One approach to improve the recovery and detection of *V. parahaemolyticus* in oyster samples could be the use of Immunomagnetic separation (IMS). This technique uses small super-paramagnetic beads which are coated with antibodies and the desired cells will attach to the antibodies then the magnetic bead suspension can be separated in a strong magnetic field. The trapped bacteria are then lysed and the supernatant containing the bacterial DNA, is subjected to q-PCR. The immunomagnetic separation and PCR assays combine selective extraction of bacteria by specific antibodies with primer-specific PCR amplification (Jenokova et al., 2000). IMS could be used as a pre-PCR step to concentrate and separate the organism of interest from oyster homogenate. IMS coupled with PCR has been used by researchers to isolate *Listeria* from cheese, *Salmonella* from fecal sample, *Campylobacter* from enriched food samples (Fluit et al., 1993; Widjojoatmodjo et al., 1992; Sails et al., 2003). In comparison with the

conventional cultural methods, the IMS-q-PCR is a rapid and specific method.

Employment of IMS as a pre q-PCR step would concentrate *V. parahaemolyticus* to a suitable volume and separate it from other inhibitor elements in shellfish or seafood homogenates, thereby eliminating the need for DNA purification and enrichment cultures. The objective of this research is to use IMS with q-PCR to isolate and identify pathogenic *V. parahaemolyticus*.

## **4.2 Materials and Methods**

### **4.2.1 Preparation of Pure Culture DNA Templates for q-PCR Analysis**

*V. parahaemolyticus* ATCC 33847 was grown in 10 ml nutrient broth with 3% NaCl at 35°C for 16 hrs. After incubation the tube was centrifuged at 5,000 rpm for 7 mins and the pellet was resuspended in 1 ml of sterile millipore water. The 1 ml aliquote of the bacterial culture was heated in an eppendorf tube at 100°C for 15 min. The cellular debris were pelleted by centrifugation at 16,000 x g for 2 min. The resulting supernatant was used as a crude DNA template for q- PCR.

### **4.2.2 *V. parahaemolyticus* Real-time PCR Assay**

Forward primer: 5'-AAA CAT CTG CTT TTG AGC TTC CA-3' and reverse primer: 5'- CTG GAA CAA CAA ACA ATA TCT CAT CAG-3' (DNA technology, Aarhus C, Denmark) was used in the q-PCR assay (Previously used by Blackstone et al., 2003). Fluorogenic probe targeting a 75 base pair region of *V. parahaemolyticus* *tdh* gene : 5'-FAM- TGT CCC TTT (T)CC TGC CCC CGG-3' was used (DNA technology, Aarhus C, Denmark) (Blackstone et al., 2003). The FAM is the fluorescent reporter dye and (T) is the nucleotide attachment of the TAMARA quencher molecule.

The q-PCR assay used the TaqManprobe method and was performed using a Smart Cycler (Cepheid, Sunnyvale, CA) (Blackstone et al., 2003). q-PCR assay had a

reaction volume of 25  $\mu$ l containing 300 nM of forward and reverse primer, 50 nM of fluorogenic probe, Premix ek Taq(which contains the buffer, dNTPs, MgCl<sub>2</sub> ), distilled water and 3  $\mu$ l of DNA template. The q-PCR cycle was run using the following parameters;

- 1) 94°C initial hold for 2 min in order to denature the DNA and activate the Taq polymerase.
- 2) The run was set for 50 cycle of amplification with each consisting of denaturation at 94°C for 10 sec followed by primer annealing or extension step at 60°C for 12 s.
- 3) The cycle threshold (Ct) was set at 30, hence the positive samples generated a signal at least 30 fluorescent units above the baseline. The Ct value is defined as the first cycle at which there is a significant increase in fluorescent signal over the background signal or a specific threshold (Smart Cycler Operation manual, 2001, Cepheid).

#### **4.2.3 Standard Curve for q-PCR Assay**

*V. parahaemolyticus* ATCC 33847 was grown in Nutrient broth + 3% NaCl for 16 hrs. After incubation it was serially tenfold diluted with the highest dilution at 10<sup>8</sup> to lowest at 10<sup>0</sup> in sterile phosphate buffered saline (PBS). Triplicate spread plates of each dilution was spread on *Vibrio vulnificus* agar (VVA) plates and the plates were incubated at 37°C overnight. One ml culture from each dilution was centrifuged at 8,000 x g for 5 min. The pellet was then dissolved in 1 ml of Millipore water and heated at 100° for 15 min. The cellular debris were then pelleted by centrifuging the tubes at 16,000 x g for 2 min. The supernatant was collected and used as the crude DNA template for q-PCR. To produce a standard curve, log value of the calculated number of cells added to each reaction tube (calculated from the plate counts) was plotted vs. the Cycle threshold (Ct) value.

#### **4.2.4 Immunomagnetic Separation from Shucked Oysters**

Oysters were purchased from local harvesters, placed on ice and transferred to the laboratory. The oysters were shucked, weighed and mixed in a ratio of 1:1 (wt/wt) with sterile PBS and then stomached for 2 min. Oyster homogenate was filtered through cheesecloth (4 layers) and serial dilutions were made. The oyster homogenates and the mantle fluid were spiked with serially diluted overnight grown culture of *V. parahaemolyticus* ATCC 17802 ranging from 10,000 to 1CFU/g. One ml of each spiked homogenate dilution was subjected to IMS protocol.

#### **4.2.5 IMS from Store Bought Oysters Coupled with q-PCR**

Store bought oysters were purchased from local grocery store. Ten g of oyster meat was weighed and 10 ml of PBS was added for a 1:1 (wt/wt) dilution. The mixture was homogenized in a stomacher for 2 min. The mixture was then filtered through cheesecloth and the filtrate was spiked with different dilutions of *V. parahaemolyticus*. Overnight grown culture of *V. parahaemolyticus* ATCC 33847 ( $10^8$  cells/ml) was serially diluted from  $10^4$  to  $10^5$  and the dilutions were used for the following next steps.

To 1ml of bacterial suspension 20 $\mu$ l of antibody was added. It was kept under shaking condition for 30 mins. Twenty  $\mu$ l of Immunomagnetic beads (Dynabeads M-280, Dynal Biotech, Oslo, Norway) was added and mixed for 5 mins. The tubes were then kept on magnetic concentrator and the supernatant was plated. The magnetic beads were collected from the both dilutions ( $10^4$  and  $10^5$ ), washed three times with PBS using magnetic concentrator and suspended in 100 $\mu$ l Millipore water. The suspension was then heated at 100 °C for 15 mins. Tubes were centrifuged at 16000 x g for 10 mins and

supernatant was used as DNA template in q-PCR. The final protocol developed for IMS coupled with q-PCR is shown in Figure 4.1. The procedure was replicated three times.



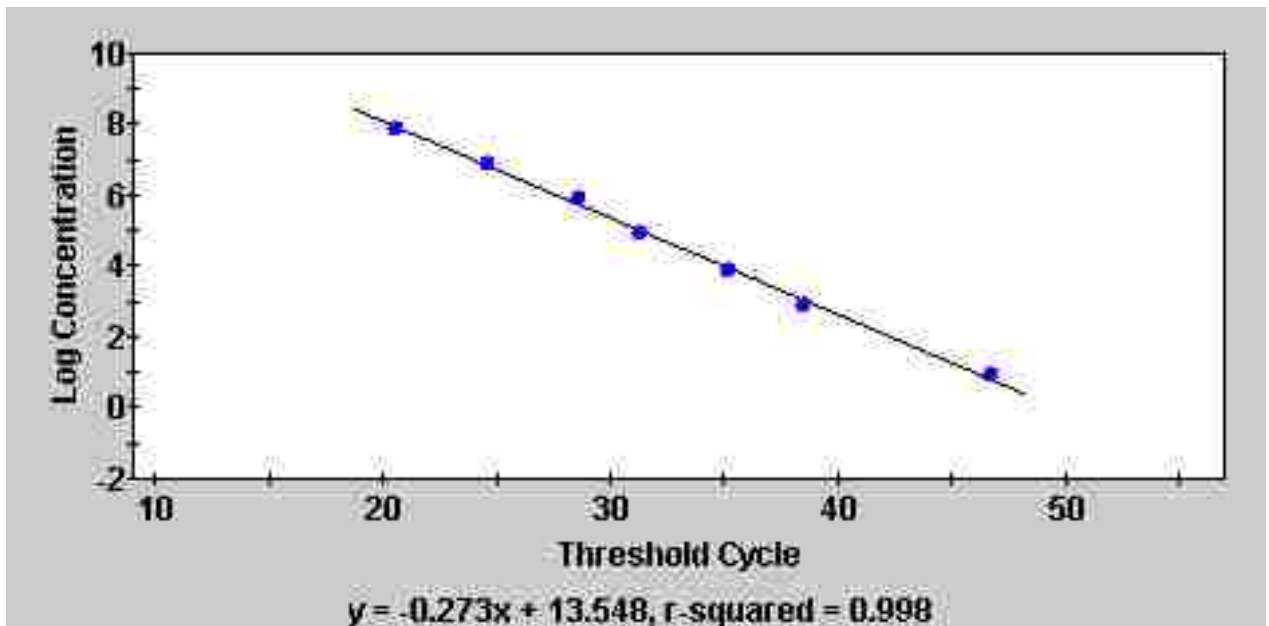
**Figure 4.1: Schematic representation of Immunomagnetic separation coupled with q-PCR protocol for the detection of pathogenic *V. parahaemolyticus* from oyster homogenate**

## **4.3 Results and Discussion**

### **4.3.1 Real-time PCR Assay (q-PCR) Standard Curve**

Previous studies have used q-PCR for the detection of pathogenic *V. parahaemolyticus* in oyster homogenate (Kim et al., 2008; Blackstone et al., 2003 and Bej et al., 1999). This is the first report showing the use of Immunomagnetic separation (IMS) coupled to q-PCR in detecting (*tdh* +) *V. parahaemolyticus* from oyster homogenate. Our study has shown that IMS coupled with q-PCR assay shows both sensitivity and specificity for *V. parahaemolyticus* detection and at the same time it could be used for quantification unlike the conventional PCR.

*V. parahaemolyticus* ATCC 33847 (*tdh* +) produced a fluorescent signal within 25 cycle during q-PCR cycle run (Fig 4.2). The standard curve showed a linear relationship between the log cell numbers and Cycle threshold (*Ct*) values with a strong correlation coefficient of  $r^2 = 0.99$  (Fig 4.2). The q-PCR reaction was able to detect < 10 CFU of *tdh* + *V. parahaemolyticus* within 1 hr. The range of detection ( $10^8$  to  $10^0$ ) shows that the assay retains its sensitivity over a broad range of template DNA concentrations (Blackstone et al., 2003). The *Ct* values increased as the number of CFU/ml decreased in the samples. The assay showed a good correlation between the *Ct* values and the concentration of purified DNA (Fig 4.3). The optic graph of the standard curve shows that the fluorescence was observed as early as 21 cycles and the entire process was completed within 50 cycles. Each line on the optic graph represents a dilution (Fig 4.3).

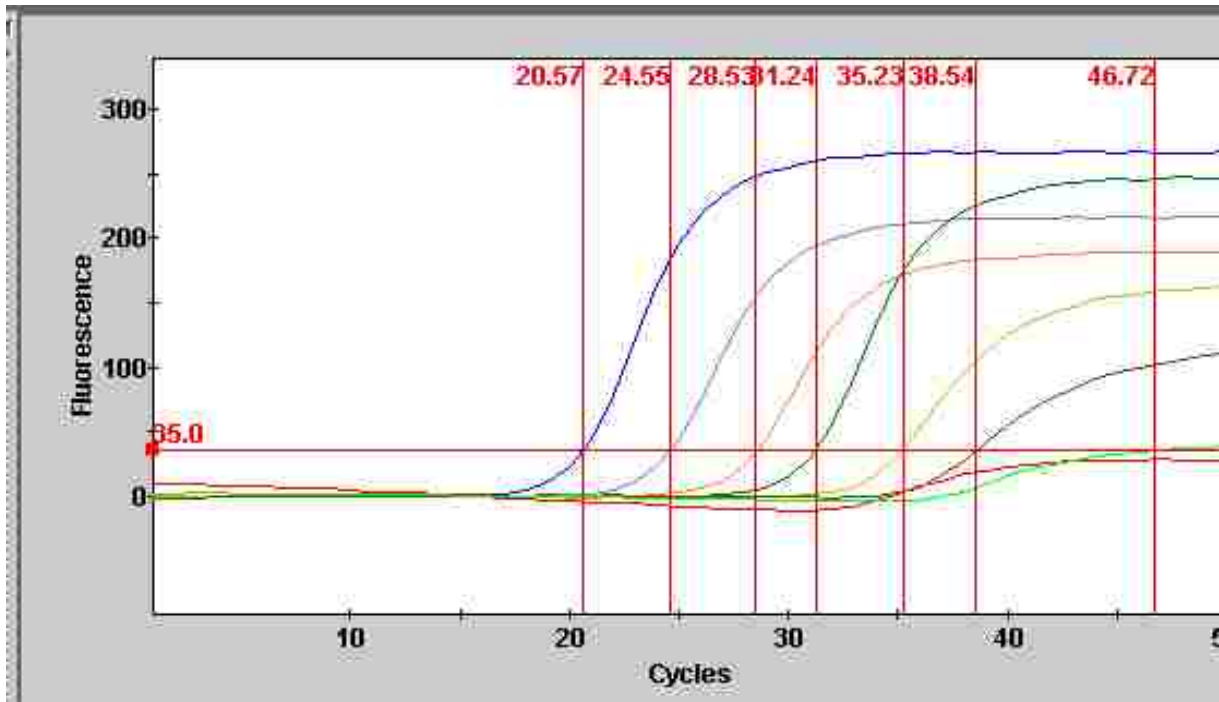


**Fig 4.2:** Standard curve showing the log cell number plotted vs. the q-PCR cycle threshold for serial 10 fold dilutions of *V. parahaemolyticus* culture.

#### 4.3.2 Evaluation of IMS from Shucked Oysters

The sensitivity of the Dynabeads was tested by IMS using decreasing numbers of *V. parahaemolyticus* in the spiked oyster homogenate ( $10^4$  to  $10^3$ ). One of the objectives in

developing an IMS method for the isolation of *V. parahaemolyticus* from the oyster homogenate was to increase the detection sensitivity. Our study showed that with the increasing number of bacteria the percentage of bacteria bound to the beads increased, thereby increasing the sensitivity (Table 4.1)



**Fig 4.3: Representation of optic graph for the number of cycles versus the number of fluorescence units for each sample used to calculate the *ct* value.**

In the IMS protocol with spiked oyster homogenate, an average binding of about 44% was exhibited at 103 *V. parahaemolyticus* CFU/ml and 69 % at 104 CFU/ml respectively. (Table 4.1). A study using IMS to recovered *Listeria monocytogenes* from food sample also showed similar results with a sensitivity for detecting 10-100 *L. monocytogenes* cells /ml of PBS was achieved, an increase in the number of bacteria in suspension resulted in an increase in the number of bacteria bound to the IMB from <



10% (100 L monocytogenes/ml) to 50% (1.5 X 10<sup>4</sup> L. monocytogenes/ml) (Skejerve, 1990).

#### 4.3.3 IMS from Store Bought Oysters Coupled with q-PCR

Real-time PCR sensitivity is affected by sample purity and volume size (Campbell and Wright, 2003). Studies have shown that there was an increase in sensitivity when q-PCR was performed in oyster homogenate seeded with *Vibrio cholerae* using magnetic bead DNA purification method (Lyon et al., 2001). However, the use of magnetic beads did not increase the sensitivity of the detection of *V. vulnificus* in seeded oysters using q-PCR (Campbell and Wright, 2003). In our study to reduce the effect of PCR inhibitors present in the oyster homogenate IMS was performed as a sample preparation step for q-PCR.

**Table 4.1. IMS of oyster homogenate spiked with *V. parahaemolyticus* using anti-H MAb**

<i>V. parahaemolyticus</i> recovered (CFU/ml) <sup>a</sup>		
Inoculum <sup>a</sup>	IMB + MAb <sup>b</sup>	% Binding
1070	635	40
1583	250	84.2
1536	306	81
640	340	53
450	167	37
507	190	42

<sup>a</sup> Number of *V. parahaemolyticus* ATCC 33847 cells in control tubes (Spiked oyster homogenate)

<sup>b</sup> Number of cells remaining in supernatant fluid after IMS. Results are averages of 3 determination.

The q-PCR detected pathogenic *V. parahaemolyticus* at levels of 1000 CFU/g of seeded oyster homogenate. Our study showed that IMS-q-PCR was more rapid than the conventional cultural methods. The IMS procedure took about 2 hrs and q-PCR about 45 minutes, hence the entire IMS coupled with q-PCR was able to detect the bacterial numbers in less than 3 hrs time compared with other conventional methods which takes more than 24-36 days (Nishibuchi et al., 1995; Nordstrom and DePaola, 2003)

During the IMS procedure a large proportion of originally bound bacteria were flushed away by each successive washing steps as was seen in earlier studies (Skjerve, 1990). The washing of the beads affects the recovery hence beads were just washed once before running the q-PCR. The washing of the IMS beads decreased the recovery rates of the bacteria from 40 % to 17% (Data not shown). Hudson et al, 2001 also showed that the number of washing steps results in reducing the recovery of *Listeria* in ham to 50%. They found that washing steps of IMS beads, results in recovery in the order of 1% (Hudson et al., 2001). So they used a single washing step for the recovery of *Listeria monocytogenes* from ham samples using IMS +PCR method (Hudson et al., 2001). Similar recovery rates of 5%-15% were seen from enrichment samples during the detection of *Listeria* from other food samples (Fluit et al., 1993; Uyttendaele et al., 2000). In our study, due to the high viscosity of the oyster homogenate the recovery of the magnetic concentrator could have been impaired; thereby allowing partial recovery of the magnetic beads as it was seen when concentrated bacteria and small food particles impaired the partial recovery of Dynabeads (Uyttendaele et al., 2000).

Our study found the sensitivity of IMS using paramagnetic beads coated with sheep anti-mouse IgG coupled with q-PCR to be  $10^3$  CFU/ml. Widjoatmodjoi and others, 1992 developed a magnetic separation method in combination with PCR for

detection of *salmonella* in feces with a sensitivity of  $10^5$ CFU/ml (Widjoatmodjo et al., 1992). Similarly, direct IMS +PCR showed sensitivity of  $10^5$  CFU/g for detection of *Salmonellae* in poultry (Fluit et al., 1993).

Including an enrichment broth cultivation procedure with IMS-qPCR the sensitivity of the assay increased from  $10^3$  to  $10^2$  CFU/ml (Stone et al., 1994). Stone found a brief enrichment of 2-4 hrs was needed to increase the sensitivity of the entire method. Our study did not have an enrichment step and the sensitivity was  $10^3$  CFU/ml. Percentage recovery of IMS could be improved by enriching the sample with Dyanabeads. Studies have shown that with an enriched sample, the % recovery of the beads increases thereby increasing the sensitivity of the detection  $< 10$  CFU/ml.

Although the recovery of cells by IMS is not sufficient, inclusion of IMS was essential to remove the inhibitors of PCR thereby providing suitable DNA for amplification (Hudson et al., 2001). This study showed that Immunomagnetic separation (IMS) when coupled with q-PCR assay could be used to detect pathogenic *V. parahaemolyticus* from oyster homogenate. The use of monoclonal antibodies coupled with immunomagnetic particles, helps to concentrate the specific bacteria, thereby removing the PCR inhibitors.

Our study showed that IMS coupled with q-PCR was sensitive and specific for detecting pathogenic *V. parahaemolyticus* from oyster homogenate. IMS helped to concentrate *V. parahaemolyticus* from oyster homogenate with a sensitivity of  $10^3$  CFU/ml and the primers used during q-PCR were *tdh* + thus it helped to detect pathogenic *V. parahaemolyticus*. The total time needed for detection of pathogenic *V. parahaemolyticus* with our IMS-qPCR technique was less than 3 hrs.

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## **CHAPTER 5**

### **DEVELOPMENT OF DIRECT COLONY IMMUNOBLOT (DCI) FOR ENUMERATION OF + TDH *VIBRIO PARAHAEMOLYTICUS* USING ANTI- HEMOLYSIN SERUM**



## 5.1 Introduction

*Vibrio parahaemolyticus* is an organism of public health concern causing acute gastroenteritis and septicemia as a result of consumption of raw or undercooked seafood (Ward and Bej, 2005). The pathogenic strain of *V. parahaemolyticus* is of a particular concern with molluscan shellfish such as oysters because they are commonly consumed raw.

Pathogenic *V. parahaemolyticus* strains produce a thermostable direct hemolysin (TDH) that is encoded by *tdh* gene (DePaola et al., 1990; Kaysner et al., 1990). 90% of clinical *V. parahaemolyticus* are *+tdh*, whereas less than 1% of food and environmental isolates are *+tdh*. Hence a method is needed which would be sensitive enough to detect this small number of pathogenic *V. parahaemolyticus* using a user-friendly method.

The conventional bacteriological methods for detection of total *V. parahaemolyticus* in raw oysters involve selective enrichment, followed by isolation on selective agar plates and biochemical identification of the isolates (Kaysner and Depaola, 1998). The recovery, identification and enumeration of *V. parahaemolyticus* in oysters can be time consuming and overwhelming in logistics. In 1992, Yamamoto et. al., developed alkaline phosphatase-labeled oligonucleotide probes to detect *tdh* and *trh* genes of *V. parahaemolyticus*. Ellison et. al in 2001 compared the standard MPN method to the DNA hybridization method that uses a direct plating protocol employing alkaline phosphatase-labeled DNA probe to determine the levels of *V. parahaemolyticus* in retail oysters. A good correlation between the two methods were observed. This method using an enzyme -labeled DNA probe greatly reduced the time and labor for *V. parahaemolyticus* identification (Ellison et al., 2001). The Interstate shellfish sanitation conference (ISSC) approved the use of the DNA hybridization method using probes to

detect *tlh* gene for total and *tdh* gene for pathogenic strains of *V. parahaemolyticus* (Cook et al., 2000). Since then nonradioactive probes for *tlh* and *tdh* have been used successfully and routinely employed for detection and enumeration of *V. parahaemolyticus* in retail oysters (Cook et al., 2002), shellfish and sediments (DePaola et al., 2003) and freshly harvested oysters (DePaola et al., 2003; Kaufman et al., 2003). But the DNA hybridization method requires expensive reagents, stringent temperature requirements and the entire procedure could take 2 days to have results.

Studies have shown that *Vibrios* express flagellar antigens unique to the species (Bhattacharyya, 1975). Anti-*V. vulnificus* H antibodies have been developed and employed in a direct colony immunoblot (DCI) protocol for detection and enumeration of *V. vulnificus* in oysters (Senevirathne et al., 2009). Direct colony immunoblot proved to be a faster, user-friendly method and showed good correlation with DNA hybridization method (Senevirathne et al., 2009). For *V. parahaemolyticus* a method needs to be developed that can detect pathogenic strains from non-pathogenic. As the percentage of pathogenic *V. parahaemolyticus* is so less compared to a non-pathogenic strains.

The ability of a pathogenic *V. parahaemolyticus* to cause a disease is attributed to the amount of hemolysin it produces. Hemolysin is only produced by the pathogenic *V. parahaemolyticus* which is due to the presence of (*tdh*+) genes. Antibodies against the anti-hemolysin serum could be employed in DCI method to detect and enumerate pathogenic *V. parahaemolyticus*. The DCI procedure takes less than 4 hrs and it doesn't require use of any expensive reagents or equipment making it an easy user-friendly method which could be used for routine analysis. The objective of this study was to examine the use of a DCI method to detect and enumerate pathogenic *V. parahaemolyticus*.

## **5.2 Materials and Methods**

### **5.2.1 Bacterial Cultures and Media Used**

*Vibrio parahaemolyticus* ATCC 33847 + *tdh* and *V. parahaemolyticus* 33z4 –*tdh* (provided by NC state food micro lab) were maintained in semi solid maintenance medium (8g typtone, 20g NaCl, 4g nutrient broth, 4g MgCl<sub>2</sub>, 4g KCl, and 4g agar). The cultures were transferred monthly throughout the study to maintain there viability.

### **5.2.2 Production of Crude and Partially Purified Thermostable Direct Hemolysin**

*Vibrio parahaemolyticus* ATCC 33847 was grown for 16 h in 3 L of broth containing 30 g NaCl, 10 g peptone, 5 g Na<sub>2</sub>HPO<sub>4</sub> and 5 g glucose per liter (pH 7.6-7.8) on a shaker at 35°C. The cells were removed by centrifugation and ammonium sulfate (35.1g/100ml) was added slowly to the culture supernatant fluid. The resulting precipitate was dissolved in 100 ml of 0.01 M phosphate buffer, pH 7.0 followed by several buffer changes. The crude hemolysin was then applied to DEAE sepharose column equilibrated with PBS. The column was washed with 500 ml phosphate buffer containing 0.2 M NaCl. The hemolysin was then eluted with 500 ml of phosphate buffer containing 0.5 M NaCl. The fractions were collected and the absorbance was determined at 280 nm. The fractions representing the eluting hemolysin peak were pooled and the hemolysin was precipitated by addition of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (40g/100ml), dissolved in phosphate buffer and dialysed extensively with phosphate buffer containing 0.0001% thimerosal.

### **5.2.3 Production of Anti-hemolysin Serum**

A New Zealand white rabbit was immunized by intramuscular injection of 2 mg of crude hemolysin (0.5 ml mixed with an equal volume of adjuvant (Sigma Titermax). One month later, the rabbit was given 2 intramuscular injections and 1 subcutaneous injection of 100 µg partially purified hemolysin mixed with adjuvant (2 ml total volume).

Seventeen days later, a final rabbit was exsanguinated. The bleed-out anti-hemolysin titer was 16,000 by EIA using partially purified hemolysin as antigen (10µg/well). The rabbit serum was positive by capillary precipitation at a hemolysin concentration of 10µg/ml.

#### **5.2.4 Direct Colony Immunoblot (DCI)**

The *vibrio* cultures were grown on selective media, *V. vulnificus* agar (VVA)(BAM manual; <http://www.cfsan.fda.gov>), thiosulfate-citrate bile salts agar (TCBS)(Acumedia manufactures INC, MI ,USA) and non-selective media, nutrient agar with 3% NaCl (Sigma Chemical Co., St. Louis, MO, USA) and TSA + 3% NaCl media plates to determine media which gave the best results with DCI.

The overnight grown colonies were directly transferred from these plates to polyvinylidene fluoride membranes (Pall Corporation 8780 Ely Rd, Pensacola, FL, USA) and air dried for 10 min. The membranes were notched and the plates were marked at identical positions so that proper orientation of the colonies was maintained. The membranes were then placed on a new petri-dish colony side up and washed with gentle agitation at room temperature with 20 ml 0.067 M phosphate buffered saline, pH 7.1-7.2 containing 0.05% Tween-20 (PBS T-20). After the first wash the membranes were moved to a new petri-dish and incubated with 1% bovine serum albumin (BSA) in PBS for 30 min to block non-specific binding sites. The membranes were then washed once with PBS T -20 and treated with 20 ml anti-hemolysin serum diluted 1:2000 in PBS for 30 min. The membranes were washed four times 5 min each with PBS T-20 to remove the unbound antibody and thereafter incubated with 20 ml peroxide-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO, USA) diluted 1:2000 in PBS-1% BSA for 30 min. The membranes were then again washed four times 5 min each and a color development solution was added (20 ml 0.05M Tris, pH 7.6, 10 mg 3, 3'-

diaminobenzidine tetrahydrochloride, 1 ml 8% NiCl<sub>2</sub> and 0.1ml 30% H<sub>2</sub>O<sub>2</sub>) for 5 min. The color development reaction was then stopped by washing the filters in distilled water. The washing solutions from all the steps were pooled together and treated with chlorine before disposal.

### **5.2.5 DNA Probe Hybridization (DNAH)**

The colonies were transferred from VVA plates to Whatman # 541 filter paper disks. The filter paper was then wetted in 1ml of lysis solution (0.5M NaOH, 1.5 M NaCl) and dried in microwave oven to disrupt the cells. The filters were then neutralized with 4ml of 2 M ammonium acetate buffer, rinsed twice and treated with proteinase K for 30 min at 42°C with gentle agitation on an orbital shaker at room temperature. The filters were then rinsed three times with hybridizing buffer (0.5g BSA, 1g Sodium dodecyl sulfate, 0.5 g polyvinylpyrrolidone and 100ml of 5X standard saline citrate solution (SSC) at 55°C for 30 min. Alkaline phosphatase labeled *tdh* probe (DNA Technology A/S, Denmark) was then added in the buffer and the membranes were incubated for 1 hr at 55°C. The filters were then washed five times 5 for mins each with 1X SSC and incubated with the substrate solution (2 Nitro blue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate) ready to use tablets (Roche Diagnostics, Indianapolis, IN, USA) in 20 ml distilled water. Color development was stopped by washing the filters with distilled water (Kaysner and DePaola, 2004).

### **5.2.6 Enumeration of *V. parahaemolyticus* +*tdh* Mixed with -*tdh* Strains**

*V. parahaemolyticus* ATCC 33847 +*tdh* and *V. parahaemolyticus* 33z4 -*tdh* were grown separately in 10 ml of Nutrient broth with 3% NaCl (N-3% NaCl) overnight at 37°C. Then 15 µl of the overnight grown culture was transferred to 10 ml of N-3% NaCl and incubated at 37°C for 16 h. The bacterial cells were collected by centrifugation at

4000 rpm for 5 mins and the pellet was resuspended in 10 ml of PBS. Serial ten-fold dilutions of both *+tdh* and *-tdh V. parahaemolyticus* strains were made in PBS. 100 µl of each dilution of *+tdh* and *-tdh V. parahaemolyticus* were mixed and spread on VVA plates. The plates were incubated at 35°C for 16 h and CFU/ml was determined the next day. The DCI was performed from the plates immediately followed by DNAH.

### **5.2.7 Statistical Analysis**

The DCI method was compared with DNAH by statistical comparisons of all pairs using Tukey-Kramer HSD (JUMP, SAS Institute Inc., Cary, N.C., U.S.A). All experiments were repeated 3 times with 3 replications per experiment.

## **5.3 Results and Discussion**

### **5.3.1 Production of Crude and Partially Purified Thermostable Direct Hemolysin**

The DEAE purified hemolysin (1mg/ml) exhibited 8 times more red cell lysis than the crude hemolysin using 1% sheep red blood cells. The crude hemolysin exhibited a specific activity of 0.5 hemolytic units/mg proteins while the partially purified hemolysin exhibited a specific activity of 16 hemolytic units/mg protein.

The bleed-out anti-hemolysin titer was 16,000 by EIA using partially purified hemolysin as the antigen (10µg/well). The rabbit serum was positive by capillary precipitation at a hemolysin concentration of 10µg/ml.

### **5.3.2 Detection of *+tdh V. parahaemolyticus* by DCI**

We developed a DCI method for detection of pathogenic *+tdh V. parahaemolyticus*. The entire procedure took less than 4 h to complete at room temperature without the use of any expensive instruments. In the process of developing the DCI method we tested four different selective media selective media such as *Vibrio vulnificus* agar VVA and TCBS; non-selective media such as Nutrient agar + 3% NaCl

(NA +), TSA + 3% NaCl for growing up the + *tdh* and - *tdh* *V. parahaemolyticus*. It was determined that out of the all the four medias, VVA media gave the best results. The colonies on this plate appeared to be smaller and less slimy than the rest of the medias used and prevent non-specific binding (data not shown). Kaysner and DePaola,2004 (FDA) also recommended the use of VVA plates for the direct plating of oyster homogenate while performing the DNA hybridization protocol (Kaysner and DePaola, 2004).

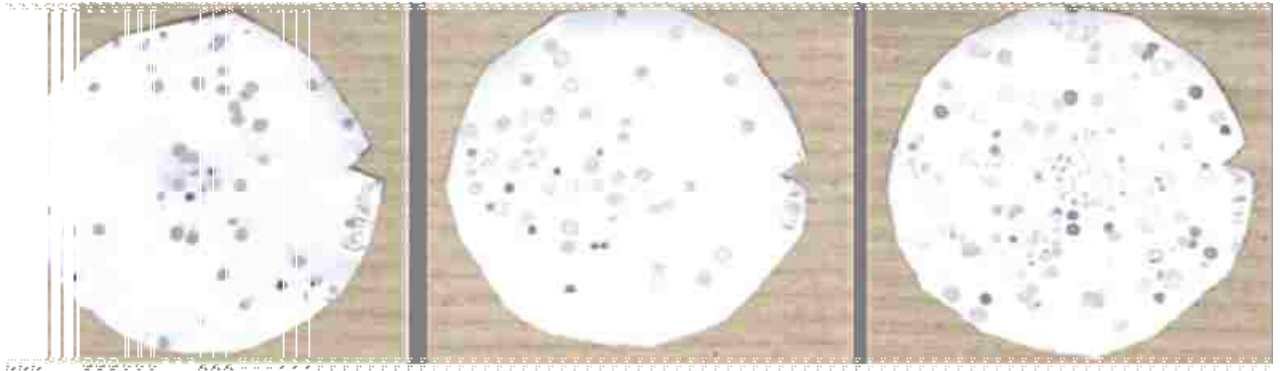
The rabbit anti-hemolysin serum was diluted 1:1000, 1:2000, 1:4000 to determine the highest dilution the DCI procedure would produce optimal color development of the + *tdh* *V. parahaemolyticus* colonies on VVA plates. The 1:2000 dilutions showed the maximum color development and this dilution was chosen for the rest of the study (Figure 5.1). Similar results were observed in the DCI optimized for detection of *V. vulnificus*, where a 1:2000 dilution gave the best color development during the DCI protocol (Senevirathne et al., 2009).

To determine the sensitivity and specificity of the DCI assay + *tdh* and -*tdh* *V. parahaemolyticus* strains were spread on VVA plates separately (Figure 5.2). The +*tdh* *V. parahaemolyticus* were easily identified from -*tdh* *V. parahaemolyticus* isolated by production of dark purple color on the membrane.

### **5.3.3 Enumeration of +*tdh* *V. parahaemolyticus* Mixed – *tdh* *V. parahaemolyticus* Cultures**

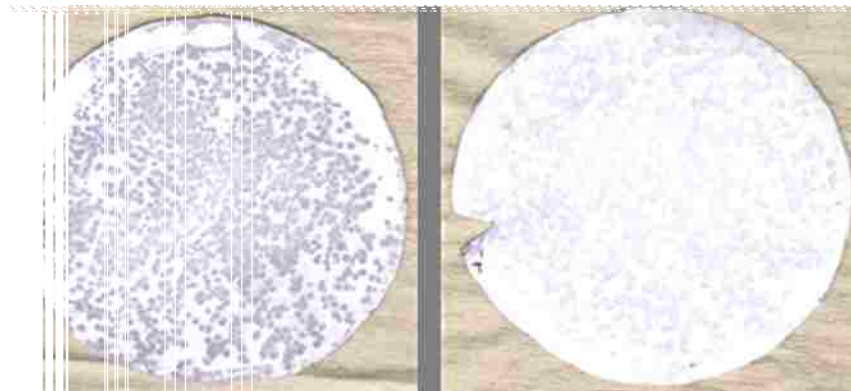
*V. parahaemolyticus* +*tdh* was easily detected and enumerated by both DCI and DNAH in mixed (+ and - *tdh*) *V. parahaemolyticus* cultures spread on VVA plates. *V. parahaemolyticus* +*tdh* colonies produced an intense purple color against the polyvinylidene fluoride membranes used during the DCI (Fig 5.3, A). On the other hand

brown color was produced on Whatman # 541 filter paper disks by DNAH as expected (Fig5.3,B).



**Figure 5.1: DCI membranes showing different dilutions of antibodies for detection of + *tdh* *V. parahaemolyticus* colonies. Antibodies were diluted to 1:1000, 1:2000, 1:4000.**

A weak cross reaction was noticed for the *-tdh V. parahaemolyticus* isolated but this was insignificant when compared to the non-specific color development by DNAH at lower hybridizing temperature (Wright et al., 1993; Senevirathne et al., 2009).



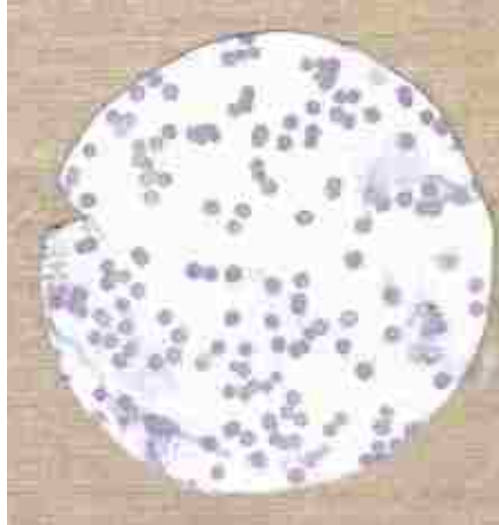
A)

B)

**Fig 5. 2: DCI membranes showing the reaction of *V. parahaemolyticus* +*tdh* (A) and *-tdh* (B) colonies against the anti-hemolysin antibodies from separate VVA**



However the color intensity produced by DCI was much more intense against the background filters than produced during DNAH which makes a DCI more easily readable method by a non-technical person (Fig 3, A; B). Similar non-specific binding was observed when *V. vulnificus* was enumerated by the DCI method in mixed cultures (Senevirathne et al, 2009).



**(A) DCI membrane, with a mixed culture of *+tdh* and *-tdh* *V. parahaemolyticus* strains.**



**(B) DNAH membrane, with a mixed culture of *+tdh* and *-tdh* *V. parahaemolyticus* strains.**

**Fig 5. 3: Detection of *+tdh* *V. parahaemolyticus* colonies using DCI and DNAH**

**Table 5.1: Enumeration of +*tdh* *V. parahaemolyticus* colonies in a mixed sample containing +*tdh* and – *tdh* *V. parahaemolyticus* cells. Bacteria counted (log CFU/ml)<sup>a</sup>**

<i>V. parahaemolyticus</i> ( <i>tdh</i> +) <sup>b</sup>	DCI <sup>c</sup>	DNAH <sup>d</sup>
2 log	2.03 ± 0.28 A	2.05 ± 0.27 A
	1.74 ± 0.65 A	1.85 ± 0.50 A
	1.88 ± 0.40 A	1.85 ± 0.39 A
	1.60 ± 0.51 A	1.55 ± 0.38 A
3 log	2.95 ± 0.40 A	2.97 ± 0.41 A
	3.15 ± 0.30 A	3.15 ± 0.29 A
	3.01 ± 0.46 A	3.02 ± 0.48 A
	3.25 ± 0.42 A	3.28 ± 0.44 A

<sup>a</sup>All analysis were based on three separate experiments with each mean ± standard deviation being average of three determinations. Means with each vertical column followed by the same letter are not significantly different ( $P \geq 00.05$ ) from each other.

<sup>b</sup>Number of +*tdh* *V. parahaemolyticus* colonies as determined by growth and appearance on VVA plates.

<sup>c</sup>Number of +*tdh* *V. parahaemolyticus* colonies as determined by Direct colony immunoblot.

<sup>d</sup>Number of +*tdh* *V. parahaemolyticus* as determined by DNA hybridization.

The sensitivity of the DCI was compared to DNAH by mixing +*tdh* *V. parahaemolyticus* cells with increasing concentration of –*tdh* *V. parahaemolyticus* cells ( $10^2$ - $10^5$  CFU/ml). The DCI and DNAH showed equal sensitivity since +*tdh* *V. parahaemolyticus* colonies could be enumerated by both the methods when the levels of +*tdh* *V. parahaemolyticus* cells were inoculated at 2 and 3 log CFU/ml and –*tdh* *V. parahaemolyticus* cells were increased (Table 1). There were no significant differences in the results when DCI was compared with DNAH at different –*tdh* *V. parahaemolyticus* levels. Similar results were observed when DCI and DNAH was used to detect 1-2 log CFU/ml of *V. vulnificus* mixed with 4 log CFU/ml of *V. parahaemolyticus* (Senevirathne et al, 2009).

Results show that DCI and DNAH methods were comparable and demonstrated no significant statistical differences when enumerating pathogenic *V. parahaemolyticus*

+*tdh* in a mixed culture of non-pathogenic *V. parahaemolyticus*. DCI is a faster (4h) and simpler method which showed good correlation with the currently used method, DNAH. In addition DCI would require less equipment and stringent laboratory conditions than DNAH.

The DCI method using anti-hemolysin serum could be an useful addition to the methods available for enumerating pathogenic *V. parahaemolyticus*.

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**CHAPTER 6**  
**CONCLUSIONS**

We successfully produced anti-H monoclonal antibodies against the polar flagella of *V. parahaemolyticus*. The serological specificity and potential reactivity of anti-H monoclonal antibody was examined by slide agglutination. An Immunomagnetic separation (IMS) protocol was then optimized to assess the efficiency of the anti-H MAb to isolate and concentrate *V. parahaemolyticus*.

Our study showed that IMS coupled with real-time PCR (q-PCR) was sensitive and specific for detecting pathogenic *V. parahaemolyticus* from oyster homogenate. IMS helped to concentrate *V. parahaemolyticus* from oyster homogenate with a sensitivity of  $10^3$  CFU/ml and the primers used during q-PCR were *+tdh* that to detects pathogenic *V. parahaemolyticus*. The total time needed for detection of pathogenic *V. parahaemolyticus* with our IMS-qPCR technique was less than 3 hrs.

The ability of a pathogenic *V. parahaemolyticus* to cause a disease is attributed to the amount of hemolysin it produces, antibodies against the anti-hemolysin serum was employed in direct colony immunoblot (DCI) method to detect and enumerate pathogenic *V. parahaemolyticus*. Our study showed that DCI is a faster (4h) and simpler method which showed good correlation with the currently used method, DNAH. In addition DCI requires less equipment and stringent laboratory conditions than DNAH.

From our results we have found that IMS+q-PCR or DCI could be useful addition to the methods available for enumerating pathogenic *V. parahaemolyticus* in seafood.

## APPENDIX 1

### LIST OF DIFFERENT *VIBRIO* SPECIES USED

Species	Culture Number	Type of Strain	Source <sup>a</sup>
<i>Vibrio parahaemolyticus</i>	ATCC 17802	Clinical	ATCC
<i>Vibrio parahaemolyticus</i>	ATCC 33847	Clinical	ATCC
<i>Vibrio vulnificus</i>	ATCC 27562	Clinical	ATCC
<i>Vibrio vulnificus</i>	Vv 1001	Clinical	LDHH
<i>Vibro mimicus</i>	ATCC 33653	Clinical	ATCC
<i>V. fluvialis</i>	ATCC 33809	Clinical	ATCC
<i>V. natriegens</i>	ATCC 14048	Clinical	ATCC
<i>V. alginolyticus</i>	ATCC 33787	Clinical	ATCC
<i>V. harveyi</i>	ATCC 14126	Clinical	ATCC
<i>V. harveyi</i> <sup>b</sup>	ATCC 35084	Clinical	ATCC
<i>V. campbellii</i>	ATCC 25920	Clinical	ATCC
<i>Vibrio parahaemolyticus</i>	132f4	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	212 t1	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	212 F4	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	212 T10	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132 y2	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132e2	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	212 T3	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132x2	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132e3	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132 e31	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132a1	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	212 t12	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132a2	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132y1	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132 f3	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132f2	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132e6	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132e12	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132y3	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132 a3	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132z5	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132e4	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132ea	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132e1	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132 e21	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132x5	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132e7	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	121z13	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132f1	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132 s11	Environmental	NC State

<i>Vibrio parahaemolyticus</i>	132x1	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132e11	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132s6	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132 e8	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132z4	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132e7	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132x3	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132s2	Environmental	NC State
<i>Vibrio parahaemolyticus</i>	132 s11	Environmental	NC State
<i>Vibrio vulnificus</i>	132B8	Environmental	NC State
<i>Vibrio vulnificus</i>	132B5	Environmental	NC State
<i>Vibrio vulnificus</i>	132A1	Environmental	NC State
<i>Vibrio vulnificus</i>	132y3	Environmental	NC State
<i>Vibrio vulnificus</i>	132e10	Environmental	NC State
<i>Vibrio vulnificus</i>	132T5	Environmental	NC State
<i>Vibrio vulnificus</i>	132Z2	Environmental	NC State
<i>Vibrio vulnificus</i>	342e3	Environmental	NC State
<i>Vibrio vulnificus</i>	342e4	Environmental	NC State
<i>Vibrio vulnificus</i>	342e6	Environmental	NC State
<i>Vibrio vulnificus</i>	342e7	Environmental	NC State
<i>Vibrio vulnificus</i>	212B6	Environmental	NC State
<i>Vibrio vulnificus</i>	212F11	Environmental	NC State
<i>Vibrio vulnificus</i>	212S8	Environmental	NC State
<i>Vibrio vulnificus</i>	212y10	Environmental	NC State
<i>Vibrio vulnificus</i>	212F15	Environmental	NC State
<i>Vibrio vulnificus</i>	212F18	Environmental	NC State
<i>Vibrio vulnificus</i>	212e14	Environmental	NC State
<i>Vibrio vulnificus</i>	212S7	Environmental	NC State
<i>Vibrio vulnificus</i>	212e12	Environmental	NC State
<i>Vibrio vulnificus</i>	212y13	Environmental	NC State
<i>Vibrio vulnificus</i>	212T13	Environmental	NC State
<i>Vibrio vulnificus</i>	212e21	Environmental	NC State
<i>Vibrio vulnificus</i>	212F14	Environmental	NC State
<i>Vibrio vulnificus</i>	212e15	Environmental	NC State

<sup>a</sup> ATCC- American Type Culture Collection; LDHH- Louisiana Department of Health and Hospitals; NC State- North Carolina State University, Department of Food Science

<sup>b</sup>Originally deposited as *V. carchariae*



## APPENDIX 2

### LIST OF MEDIAS AND REAGENTS USED

- 1) Phosphate Saline buffer (PBS)- (NaCl-7.65g, Na<sub>2</sub>HPO<sub>4</sub>, anhydrous-0.724 g, KH<sub>2</sub>PO<sub>4</sub>-0.210 g, d. water- 1000 ml) Dissolve the ingredients in d. water and adjust the pH to 7.4 (with 1 N NaOH)
- 2) Alkaline peptone Water (APW)- (Peptone- 10g, NaCl- 10 g , d. water- 1000ml). Dissolve the ingredients and adjust the pH to 8.5. Autoclave at 121 C for 15 min.
- 3) VVA Agar- Solution 1 - Peptone-20 g, NaCl-30 g, 100X-Dye stock solution\*-10 ml. Agar-25 g, Distilled water 900 ml) Adjust to pH 8.2. Boil to dissolve agar. Autoclave for 15 min at 121°C. Bring it back to 50°C. \*100X Dye stock solution- Bromthymol blue -0.6 g, 70% Ethanol -100ml. Solution 2- Cellobiose- 10 g , d. water -100ml. Dissolve cellobiose in d .water by gently heating. Add Solution 2 to cooled Solution 1, mix, and dispense into petri dishes. Final color of the media is light blue.
- 4) TCBS Agar – Made according to manufacturer’s instructions (Troy Biologicals Inc. Troy, MIL).
- 5) Tris Buffer solution- Add 0.788g of Tris in hydrochloric acid to 90 ml of distilled water, adjust the pH to 7.6. with NaOH and bring the volume to 100 ml.
- 6) Nutrient Agar with 2% NaCl- Made according to manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO 63103).
- 7) Alkaline Peptine broth- (Bacto peptone- 10 g, NaCl- 20 g, Yeast extract- 2 g, KCl- 2 g, MgCl<sub>2</sub>.6H<sub>2</sub>O-2g, d. water- 1000ml). pH was adjusted to 7.2.
- 8) Vibrio Maintenance medium- Tryptone-8g, NaCl- 20 g, Nutrient Broth- 4g, MgCl<sub>2</sub>- 4g, KCl- 4g, Agar- 4 g, d.water- 1000 ml)

- 9) Coupling buffer – ( $\text{Na}_2\text{CO}_3$ -1.6 g,  $\text{NaHCO}_3$ -2.9g,  $\text{NaN}_3$ - 0.9 g, d. water -1000ml)
- 10) Washing Buffer- ( PBS- 500 ml,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ -1.5mM, Mercaptoethanol- 2mM, BSA- 0.5 g)
- 11) Diethanolamine buffer- Diethanolamine- 97 ml,  $\text{NaN}_2$ - 0.2g,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ - 100mg, d. water- 800 ml) Dissolve the ingredients and adjust the pH to 9.8. Bring the volume to 1 l with d. water.
- 12) Lysis Solution (0.5 M NaOH, 1.5M NaCl)- NaOH- 20g, NaCl- 87g , d.water- 1000ml.
- 13) Ammonium acetate buffer (2M)- Ammonium acetate- 154g, d. water- 1000ml
- 14) Standard Saline Citrate Solution (SSC)- For making 20 X SSC- (NaCl- 175.4 g, Sodium Citate.  $2\text{H}_2\text{O}$ -88.2 g, d. water- 1000ml). Dissolve the content and adjust the pH to 7 with 10 N NaOH. Bring the volume to 1000ml.
- 15) 5 X SSC- 20 X SSC- 25 ml, d.water- 75 ml
- 16) 3 X SSC- 20 X SSC-150 ml, d. water- 850 ml
- 17) 1 X SSC- 20 X SSC -50 ml m d. water- 950 ml
- 18) Hybridization buffer- BSA- 0.5g, Sodium Lauryl Sulfate-1.0g, Polyvinylpyrrolidone -0.5 g, 5 x SSC-100 ml. Warm to  $54^\circ\text{C}$  before use.
- 19) NBT/BCIP solution- Just before use, 2 NBT/ BCIP ready- to-use tablets (Boehringer Mannheim, Cat. No. 169771) in 20 ml d. water

## APPENDIX 3

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## VITA

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