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Application of Anti-H Monoclonal Antibodies to Develop Rapid Immunoassay for *Vibrio vulnificus*

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**APPLICATION OF ANTI-H MONOCLONAL ANTIBODIES TO
DEVELOP RAPID IMMUNOASSAY FOR *VIBRIO VULNIFICUS***

**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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December 2010,**

**This work is dedicated to
my major professor Dr. Marlene Janes**

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ABSTRACT

In the US there are about 76 million foodborne illness cases reported every year, in spite of initiatives by federal agencies. This emphasizes the need for the development of novel detection techniques which are sensitive, specific and rapid. A detection technique should be rapid enough to give results same day, allowing companies to release food lots without delay and decreasing storage costs. This kind of screening would also allow immediate measures, if needed, before releasing the lots. In the case of *Vibrio vulnificus* (*V. vulnificus*), conventional methods are available to identify and enumerate this pathogen in oysters, but they are labor-intensive and time consuming. To maintain a constant supply of safe oysters, methods need to be developed that are sensitive and rapid. Application of species-specific monoclonal antibody (MAB) can increase the sensitivity and speed of *V. vulnificus* detection by eliminating enrichment steps, hence the objective of our study was to develop antibody based rapid and sensitive *V. vulnificus* detection methods. In the first method monoclonal antibodies were utilized in the development of an immunomagnetic separation (IMS) protocol, which was then combined with rt-PCR to develop rapid method which can detect presence of *V. vulnificus* in oysters within 3 h with a sensitivity of 10^2 CFU/ml oyster homogenate. We have also used our anti *V. vulnificus* -H species specific monoclonal antibodies to develop a lateralflow detection device (dip stick) which when combined with a short 5 h enrichment step was successfully able to identify less than 10 CFU/ ml of *V. vulnificus* from oyster homogenate. Our IMS rt-PCR and dip stick assay could be an answer to seafood industries rapid pathogen detection needs.

CHAPTER 1
INTRODUCTION

1.1 Introduction

Louisiana is the largest oyster producing state in the USA with a contribution of about 30% of the total volume of production in the country. *Vibrio vulnificus* (*V. vulnificus*) infection is a disease with serious health implications but, it is comparatively an uncommon illness. (Matui, 2004). Notably there are only about 50 confirmed cases of *V.vulnificus* per year (Strom and Paranjpye, 2000) but, the severity of the disease that is caused by this organism makes it the most pathogenic organism that causes the majority of the fatalities linked to seafood in the USA. (Morris et al, 1985; Morris et al, 1988). The organism is infamously associated with shellfish especially raw oysters and this could lead to the abated consumer confidence regarding the safety of the shellfish and could directly impact the shellfish industry (Mackenzie, 1997).

The CDC in 1964 first isolated *V. vulnificus* but it was misinterpreted as virulent strain of *Vibrio parahaemolyticus*. Due to many clinical cases of foodborne septicemias and wound infections with characteristics distinctive from other *Vibrio* species it was later recognized as a separate species only in the 1970's. (Morris et al, 1988; Blake et al, 1979; Hollis et al, 1976). Out of 422 infections reported between 1988 and 1996, 45% were wound infections, 43% primary septicemia, 5% gastroenteritis, and the remaining 7% from undetermined exposure. (CDC, 1998) *V.vulnificus* is very susceptible to heat and many thermal treatments have been shown to be effective in reducing the bacterial load (Kim et al., 1997), but the biggest drawback of such methods is the loss of sensory attributes which consumers like in raw oysters. Hence in order to balance the food safety aspect of raw oysters while ensuring the supply of raw oysters, it is necessary that there is an assured distribution of raw oysters with levels of pathogens at very low levels as recommended by the Interstate shellfish sanitation conference (ISSC). In order to

achieve this there is a crucial need for development of rapid detection methods that would enable the processors to evaluate the microbiological quality of the pathogens within the given lot.

Currently there are many detection methods available that are approved by agencies like the FDA, but the majority of them are time consuming conventional methods that would require long enrichment periods (up to 24 h) (Kaysner and DePaola, 2005). Oyster processing companies would not be able to hold the batches for long times until they get the results of the tests due to various reasons such as storage and logistics etc. Hence, the current study was aimed at developing rapid detection methods for in lab and onsite use. Utilizing the serological and immunological methods already available and developing novel techniques to improve the sensitivity and selectivity of the rapid detection methods was the main aim of the study.

In order to develop rapid detection techniques utilizing species specific monoclonal antibody, the prime objectives of this study were to:

- (1) Standardize the IMS protocol to concentrate *V. vulnificus* from environmental samples
- (2) Develop a detection method which can identify *V. vulnificus* from oyster homogenate within 3 hours by combining IMS with RT- PCR
- (3) Develop a lateral flow chromatography test strip utilizing monoclonal antibody for point of care (POC) detection of *V. vulnificus*

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CHAPTER 2
LITERATURE REVIEW

2.1 General Information

Vibrios' are notorious for causing many deaths worldwide and members of this genus can cause infections in various forms ranging from gastroenteritis, septic shock to soft tissue necrosis. According to the CDC (2007) last year all of the *Vibrio* spp. together were responsible for causing 568 illnesses and 36 deaths in the United States which is a substantial increase compared to 2006 with 175 illnesses and 17 deaths. There are at least 12 out of 76 known *Vibrio* spp. that are recognized as human pathogens. The most common pathogenic *Vibrio* species include *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. fluvialis*, *V. furnissii*, *V. hillisae*, *V. metschnikovii*, *V. damsela* and *V. mimicus* (Pruzzo et al., 2005). Warm and halophilic marine environments which are very important for the production of good tasting and large oysters also favor the accumulation and growth of vibrios (DePaola et al., 1994).

V. vulnificus is considered as one of the most lethal of all pathogenic vibrios with fatality rates of up to 60% (ISSC, 2003, Linkous and Oliver, 1999). A close look at the CDC data suggests that *V. vulnificus* was the cause of 31 out of 36 vibrio related deaths, these statistics show how important it is to study and prevent *V. vulnificus* infections. *V. vulnificus* like other vibrios is naturally present in warm estuarine environments around the globe. They are gram-negative, slightly curved rod shape motile bacteria found in aquatic habitats (Panicker et al., 2004). Temperature is one of the most critical factors associated with the presence of *V. vulnificus* in seawater and shellfish, various studies reported the linear relationship between bacterial number and temperature and also observed that as the temperature dropped below 15⁰ C, levels of *V. vulnificus* decreased to undetectable levels (Pfeffer et al., 2003). Salinity also has a significant effect on *V. vulnificus* survival preferring a low to intermediate salinity (5-25%) and

inhibitory effect of elevated levels of salinity (>25‰) have been reported (Motes et al., 1998). The distribution of *V. vulnificus* in seafood and environment is closely related to reported illnesses (Motes et al., 1998, Cook et al., 2002). During winter months (November to March) *V. vulnificus* counts are low, typically less than 10³ CFU/g of Gulf Coast harvested oysters, but bacterial count increases with temperature, by the end of April *V. vulnificus* density usually exceeded 10³ CFU/g (Motes et al., 1998). An average of 10⁴ CFU/g or more of *V. vulnificus* have been reported for oysters during the summer months (Oliver and Kaper, 1997). If not refrigerated rapidly *V. vulnificus* multiply rapidly in oysters and hence, levels at market can be >1 log greater than at harvest (Cook 1997). Beside shellfish *V. vulnificus* is also found in coastal and estuarine waters worldwide (Kaysner et al., 1987, Oliver and Kaper 2001). The levels of *V. vulnificus* in estuarine water are usually in range of 1-50 CFU/ ml of water (Pfeffer et al., 2003, Tamplin et al., 1982) but occasionally levels up to 10⁴ CFU/ml also have been reported (Vanoy et al, 1992). Other than water and shellfish high levels of *V. vulnificus* are also found in sediments, nonmolluscan shellfish and fish (DePaola et al., 1994).

The organism was initially reported as lactose fermenting vibrios but, further study of biochemical properties of different isolates revealed that some isolates of *V. vulnificus* cannot ferment lactose, hence lactose fermentation varies (Bisharat et al., 1999). Three biotypes of *V. vulnificus* have been identified based on biochemical characteristics, molecular typing and serological characters. Biotype 1 and 3 can infect humans through consumption of contaminated seafood or skin lesions, while biotype 2 is not a human pathogen (Levin 2005).

2.2 Virulence Factors

There are various factors that contribute to the pathogenicity of *V. vulnificus* such as low pH survival, polysaccharide capsule, lipopolysaccharide (LPS), extracellular virulence factors,

iron acquisition, attachment and adhesion protein expression (Levin, 2005). The brief summary of these important virulence factors are given below.

To cause illness *V. vulnificus* needs to survive the host's first defense line- highly acidic gastric environment. One common approach utilized by *V. vulnificus* to neutralize acidic environment is through breakdown of amino acids to yield amines and carbon dioxide (Rhee et al., 2002) suggested that enzyme lysine decarboxylase produced by *V. vulnificus*, breakdown lysine to produce cadaverine, which not only provide protection against low pH but also acted as superoxide radical scavenger, providing oxidative stress tolerance (Kim et al., 2006; Kang et al., 2007).

The Polysaccharide capsule of *V. vulnificus* is probably one of the most important and studied virulence factors.(Tamplin et al., 1983 ; Tamplin et al., 1985) It is believed that the capsule protects an organism from host's defense mechanism and provide resistance to opsonization by complement and therefore, escaping phagocytosis (Robert, 1996). Capsule formation also provides some level of protection against bactericidal effects of serum and also reduces the nonspecific host responses by masking immunogenic structures. Animal experiment comparing virulence of unencapsulated and encapsulated *V. vulnificus* found that the encapsulated strain significantly reduced the LD 50 Value in the experiments indicating it was more virulent (Write et al., 1981).

Lipopoly saccharide (LPS) is associated with primary septicemia while extracellular enzymes exhibit the elastolytic and collagenic actions. LPS is the factor that can cause shock and death associated with *V. vulnificus* infection. The major symptoms are fever, swift decrease of blood pressure and heart rate and hemorrhage are also typical symptoms of endotoxic shock. Mcpherson et al (1991) reported that injection of purified *V. vulnificus* LPS resulted in rapid

decrease of heart rate and blood pressure in rats, with death resulting within in an hour. A successive study by Elmore et al., (1992) utilized an inhibitor of LPS induced enzyme and found complete inhibition of these symptoms. A subsequent study reported that female hormone, estrogen protects female rats from *V. vulnificus* LPS and hence provided the evidence of role of LPS and also explained that why 80% of primary septicemia cases occur in males (Merkel et al 2001).

An elevated serum iron level in infected person is greatly associated with *V. vulnificus* disease. In two different studies Wright et al., (1991) and Stelma et al., (1992) reported that injecting mice with iron before infecting significantly increased mortality rate and decreased LD50. Based on these finding they have concluded that infectious dose of *V. vulnificus* and amount of iron available in serum are highly correlated. The exact relation between high mortality rate and elevated serum iron levels is still unclear but it is believed that high serum iron level increases the growth rates of this pathogen and decreases neutrophil activity (Starks et al., 2006). All these studies indicate that iron is crucial for *V. vulnificus* pathogenicity and hence explained the high infection and mortality rate among individuals with elevated serum iron levels. Typically, in human system most of the serum iron is bound to transferrin and not available to the organism. In order to survive in the human host *V. vulnificus* has developed various iron acquisition mechanisms, primarily siderophore based mechanisms.(Wehster and Litwin, 2000) The catechol siderophore is the major siderophore which scavenge iron from transferrin and holotransferrin for *V. vulnificus*.

Attachment through various surface receptors is one of the major factors required for virulence of the bacterium. Many Gram negative bacteria including *V. vulnificus* utilize pili for adherence to host cell, various studies emphasized the importance of pili in *V. vulnificus*

infection and reported without the pili the bacteria was unable to attachment to epithelial cells and increases LD 50 value up 2 logs. (Kim et al., 2006). Two other proteins, OmpU and IipA, are also believed to be involved in adherence. Studies involving OmpU and IipA mutants showed small increase in LD50 value and reduced cytotoxicity in mice (Goo et al., 2007) They concluded that these proteins are important for local cytotoxic damage but not for lethality. In order to determine the role and importance of flagella based motility in bacterial pathogenesis, several flagellar genes were mutated. The mutated *V. vulnificus* strains showed significant decrease in cytotoxicity, cellular adhesion, motility with a 3 log increase in LD50 value (Kim and Rhee 2003). The same studies also suggested that decrease in motility may lead to reduction in adhesion and hinders the cytotoxin release. In conclusion, studies involving the importance of attachment and motility reported that host cell contact is vital for *V. vulnificus* cytotoxicity and pathogenicity.

Extracellular enzymes: *V. vulnificus* produces various extracellular factors which are believed to contribute in the pathogenicity of the bacterium. The hemolysin encoded by *vvh* gene contributes to the cytotoxicity of bacterium and also believed to have a role in iron acquisition by releasing the iron form hemoglobin (Helms, 1984). Some other extracellular factors suggested to be involved in *V. vulnificus* pathogenicity are protease, collagenase, elastase, lipase, mucinase, RTX toxins and hyaluronidase.

2.3 Disease and Infection

V. vulnificus is believed to be responsible for three different type of human infections; gastroenteritiss, wound infection and primary septicemia.

2.3.1 Gastroenteritis This is the least severe of the three infections caused by *V. vulnificus*. Two major symptoms of this form of infection include diarrhea and abdominal cramps which typically subside without antibiotic treatment or hospitalization. Many studies associated *V. vulnificus* infection with consumption of raw oysters (Levine et al., 1993) other possible factors involved in this infection development includes chronic alcoholism and routine antacid use (Johnson et al., 1984).

2.3.2 Wound Infection This type of *V. vulnificus* infections has fatality rates up to 25% (Oliver 1989). The bacterium can infect the preexisting wound or wound incurred during seawater related activities. Various studies on *V. vulnificus* wound infection reported the incubation time ranging from 3 hours to 12 days, but in the majority of cases symptoms began within 24 hours. (Oliver 2005). The common symptoms for *V. vulnificus* wound infection include pain, erythema and edema at the wound site. The infection, if untreated, rapidly proceeds to deeper tissue causing necrotizing fasciitis. Severe wound infection often requires amputation of the limbs or at least surgical removal of affected tissue.

2.3.3 Primary Septicemia This is the most important among all three foodborne disease syndromes caused by *V. vulnificus* (Strom and Paranjpye, 2000). Primary septicemia is responsible for almost all seafood associated deaths in the US and a majority of the time is due to consumption of raw or undercooked oysters (Hlady 1997). Shapiro et al 1998 reported that in all most all primary septicemia cases that occurred in the US during 1995 to 2001 were associated with consumption of raw oysters from the Gulf Coast. Majority of *V. vulnificus* infection cases occur during warm water months of April to November (Shapiro et al 1998).

Development of primary septicemia associated with *V. vulnificus* requires some underlying and chronic diseases in almost all cases. Most commonly conditions such as liver disease, chronic alcohol abuse which lead to liver damage and causes elevated serum iron level are found responsible for severe *V. vulnificus* infection and primary septicemia development.

The Common symptoms of primary septicemia include fever, nausea hypotension which are typically develop within 36 hours of raw oyster consumption. Another symptom associated with severe primary septicemia is development of blisters that lead to tissue destruction and limb amputation. Primary septicemia infection typically develops very quickly and persons who do not receive treatment within 72 hours after the 1st sign of symptoms will generally dies (Ref).

2.4 Detection of *V. vulnificus*

2.4.1. General Identification Methods Two major analytical processes for *V. vulnificus* identification are suggested in BAM (Bacteriological Analysis Manual). The first one is MPN (most probably number) coupled with biochemical profiling of suspected isolate while the other suggested method include direct plating and DNA hybridization (BAM).

2.4.2 Serological Identification It is well known that *V. vulnificus* possesses unique species specific H antigens which are present on flagella core. Because of this species specific H antigen flagellar antiserum was successfully use to distinguish *V. vulnificus* from other vibrios by slide agglutination. Simonson and Siebeling (1986) raised the polyclonal antibody against *V. vulnificus* flagellar core and used it for development of species specific coagulation assay for *V. vulnificus* with about 99% detection rate. In order to improve sensitivity of the coagulation test Simonson and Siebeling (1988) used anti-flagellar monoclonal antibody which successfully identified all *V. vulnificus* clinical and environmental isolates tested and did not react with any

other *Vibrios*. Various distinctive cell surface antigens also have been utilized to identify *V. vulnificus* but they were not as specific as anti-flagellar monoclonal antibody and had about a 1% false positive result (Gray and Kreger, 1985). Because of high specificity and sensitivity of anti-flagellar monoclonal antibody it could be use in development of various immunoassays for *V. vulnificus* such as immunomagnetic separation and lateral flow immunochromatography.

2.4.2.1. Antibody Structure Antibodies are proteins produce by the immune system in response to the presence of a foreign molecule in the body. Antibodies are glycoproteins with high specificity and affinity toward their targets. These molecules were initially identified in serum and also known as immunoglobulins. Higher mammals have five classes of immunoglobulin, named IgG, IgM, IgA, IgE and IgD (fig.2.1).

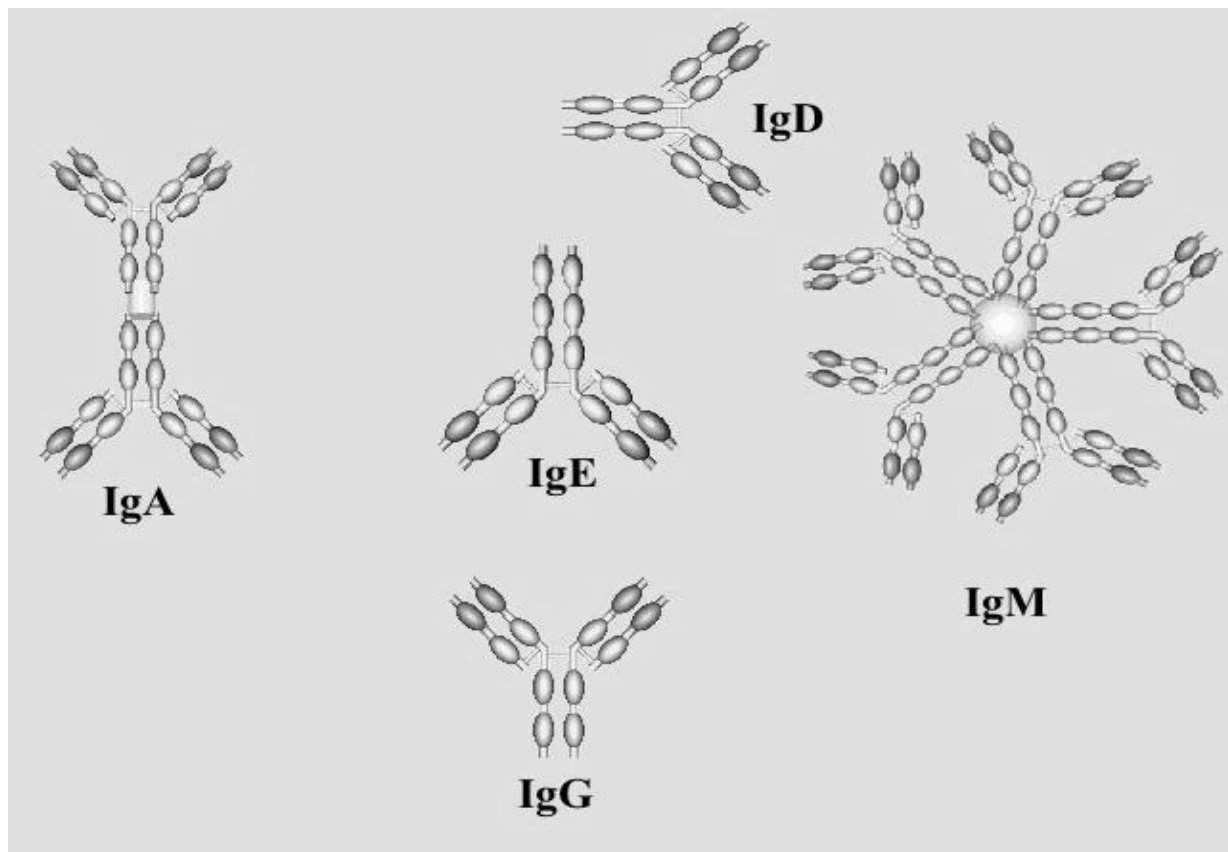


Figure 2.1: Types of Antibodies Source: Kuby 2006

All antibodies have the same four polypeptide chain units two light (L) and two heavy (H) chains with molecular weights of 25kDa and 50kDa respectively. The light chain is bound to the heavy chain by noncovalent interactions and disulfide bridges, while two heavy chains are bound with each other by disulfide bridges as well as noncovalent hydrophilic and hydrophobic interactions. Heavy and light chains have intrachain disulfide bridges about 90 amino acid, which creates polypeptide loops, domains of 110 amino acids. Each light chain consists of each of one variable domain (VH) and constant domain (CL), while each heavy chain is made up of one variable domain (VH) and three constant domains (CH1, CH2 and CH3) (fig. 2.2). The N terminal half of the heavy chain and all of the light chain together make Fab fragment of antibody which contains the antigen binding site. The amino acid sequence of this region is specific to that particular antibody and differs from one antibody to another, thus called variable region while rest of the antibody molecule is made of constant regions. The structures of constant regions are same for all antibodies of same class.

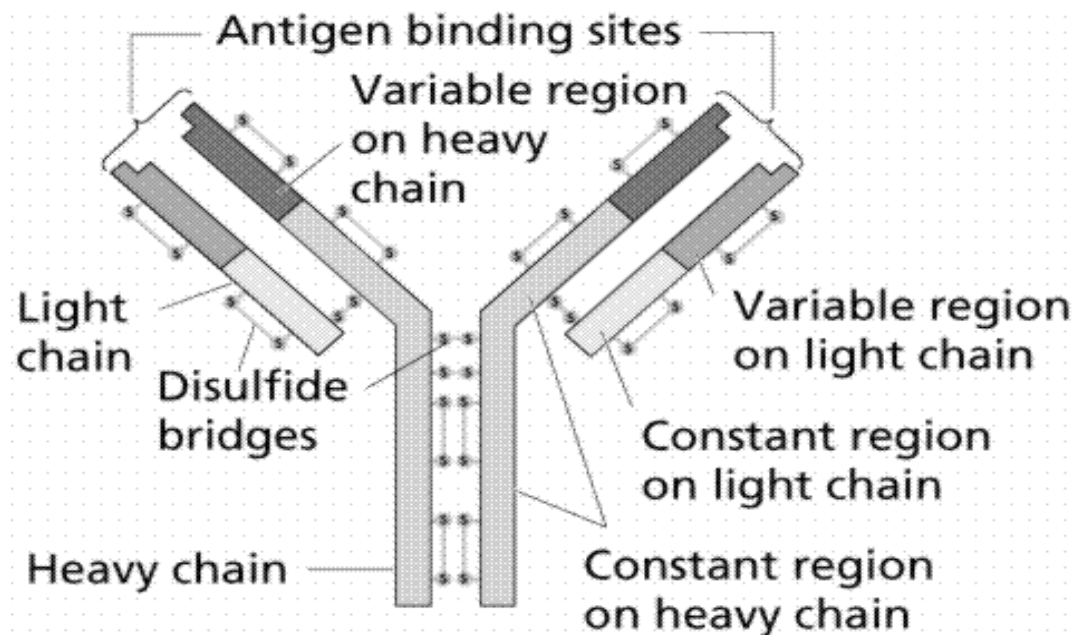


Figure 2.2 : Basic structure of antibody Source: cartage.org.lb

There are five different types of heavy chains known as α , δ , ϵ , γ , and μ which determine the class of antibody IgA, IgD, IgE, IgG and IgM respectively. There are also two different kinds of light chains κ and λ found in antibody but each antibody can only have one of the above.

Antibody class IgG is most abundant of all antibody found in body, which is also used in most antibody applications. Depending on structure and affinity toward antigen IgG is further divided in to four different subclasses referred as IgG1, IgG2a, IgG2b and IgG3 in mice while IgG1 to IGg4 in humans (Kuby 2000).

2.4.2.2. Monoclonal Antibody Production

The conventional technique use for production of MAbs –the hybridoma technology, was developed by Kohler and Milstein (1975). Since first reported hybridoma technology was successfully utilize numerous time to produce rodent antibodies of required specificity to vast variety of antigens. As each individual B cell produces an antibody with single specificity it is important to isolate each antibody producing B cell. However, generally it is not possible to grow antigen producing B cells in culture and thus direct utilization of B cell to produce desire antibody is not possible. Hybridoma technology allows production of hybrid cell lines from MAb secreting B cells and which can potentially utilize to *in vitro* mass production of specific antibodies (Kube, 2000, Atbitar 2003).

The general scheme for MAbs production is shown in figure 2.3. Briefly, mice are immunized by injecting antigen specific to the required antibodies. When an animal exhibits elevated level of specific antibodies, B cells are harvested from spleen and fused with myeloma cells to produce hybridomas. The process of hybridoma production can be divided into three major parts, immunization of animal, fusion and selection of antibody producing hybridoma.

Immunization is the first crucial step in production of MAbs, different antigens vary greatly in their immune response generation capacity or immunogenicity and thus utilization of specific immunization protocol for that specific antigen is necessary to produce optimal immune response. Other factors such as nature of antigen molecule, dose and route of immunizations, antigen carrier, adjuvant and type of animal use need to be considered in protocol development. The type of antibody produced is also depend on this first step, e.g if IgM are antibodies of interest only one immunization is carried out before sacrificing the animal in contrary IgG production requires multiple shots at intervals of 3-4 weeks to allow sufficient secondary response. (Atbitar 2003) (Fig 2.3).

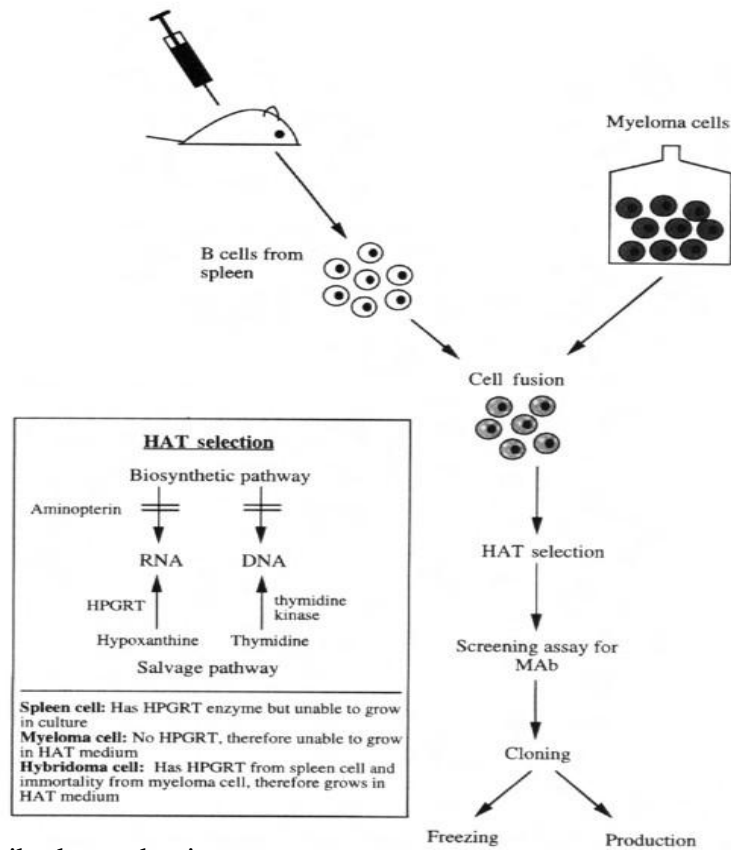


Figure 2.3: Monoclonal antibody production process
 Source: (Atbitar 2003)

At the end of the immunization period to ensure that the animal has exhibited adequate immune response to the injected antibody a blood sample from the animal should be tested for the presence of specific antibodies. The second step in production of MAbs is fusion of antibody producing B cells with myeloma cells and this is usually accomplished by utilizing membrane fusion inducer such as polyethylene glycol. The resulting hybridoma cell will possess the antibody production ability of B cells and good growth characteristics of myeloma cells.

After the fusion process is completed a mixture of hybridoma cells, B cells and myeloma cells are present and selection of hybridoma cells over other cell type is required, which leads to the screening process. B cells cannot grow in cell culture media so growth of the cell mixture for few days will readily remove B cells, on the other hand myeloma cells will grow rapidly and would make hybridoma selection very difficult. For this reason typically hypoxanthinephosphoribosyltransferase deficient myeloma cells are used for hybridoma production as they are not able to use the salvage pathway for RNA synthesis. The further selection of the hybridoma cells is achieved by utilizing HAT medium with aminopterin which blocks RNA and DNA synthesis through de novo pathway thus hinders the growth of myeloma cells. However hybrid cells possessing HPRT enzyme from B cells, will able to use hypoxanthine and thymidine from HAT media to produce RNA via salvage pathway and survive. Finally, the hybridomas have been screened through HAT media, are screened again through ELISA to determine antigenic specificity (Kube 2000).

2.4.3. Lateral Flow Chromatography Test (Dipstick Test) Dipstick test is very popular technique among all membrane based Immunoassay's and widely used for a number of point of care and field use applications. Basic technology for the development of a dipstick has been available since early 70's but the first assay was not developed until Towbin et al., (1979)

reported that protein molecule can pass through microporous hydrophobic membrane such as nitrocellulose membrane and can be detected using specific antibodies. Since, the first dipstick was developed for detection of Human chorionic gonadotropin (hCG) hormone, use of the dipstick increased because of simplicity and versatile nature of the test. This technology was used to develop a wide variety of tests for food industry, microbial analysis, clinical analysis and environmental applications.

The dipstick test is an immunoassays that employs the basic principle of capillary flow movement of the test sample along the strip which is pre-treated with an antigen or antibody, that results in the reaction between colored substrate and transforms the substrate and depending on the presence or absence of certain analytes in the sample the colored reagent will bind to the test line or zone that results in development of visible colored zone.

Table 2.1: Some of Recently Developed Dipstick Assays for Variety of Analytes

Classification of analytes	Analyte	Assay type	References
Bacteria	<i>Vibrio harveyi</i>	Non- competitive	Sithigorngul et al.,
	<i>Legionella pneumophila</i>	Non- competitive	Horng et al.,
Viruses	Canine distemper	Non- competitive	An DJ et al,
	White spot syndrome virus	Non- competitive	Sithigorngul et al.,
Hormones	Clenbuterol	Competitive	Zhang et al.,
	19-Nortestosterone	Competitive	Liu et al.,
Toxins	Aflatoxin B1	Competitive	Delmulle et al.,
	Microcystins	Competitive	Kim et al,
Insecticides	Carbaryl and endosulfan	Competitive	Zhang et al.,

2.4.3.1 Major Components of the Dipstick

A typical dipstick assay has various components such as sample pad, reagent pad, reaction membrane and waste reservoir (Fig 2.4). These components are generally attached to backing material or plastic housing with sample port and reaction window exhibiting the test and control zones. A brief description of each component of dipsticks is given below.

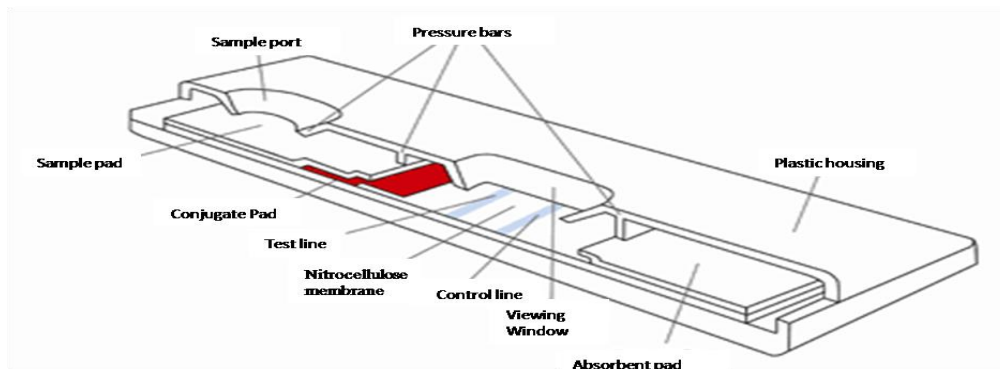


Figure 2.4: Components of typical dipstick

Source: <http://www.azonano.com/details.asp?ArticleID=1898>

Sample Pad Absorbant pad onto which the test sample is applied

Reagent Pad or conjugated pad This pad contain the analyte specific antibodies which are generally conjugated with colored particles such as latex microspheres or colloidal gold

Reaction membrane This is one of the most important components of dipstick. The selection of membrane greatly influenced the nature of analyte; pore size, protein binding capacity and strength are major factors that need to be consider in membrane selection. Typically hydrophobic cellulose acetate or nitrocellulose membrane with target specific antibodies immobilized in test line and control zone with anti-antibodies are utilized in dipstick test preparation.

Absorbent pad Present on the opposite end of sample pad, this pad helps to draw the sample across the reaction membrane for collection.

Generally one of the two major formats; competitive or non- competitive are used to develop the dipstick assay. A non- competitive or sandwich format dipstick utilizes two antibodies and is particularly useful in detection of larger molecules with more than one epitope or more specifically analytes with more than one antibody binding sites, e.g bacteria, virus. Briefly in this format once the sample is applied to the sample pad it will travel to the reagent pad where specific conjugated antibodies binds with the analyte.(Figure-A) The sample containing analyte-antibody complex continues to travel across the membrane until it reaches the test line where this complex will combine with immobilized antibodies to produce a visible signal. The sample will then travel further on the membrane until it reaches the control line where excess of conjugated antibody will bind with anti-antibodies and produces the visible signal. Typically in this non-competitive format two visible lines in the test and control zone will indicate a positive result while a single visible line in the control zone indicates a negative results.

The dipstick competitive format assay is typically used for detection of smaller molecules with one antibody binding site. In the competitive format, the reaction pad already contains analyte-antibody conjugate complex, if target analyte is present in the sample it will not bind with the antibody conjugate and compete with the analyte-antibody conjugates complex to bind with the immobilized antibodies present on the test line therefore very faint or no visible line will develop at the test line, while unbound conjugates will bind to the control line and will produce a visible signal.(Figure-2.5 B) Therefore in the competitive format single a visible line at the control zone indicates the positive results with two visible lines on the test whereas the and control zone indicates the negative test. This type of format is generally used for detection of toxins such as aflatoxin B1 and EHEC Shiga toxins.

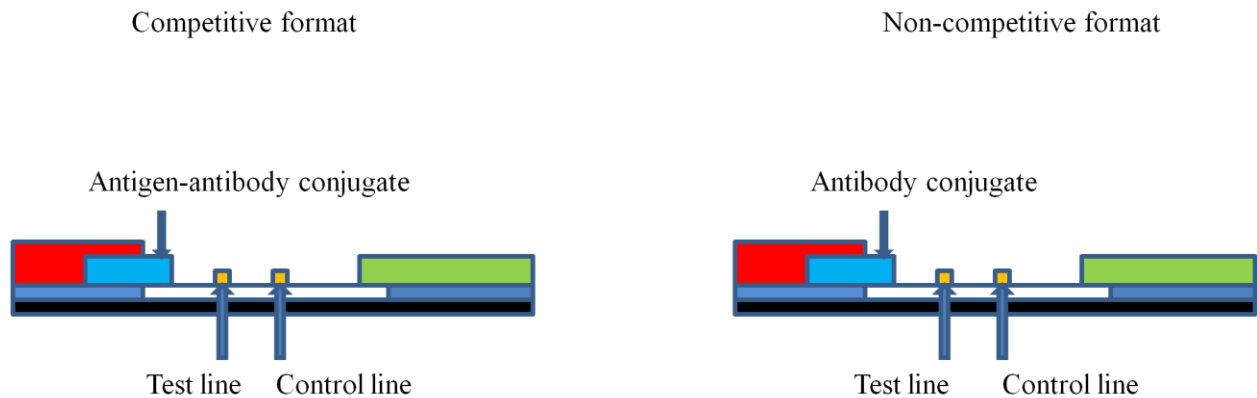


Figure 2.5: Schematic representation of Competitive (A) and Non-competitive (B) dipstick formats.

There are several advantages for the dipstick test assay firstly, it is very simple and requires minimal user dependent steps, secondly the dipstick test is compact and shelf stable hence, suitable for many field use applications and finally, relatively low cost and short assay development time (O' Farrell, 2009).

Since its first use in home pregnancy test strip, the dipstick test has been used to develop tests for a wide range of analytical procedures including major shrimp pathogens such as *Vibrio harvei* and White spot syndrome virus (Sithigorngul et al 2007, Sithigorngul et al., 2006).

Species specific anti- *H. V. vulnificus* monoclonal antibodies could be utilize to develop a lateral flow assay for *V. vulnificus* that will have several advantages over other rapid assays, such as, it is very simple and requires minimal user dependent steps, it is compact and shelf stable hence, suitable for many field use applications and finally, relatively low cost and short assay development time (O' Farrell, 2009).

2.4.4. Immunomagnetic Separation (IMS) IMS is a widely used immunoassay to isolate and concentrate variety of targets. IMS utilizes minute paramagnetic particles coated with target

specific antibodies and relies on antigen –antibody interaction and an external magnetic field to separate target cells from the sample. IMS provides a promising tool to remove small particles from sample and concentrate target organisms by altering the ratio of target to non- target organisms in favor of target organisms. Schematic representation of IMS process is exhibited in Fig. 2.6. Nowadays IMS techniques are extensively used in food diagnostics (Jadeja et al., 2010, Fu et al, 2005). There are various factors that can affect the efficiency of IMS protocol, such as type and size of magnetic beads, antibody selection, target organism, competitive flora and food or environmental matrix.

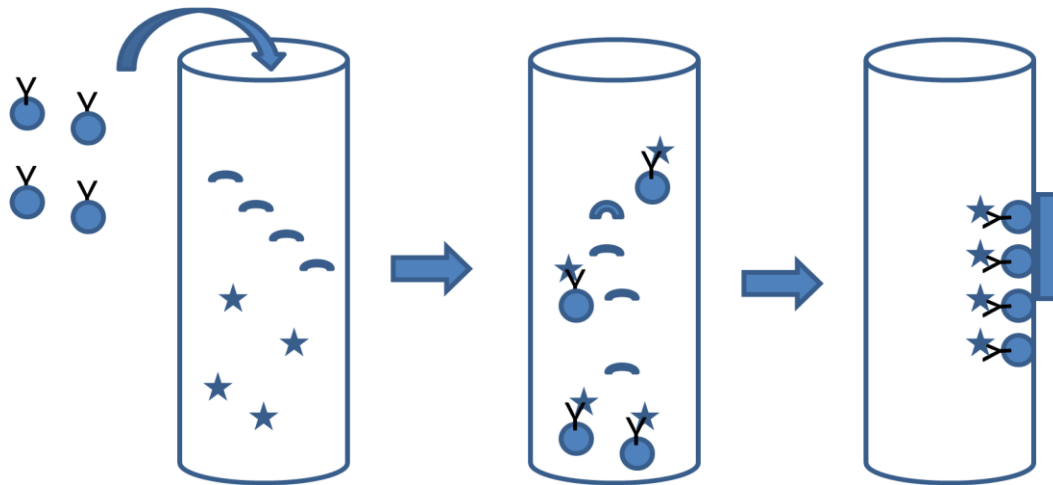


Figure 2.6 :Schematic representation of IMS process

Source: http://oh.water.usgs.gov/micro_ims_atp_method.htm

IMS has been successfully utilized to isolate and concentrate various pathogens such as *E.coli O157: H7*, and *V. parahaemolyticus* from the food matrixes and complex environmental samples (Fu et al., 2005). As such IMS has become an important tool for preliminary screening for the presence of pathogens in food products. This technique also became an essential part of various conventional and rapid pathogen detection methods. A successful IMS protocol not only increases the specificity and speed of different pathogen detection methods by eliminating

the pre-enrichment but also removes the PCR inhibitors and bacterial growth inhibitors present in the sample (Fu et al., 2005).

2.4.5. Real -time PCR Polymerase chain reaction (PCR) is a very sensitive technique to amplify target nucleic acid and can be used for a variety of applications in the field of molecular biology. PCR technique is extremely versatile and used for rapid detection of various pathogenic and non-pathogenic organisms (Panicker and Bej, 2005). Hill et al. (1991) were developed the first PCR protocol for *V. vulnificus* detection and since then various researchers have developed many PCR processes targeting single or multiple genes to identify *V. vulnificus* from oyster meat (Brasher et al. 1998, Aono et al. 1997). Though, PCR is relatively rapid and sensitive detection technique, it requires time consuming post PCR procedures such as electrophoresis. The requirement of post PCR procedure can be eliminate by using real-time PCR (rt-PCR), which allows the detection of PCR amplicons during the early phase of reaction. In 1993 Higuchi et al., introduced Real time monitoring of DNA amplification by inclusion of fluorescent dye that binds amplicons as they are made (fig.2.7). Several different methods can be used to detect DNA amplification under fluorescent detection. The TaqMan PCR is one of the most popular techniques which utilizes fluorescent oligo probe. This oligo probe binds target DNA sequence, internal to primer binding sites and possess a reporter dye (fluorescence dye) and a suppressor dye (quencher dye) that prevent fluorescent activity via fluorescence resonance energy transfer. In rt-PCR when double standard DNA products are made, a measure of fluorescence is taken after the fluorogenic probe is hydrolytically cleaved from amplicon by the exonuclease activity of the *Thermus aquaticus* DNA polymerase. Once cleaved the probe's fluorescent activity is no longer suppressed by quencher dye. This type of rt-PCR is also referred as 5' exonuclease-based rt-PCR.

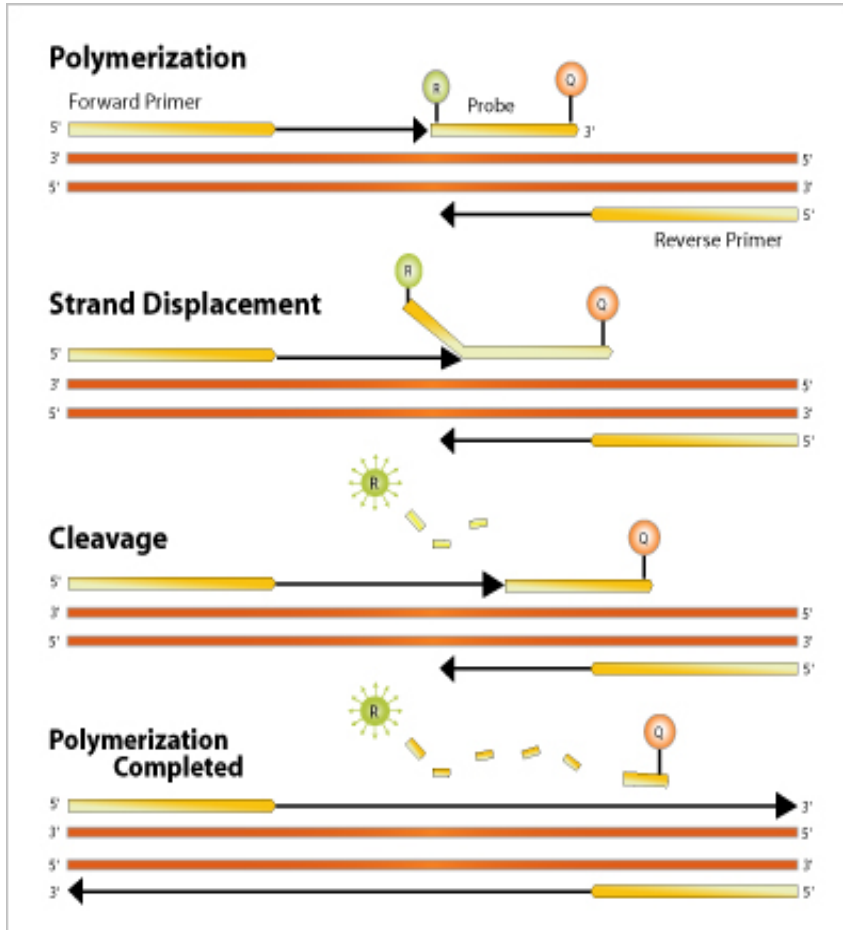


Figure 2.7 The oligoprobe contains a fluorescent marker and a chemical group that quenches fluorescence of the oligoprobe until the dye is liberated by exonuclease activity of the Taq DNA polymerase. This can only occur if the oligo binds the complementary sequences present in the target gene and amplicon

Source: <http://www.servicexs.com/servicexs+is+powered+by/applied+biosystems/taqman+rt-pcr+assay/>

In recent years the rt-PCR amplification method based on *V. vulnificus* hemolysin gene (*vvhA*) has been successfully employed for qualitative and quantitative rapid detection of *V. vulnificus* from variety of matrixes (Panicker and Bej 2005). However, rt-PCR is sensitive to

inhibition by various PCR inhibitors present in complex environmental samples such as oyster meat (Kafuman et al., 2004). One approach to overcome this problem is the use of IMS with species specific monoclonal antibody that can increase the sensitivity and speed by eliminating enrichment step further more it also removes PCR inhibitors (Jadeja et al., 2010).

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CHAPTER 3

IMMUNOMAGNETIC SEPARATION OF *VIBRIO VULNIFICUS* WITH ANTI-FLAGELLAR MONOCLONAL ANTIBODY

3.1 Introduction

Vibrio vulnificus, a gram negative, halophilic bacterium, is responsible for the majority of seafood-related deaths in the United States (Hlady, 1997, Mitra, 2004, Oliver, 1989.) The majority of *V. vulnificus* infections in the United States are due to consumption of raw molluscan shellfish, in particular oysters harvested during April to October from the Gulf of Mexico (Panicker, et al., 2004). Primary septicemic infections caused by this organism are documented to have a fatality rate as high as 60% (Cohen, 2000, Hlady, 1997, Levin, 2005). Conventional *V. vulnificus* bacteriological detection and enumeration methods are very labor-intensive and time-consuming (Peeler, et al., 1992). This emphasizes the need for the development of novel detection techniques which are sensitive, specific and rapid. A detection technique should be rapid enough to give results within a short period (< 4 hr), allowing commercial processors to release food lots without delay thereby decreasing storage costs. PCR using *V. vulnificus*-specific probes for the hemolysin gene (*vvh*) can be employed as rapid methods for the detection of the organism (Panicker, et al., 2004, Wright, et al., 1985). These methods are comparatively rapid (2-3 hr) but, in some instances, they may require considerable time (>6 hr) for pre-enrichment to achieve high sensitivity. A real-time PCR (Rt PCR) amplification method targeting the *V. vulnificus* hemolysin gene has been developed for qualitative and quantitative detection of *V. vulnificus* from sea water and oyster tissue (Panicker, et al., 2004). Although this method is rapid, detection is compromised in cases of low bacterial number or the presence of polymerase inhibitors in complex environmental samples, such as shellfish (Hill, 1996).

To improve the sensitivity of *V. vulnificus* detection and recovery of the organism from complex environmental samples, immunomagnetic separation (IMS) might be a very useful approach. Immunomagnetic separation, which has been successfully used to concentrate and

isolate numerous pathogens (Datta, et al., 2008, Khare, et al., 2004), could be used to isolate *V. vulnificus* from PCR inhibitors in shellfish homogenates. Immunomagnetic separation might also serve to shorten or eliminate the pre-enrichment step before rt -PCR. A satisfactory IMS protocol for concentration of *V. vulnificus* would improve the recovery of the bacterium from complex environmental samples and serve to isolate the organism from other bacteria. In order to optimize an IMS protocol, development of a *V. vulnificus* specific antibody is crucial.

Many species in the genus *Vibrio* possess species-specific H antigens (Datta, et al., 2008, Tassin, et al., 1984). Based on knowledge of this unique H antigen expression in the flagellar core protein, serological techniques can be employed to differentiate and identify various *Vibrio* spp. Since Simonson and Siebeling (1988) documented that anti-*V. vulnificus* H monoclonal antibodies were highly specific, the use of these antibodies would provide the species-specificity required for a successful IMS protocol. This study describes the production and analysis of six anti-*V. vulnificus* H monoclonal antibodies (MAbs) and the employment of the MAbs in an optimized IMS protocol.

3.2 Materials and Methods

3.2.1 Flagellar Core Purification

Flagellar cores were purified from a motile strain of *V. vulnificus* ATCC 27562 by methods previously described (Simonson and Siebeling, 1986). Briefly, *V. vulnificus* cells were grown overnight on nutrient agar supplemented with 2% NaCl (NA+) at 37°C. The cells were harvested from the agar surface in 0.15M NaCl and were homogenized for 90 s in a Waring blender at medium speed. The bacterial cells were sedimented by centrifugation at 10,000 X g for 10 min and the sheared flagella were obtained by centrifugation of the remaining supernatant fluid at

30,000 X g for 2 h. The flagella were suspended in 0.1 M Tris buffer, pH 7.8, containing 0.1 mM EDTA, 1% Triton X-100 and 0.001% thimerosal (TET) and the differential centrifugation cycle was repeated 3 times. The purified cores were negatively stained with uranyl acetate and 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 (bicinchoninic acid) protein assay (Pierce, Rockford, IL).

3.2.2 Polyacrylamide Gel Electrophoresis

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

3.2.3 Immunization

BALB/c mice were immunized at 2-week intervals for 4 to 8 weeks by intraperitoneal injection of 50 µg purified *V. vulnificus* polar flagellar core protein. Mice exhibiting elevated anti-H flocculation titers (Tassin, et al., 1984) were boosted with 50 µg of flagellin and their spleen cells were collected after 3 days.

3.2.4 Hybridoma Production

The method used to promote cell fusion was modified from that described previously (Simonson and Siebeling, 1988). Briefly, 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 solution (Hybrimax, Sigma Chemical Co., St. Louis, MO). The cells were

sedimented and resuspended in RPMI 1640 containing 15% fetal bovine serum, 2 mM glutamine, 1% nonessential amino acids, 100 units/ml penicillin-streptomycin, 100 μ M hypoxanthine, 4×10^{-7} M aminopterin, 1.6×10^{-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 maintained in RPMI 1640 at 37° C in a humidified atmosphere of 5 to 7 % CO₂.

3.2.5 Anti-H ELISA

Supernatant fluid from each well exhibiting hybridomal growth was tested by ELISA for the presence of anti-*V. vulnificus* H antibody using *V. vulnificus* polar flagellar cores (2 μ 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, J.G., and R.J. Siebeling. 1988). Culture 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

Six hybridomas secreting *V. vulnificus* 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 (NH₄)₂SO₄ 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 μ m filter, IgG

was purified by affinity chromatography on protein A-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 Immunoelectron Microscopy

Carbon-coated grids were sequentially floated on drops of the following reagents at room temperature: purified *V. vulnificus* flagellar cores (5 min), 1% bovine serum albumin (BSA) in PBS (5 min), MAb 8-D-4 (360 µg IgG/ml PBS, 10 min), 3 PBS washes, anti-mouse IgG-conjugated with 10nm colloidal gold particles (Sigma Chemical Co, St. Louis, MO) diluted 1:10 in BSA/PBS (10min), 3 PBS washes and 2 water washes. The liquid was drained from the grids with filter paper after each step. Negative control grids were placed on drops of the same reagents with the exception of the primary antibody (8-D-4 IgG).

3.2.8 Coagglutination

Previously established methods were employed for preparing formalin-fixed *S. aureus* Cowan 1 ATCC 12598 cells (Simonson and Siebeling, 1986). The three Mab's (6-G-10, 3-D-10 and 8-D-4) which demonstrated the highest anti-H ELISA titers were selected to prepare anti-H coagglutination reagents by mixing 50 µg of each MAb with 1 ml of *S. aureus* cells. Each of the three anti-H coagglutination reagents was tested against *V. parahaemolyticus* ATCC 17802, *V. vulnificus* ATCC 27562, *V. cholerae* ATCC 14035, *V. mimicus* ATCC 33653, *V. fluvialis* ATCC 33809, *V. natriegens* ATCC 14048, *V. harveyi* ATCC 14126, *V. harveyi* ATCC 35084, *V. campbellii* ATCC 25920, *V. damsela* ATCC 35083 and *V. alginolyticus* ATCC 33787. The fastest agglutinating MAbs, 3-D-10 and 8-D-4, were also used for slide coagglutination tests

with 40 environmental and clinical isolates of *V. parahaemolyticus* and 70 environmental and clinical strains of *V. vulnificus*.

MAbs 3-D-10 and 8-D-4 were also selected to prepare IMS reagents by coating IMB (Dynabeads M-280 sheep anti-mouse IgG, Dynal Biotech, Oslo, Norway) by the process described by Skjerve et al (1990) with some modification. Briefly, IMB were incubated with MAb for 1 h at 25⁰C, followed by an overnight stationary incubation at 4⁰C at a concentration of 4 µg IgG/10⁷ IMB. These IMS reagents were used for further coagglutination studies involving the 11 different *Vibrio* ATCC species, 40 *V. parahaemolyticus* and 70 *V. vulnificus* isolates mentioned above.

All *Vibrio* isolates tested by coagglutination were grown on peptone agar slants (1% peptone, 2% NaCl, 0.2% yeast extract, 1.5% agar) for 24 h at 35° C and the bacterial cells were harvested in 1 ml of TET buffer. One drop of cell suspension was mixed with 30 µl of each coagglutination reagent (MAb-coated *S. aureus* cells or MAb-coated IMB) on a clean glass plate. The plate was observed for 3 min over a light box for evidence of agglutination.

3.2.9 Immunomagnetic Separation

IMS reagents were prepared by adding 5 µg of MAb to 20 µl (10⁷) IMB by the method described previously. The binding capacity of each IMS reagent was tested against *V. vulnificus* ATCC 27562 and two clinical isolates, *V. vulnificus* 1007 and C7184. Serial 10-fold dilutions of a 16-18 hr *V. vulnificus* ATCC 27562, C7184 or 1007 culture grown in NB+2% NaCl were made in PBS to reach 10²-10³ cells/ml. Each bacterial suspension (500 µl) was mixed with 20 µl IMS reagent in a sterile, 1.5 ml microcentrifuge tube. After 30 min incubation at 25⁰C on a rocker, the IMB were isolated by placement of the tubes in a magnetic particle concentrator (Dynal

Biotech ASA, Norway). The supernatant fluid from each tube was diluted when needed and plated on NA+ plates to determine CFU/ml. This procedure was repeated with spiked oyster homogenates to determine the binding capacity of the IMS reagent in complex environmental samples. Oyster homogenates were prepared by stomaching 10 g of oyster meat with 20 ml of sterile PBS. The homogenates were then filtered through cheese cloth and spiked with *V. vulnificus* to reach a bacterial concentration of 10^2 and 10^3 CFU/ml and 500 μ l of homogenate was then mixed with 20 μ l IMS reagent in sterile tubes, followed by 30 min incubation on a rocker at room temperature. The procedure described earlier was employed to isolate the IMB and CFU/ml was determined by plating the supernatant fluid on NA+ plates.

3.3 Results and Discussion

3.3.1 Production and Characterization of MABs

Before mice were injected, purified *V. vulnificus* flagella were examined by TEM to ensure that flagellar cores, free from cell debris and flagellar sheath, were obtained (Figure 3.1A). When subjected to SDS-PAGE, the purified flagellar cores exhibited a major protein band corresponding to approximately 41 kDa (Figure 3. 2). Two separate fusion experiments produced 6 stable, rapidly growing hybridomas producing IgG that consistently resulted in absorbance readings ≥ 1.0 when the cell culture fluid was tested by anti-H ELISA.

After antibody purification and subclass determination of each MAb, the anti-H ELISA titers were determined and defined as the reciprocal of the highest dilution of MAb, starting with 25 μ g of IgG in well 1 of a microtiter plate, that gave an absorbance reading ≥ 0.2 in three separate experiments (Table 3.1). The affinity of MAb 8-D-4 was demonstrated by immunogold TEM (Figure 3.1B).

TABLE 3.1: Analysis of Antibodies Purified from Hybridoma Cell Culture Fluid

Hybridoma designation	IgG Concentration	Anti H-ELISA titer ^a	Subclass Designation
3-D-10	1100 µg/ml	>2048	IgG2a
1-C-6	1960 µg/ml	256	IgG1
2-D-3	200 µg/ml	256	IgG1
6-G-10	1240 µg/ml	1024	IgG2b
8-D-4	1290 µg/ml	1024	IgG2b
5-G-8	275µg/ml	512	IgG2a

^a The greatest dilution of MAb which gave an absorbance reading of ≥ 0.2 with a beginning IgG concentration of 25µg/well and 1 µg *V. vulnificus* flagellin bound/well.

3.3.2 Slide Coagglutination

The serological specificities of 3 selected MAbs (6-G-10, 3-D-10 and 8-D-4) were assessed by slide agglutination. Only *V. vulnificus* coagglutinated with the three reagents within one min, while other species showed no reaction, even after 3 min. Since MAbs 3-D-10 and 8-D-4 exhibited strong coagglutination reactions with *V. vulnificus* ATCC 27562 in less than 30 s, they were then selected to study the agglutination reactions with 40 *V. parahaemolyticus* and 70 *V. vulnificus* strains. All strains of *V. vulnificus* showed reactions within 30 s, while there was no reaction with any of the *V. parahaemolyticus* strains tested, confirming the species-specificity of the MAbs assayed (data not shown). Skjerve et al. (1990) demonstrated that a slide coagglutination reaction was a simple and reliable tool to assess the ability of antibody-coated IMB to bind bacterial strains of interest in an IMS procedure. Thus, the serological specificity and potential reactivity of MAbs 8-D-4 and 3-D-10 in an IMS protocol was further examined by repeating the coagglutination study substituting MAb-coated IMB for MAb-coated *S. aureus* cells. The MAb-coated IMB coagglutinated all 70 *V. vulnificus* strains but did not react with any

of the 40 *V. parahaemolyticus* isolates tested. The results from both the studies were in agreement with the findings of Simonson and Siebeling (1988) who determined that *V. vulnificus* anti-flagellar core MAb specifically reacted with strains of *V. vulnificus* without any cross-reactions with 31 heterologous *Vibrio* spp. tested.

3.3.3 Immunomagnetic Separation

In this study, IMS reagents were prepared by coating MAbs 8-D-4 and 3-D-10 on IMB to reach a final concentration of $5\mu\text{g IgG}/10^7$ IMB. Optimal MAb concentration was determined by conducting some preliminary studies in which IMS reagents were prepared with different concentrations of MAbs that ranged from $5\mu\text{g}/10^7$ IMB to $15\mu\text{g}/10^7$ IMB. The results showed the IMS reagent with $5\mu\text{g MAb}/10^7$ IMB was optimum. Higher MAb concentrations did not alter the bacterial binding capacity of IMS reagents and these results are in accordance with previous studies conducted by Skjerve et al. (1990), which also suggested that $5\mu\text{g MAb}$ concentration exhibited the maximum *L. monocytogenes* binding.

The concentration of IMB employed for this study was approximately 10^7 , which was predetermined from previously established IMS protocols (Enroth and Engstrand, 1995, Khare, et al., 2004). Two different incubation periods of 30 and 60 min were tested for optimum separation of organisms. No significant difference was found between shorter and longer incubation periods; hence a 30 min incubation time was used for rest the study. Previous studies have shown that more than one bacterium can bind with one bead (Grant, et al., 1998, Roberts and Hirst, 1997), thus in order to achieve better accuracy, numbers of *V. vulnificus* cells bound by IMB were determined by plating unbound bacteria in aspirated supernatant fluid.

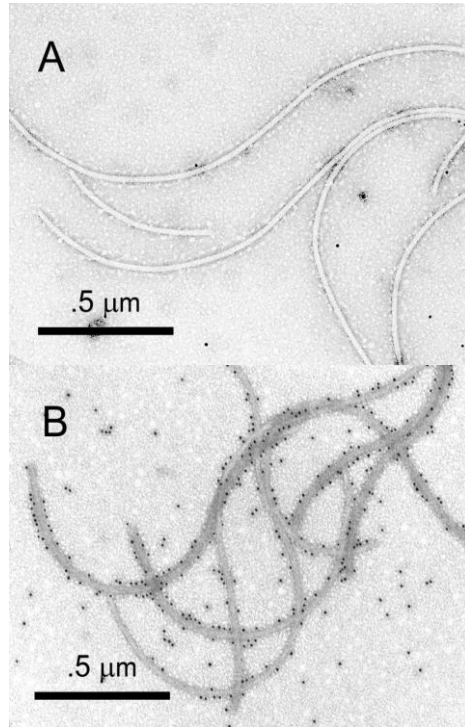


FIGURE 3. 1. Electron micrograph of *V. vulnificus* ATCC 27562 purified flagellar cores reacted with anti-mouse IgG conjugated to colloidal gold particles in the absence (A) and presence (B) of anti-flagellar MAb.

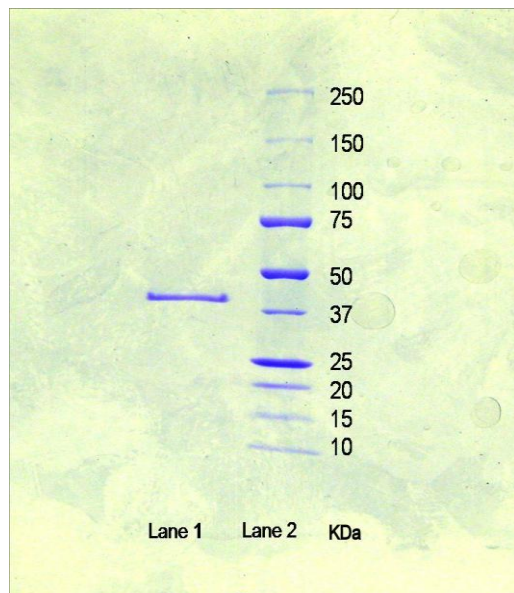


FIGURE 3.2. SDS-PAGE of purified *V. vulnificus* flagellar coreprotein. Lane 1, purified flagellin; lane 2, standard protein markers.

The inhibitory effects of food matrices on IMS have been discussed in previous studies. Fu et al. (2005) reported that cell capture efficiency of IMS was dramatically reduced due to presence of meat-associated inhibitors; hence, it is very important to know the true potential of an IMS reagent before using it with complex environmental matrices which may hinder the efficiency of IMS. In order to determine the binding capacity of our IMS reagents in the absence of IMS inhibitors we used spiked PBS and tested each IMS reagent separately with three different *V. vulnificus* strains. It was determined that 3-D-10 and 8-D-4 MAbs did not exhibit major differences in binding capacity. The highest binding in PBS of about 57% was observed between *V. vulnificus* strain C7184 and MAb 3-D-10 while the lowest binding (about 19%) was observed between *V. vulnificus* ATCC 27562 and MAb 3-D-10 (Table 3. 2).

When oyster homogenate was tested, two different concentrations of *V. vulnificus* were employed to understand the effect of different bacterial concentrations on the IMS process. We did not test cell concentrations lower than 100 CFU/ml, as it would result in <10 CFU/ plate and would not aid in projecting accurate estimates of actual bacterial count. The highest binding in oyster homogenate was observed between *V. vulnificus* strain 1007 and MAb 8-D-4 at the concentration of 10^3 CFU/ml, while *V. vulnificus* strain ATCC 27562 and MAb 8-D-4 had the lowest binding at 10^2 CFU/ml (Table 3.3).

Previous studies which focused on the effect of concentration of bacteria on the binding capacity showed that binding capacity is proportional to the concentration of the cells present in the medium (Jenikova, et al., 2000). Skjerve et al. (1990) found that an increase in the number of bacteria in suspension resulted in an increase in the number of bacteria bound to the IMB ranging from <10% (100 *L. monocytogenes*/ml) to 50% (1.5×10^4 *L. monocytogenes*/ml).

TABLE 3.2: Recovery of *V. vulnificus* Cells by IMS in PBS^a

MAb	<i>V. vulnificus</i> strain	Inoculum CFU/ml	Bacteria in supernatant fluid CFU/ml	% Binding	Std. Deviation
8-D-4	ATCC 27562	1.99 X10 ³	1.52 X10 ³	23.8	2.17
	1007	2.01 X10 ³	0.96 X10 ³	52.0	1.36
	C7184	2.73 X10 ³	1.50 X10 ³	45.0	3.78
3-D-10	ATCC 27562	3.24 X10 ³	2.61 X10 ³	19.4	3.41
	1007	2.65 X10 ³	1.21 X10 ³	54.4	2.32
	C7184	2.05 X10 ³	0.89X10 ³	56.8	2.08

^aValues are average of three independent experiments

A similar observation was made by Fratamico et al. (1992) where it was found that an increase in *E. coli* B1409 levels improved recovery of the target organism from the sample. Our study also found that as the bacterial cell concentration decreased in the spiked oyster homogenate, the binding capacity of the IMS reagents was also significantly decreased. One explanation for this decrease might be that the bacteria have less chance of interacting with IMS reagents in solution as the bacterial number decreases. Our Statistical analysis suggested that binding potentials of MAB 8-D-4 and 3-D-10 are not significantly different, on other hand cell concentration and type of bacterial strain are significant effects.

There are many studies suggesting that composition of the matrix has a great influence on the IMS reagent potential. Fu et al. (2005) found that binding capacity of IMS reagents decreased by 2 logs when IMS was performed with beef. Jeníková et al. (2000) also encountered a similar type of problem with ground beef and explained that the drop in recovery might be due

to the high fat content of beef since it may cause the loss of some beads as they become fixed to the food matrix and the magnetic field could not separate them.

TABLE 3.3: Recovery of *V. vulnificus* Cells by IMS in Oyster Homogenate^a

MAb	<i>V.vulnificus</i> strain	Inoculum CFU/ml	Bacteria in supernatant fluid CFU/ml	% Binding	Std Deviation
8-D-4	ATCC 27562	1.07 X10 ³	0.69 X10 ³	35.5	1.44
		1.09 X10 ²	0.82 X10 ²	25.1	3.61
	1007	1.19 X10 ³	0.51 X10 ³	57.0	2.09
		1.36X10 ²	1.01X10 ²	25.5	2.04
	C7184	1.89 X10 ³	0.92 X10 ³	51.3	4.13
		1.61 X10 ²	1.18 X10 ²	26.6	2.01
3-D-10	ATCC 27562	1.11 X10 ³	0.72 X10 ³	35.1	3.16
		1.10 X10 ²	0.82 X10 ²	25.2	3.14
	1007	1.26 X10 ³	0.57 X10 ³	55.0	3.10
		1.22 X10 ²	0.92X10 ²	27.8	1.44
	C7184	2.13 X10 ³	1.02 X10 ³	52.5	1.82
		1.59 X10 ²	1.11 X10 ²	28.9	2.42

^aValues are average of three independent experiments

In contrast to these studies, oyster homogenate did not show any inhibitory effect on the binding capacity of the IMS reagents tested. For example, the binding capacity of IMB coated with MAb 8-D-4 was 52% in PBS and 57% in oyster homogenate spiked with 10³ *V. vulnificus* 1007. Likewise, MAb 3-D-10-coated IMB exhibited binding capacities of 55% in both PBS and oyster homogenate spiked with 10³ *V. vulnificus* 1007. This lack of interference might be explained by the low fat content of oyster meat of about 2.4% as compared to ground beef (17%). In both PBS suspension and oyster homogenate, immunomagnetic separation of *V.*

vulnificus ATCC 27562 demonstrated lower binding in comparison to the other two strains and even a difference in the concentration of bacteria did not significantly alter binding capacity. In order to explain reduction in binding capacity of IMS reagents with *V. vulnificus* ATCC 27562, we propose that the ATCC strain might have become less motile over time. The ATCC strain may also have lost its ability to produce flagellar sheath, which would result in a single flagella binding many IMB, thus reducing overall binding capacity of the reagents.

Notzon et al. (2006) developed an IMS-rt PCR assay for rapid detection of *Salmonella* species in meat. With the help of IMS they were not only able to isolate target organisms from meat-associated PCR inhibitors but also reduced the assay time and achieved a high detection sensitivity of 10 CFU/25g meat. In an analogous type of approach, IMS reagents prepared with MAb 3-D-10 and 8-D-4 could be used to rapidly isolate *V. vulnificus* from various complex environmental samples. Combination of PCR-based detection methods with IMS could be a rapid alternative to lengthy standard methods for the detection of *V. vulnificus* in oyster meat. IMS could also be used in recovery of injured *V. vulnificus* cells which are not able to revive in enrichment broth, hence, increasing the overall sensitivity of the detection procedure.

3.4 References

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CHAPTER 4

EVALUATION OF IMS rt-PCR FOR ENUMERATION OF *V. VULNIFICUS* IN SPIKED OYSTER HOMOGENATES

4.1 Introduction

Oysters are known reservoirs of many foodborne pathogens including *V. vulnificus* hence, consumption of raw oysters has been associated with many outbreaks around the globe (Potasman et al., 2002). *V. vulnificus* is an aquatic organism and abundantly found in warm and tropical ecosystems such as Gulf of Mexico in the United States which is also the largest oyster harvesting area in the country (Panicker et al., 2004, Oliver et al., 1983). Consumption of gulf water harvested oysters, especially during summer months has been a cause of concern for many regulatory agencies.

V. vulnificus is an opportunistic pathogen and may only cause mild gastroenteritis in healthy individuals, but the same organism can infect susceptible individuals, especially a persons with a compromised immune system, alcoholism, cirrhosis and hemochromatosis, to cause serious primary septicemia with a fatality rate as high as 50% (Blake *et al.*, 1979). As *V. vulnificus* is naturally found in warm seawaters, contact of wounded or cut skin with such infected water can also cause septicemia. Because of the seriousness of infection and high mortality rate it is advisable to closely monitor the presence of *V. vulnificus* in oysters by rapid detection methods to ensure the steady supply of safe consumable oysters.

The detection processes approved by the FDA for *V. vulnificus* include MPN (most probable number) combined with use of selective media, DNA hybridization and biochemical testing (Kaysner and DePaola 2001). These methods are time consuming and laborious and can take up to 5 days to obtain confirmed results. In recent years many PCR based rapid methods have been developed for various pathogens including *V. vulnificus*. These conventional PCR based methods are comparatively rapid and specific but require post processing of samples and

they are not truly quantitative. In order to overcome these limitations researchers have developed the real-time PCR (rt-PCR) method, which completely eliminated the need for post processing by providing real time information on the amount of amplicon accumulated in the reaction tube. The rt- PCR has been successfully used for qualitative and quantitative detection of foodborne pathogens (ref) and similar to the regular DNA based methods this method also requires a species specific target gene.

In the case of *V.vulnificus* hemolysin /cytolysin gene (*vvhA*) has been successfully used for many PCR and DNA hybridization assays. Using the same target gene (*vvhA*) Paniker et al (2004) developed a multiplex PCR protocol to detect clinical and environmental strains of *V. vulnificus* in shellfish while Campbell and Wright (2003) developed TaqMan rt-PCR for detection of *V. vulnificus* from oysters and found that rt-PCR could be a rapid and sensitive alternative to conventional culture based methods for qualitative and quantitative detection of the bacterium. Panicker and Bej (2004) utilized the TaqMan based rt-PCR method with a probe designed to target *V. vulnificus* hemolysin (*vvh A*) gene at very low levels of *V.vulnificus* in oysters after 5 hr enrichment, In the same study they also discussed inhibitory effects of oyster meat on rt-PCR.

One approach to overcome the inhibitory effect of the oyster meat and reduce the enrichment period is to use Immunomagnetic separation (IMS) as a pre rt-PCR treatment which can readily separate target organisms from PCR inhibitors and other competing micro flora. The objective of this study was to develop and standardize an IMS rt-PCR protocol for rapid detection of *V. vulnificus* from oyster homogenate.

4.2 Materials and Methods

4.2.1. Bacterial Strains Used

V.vulnificus ATCC 27562 strain was used to produce standard curves. *V.vulnificus* ATCC 27562 and two other clinical isolates 1007 were used to inoculate oyster homogenate for this study. Specificity of the real-time PCR IMS assay was determined by testing 11 *Vibrio* strains from our culture collection (Table 4.1) All strains were maintained on TSA slants supplemented with 2% or 3% NaCl depending on the nutritional needs of the individual organism.

Table 4.1: Bacterial Strains Used in this Study

Bacterial strain used in the study	
<i>V. vulnificus</i> ATCC 27562	<i>V. natriegens</i> ATCC 14048
<i>V. parahaemolyticus</i> ATCC 17802	<i>V. harveyi</i> ATCC 35084
<i>V. cholera</i> ATCC 14035	<i>V. campbellii</i> ATCC 25920
<i>V. mimicus</i> ATCC 33653	<i>V. damsela</i> ATCC 35083
<i>V. fluvialis</i> ATCC 33809	<i>V. alginolyticus</i> ATCC 33787
<i>V. vulnificus</i> 1007 (clinical isolate)	

4.2.2 Sample Preparation

Oysters were obtained from local grocery stores. Oysters were carefully weighed and mixed with sterile PBS at a 1:1 wt/v ratio. Oyster tissue homogenates were obtained by stomaching samples in a stomacher for 1 min and filtering the resultant homogenate through

cheesecloth to remove large particles. Oyster homogenates were spiked with 16- 18 h old *V. vulnificus* (ATCC 27562/1007) culture to obtain a bacterial density of $10^2 - 10^3$ CFU/g in spiked homogenate. One ml of spiked homogenate was used for the IMS rt- PCR assay.

4.2.3 Immunomagnetic Separation

Oyster homogenate was prepared as described earlier. One ml of spiked oyster homogenate was mixed with 30 μ l of IMB coated with anti *V. vulnificus*-H monoclonal antibodies 3-D-10 ($5\text{mg}/10^7$). The suspension was incubated at room temperature for 30 min on a rotating platform. At the end of the incubation period IMB- bacterial complex was removed by placing the tube in a magnetic particle concentrator. The beads were washed once with PBS to remove nonspecifically bound oyster tissue particles and resuspended in 500 μ l sterile Millipore water. This step followed by IMS sample preparation and rt- PCR.

4.2.4 DNA Templates Preparation

Pure culture sample preparation: *V. vulnificus* ATCC 27562 was grown in TSB+2% NaCl for 16-18 h. At the end of the incubation period the cell pellet was obtained by centrifugation of tube at 3000 X g for 5 min. The pellet was resuspended in 500 μ l sterile milliQ water and heated in a boiling water bath for 15 min. Cellular components and paramagnetic beads were pelleted by centrifugation at 16000 X g for 2 min and the supernatant was used for rt-PCR for quantitative detection.

4.2.5 IMS Sample Preparation

At the end of the IMS process beads were obtained and resuspended in 500 μ l sterile millipor water than heated in boiling water bath for 15min. Cellular components and

paramagnetic beads were pelleted by centrifugation at 16000 X g for 2 min and the supernatant was processed to obtain bacterial counts.

4.2.6. rt-PCR

rt- PCR assay was carried out with forward primer - TGT TTA TGG TGA GAA CGG TGA CA (DNA technology, Aarhus C, Denmark) reverse primer TTC TTT ATC TAG GCC CCA AAC TTG (DNA technology, Aarhus C, Denmark) and probe CCG TTA ACC GAA CCA CCC GCA A-3 (DNA technology, Aarhus C, Denmark) (Campbell and Write, 2003) that was tagged by a reporter dye FAM (6-fluorescein) and BHQ-1, Black Hole-1 quencher dye. The Smart Cycler (Cepheid, Sunnyvale, CA) was used to perform rt-PCR. The rt-PCR amplification mixture was prepared by mixing 3 µl DNA template, (0.90 µm primers, 0.25 µm fluorogenic probe and PrimeX Taq premix (Takara, Japan). Reactions were performed in specifically designed thin walled reaction tubes. The PCR parameters used were: holding samples at 50°C for 2 min followed by denaturation at 95°C for 10 min and amplification at 95°C for 15 s and 60°C for 1 min (40 repetition). The crossing threshold value (Ct) was set at 30 for all reactions.

4.2.6. Specificity Detection

Overnight grown cultures of different *Vibrio* strains were serially diluted with PBS to reach 10³ cells/ ml. The IMS rt- PCR assay was carried out on each strain utilizing the protocol described in this section

4.2.7 Enrichment

Oyster meat homogenate was prepared by stomaching 10 gm of oyster meat with 20 ml of TSB +2% NaCl. The oyster homogenate was then filtered through cheesecloth to remove large particles and spiked with *V. vulnificus* ATCC 27562 to reach concentrations of 10¹

CFU/ml. One ml sample was collected every hour for 6 hours and immediately tested with IMS rt-PCR to determine the sensitivity of the assay.

4.2.8 Standard Curve Preparation

V. vulnificus ATCC 27562 were grown in TSA+2% salt for 16-18 hours. At the end of incubation the bacterial suspension was serially diluted to obtain dilutions 10^0 to 10^7 in sterile phosphate buffer saline (PBS). Total plate count of each dilution was performed by the standard spread plate method utilizing Nutrient agar +2% NaCl plates. One ml of each serially diluted culture was collected in a 1.5 ml centrifuge tube and prepared by the method described in the sample preparation.

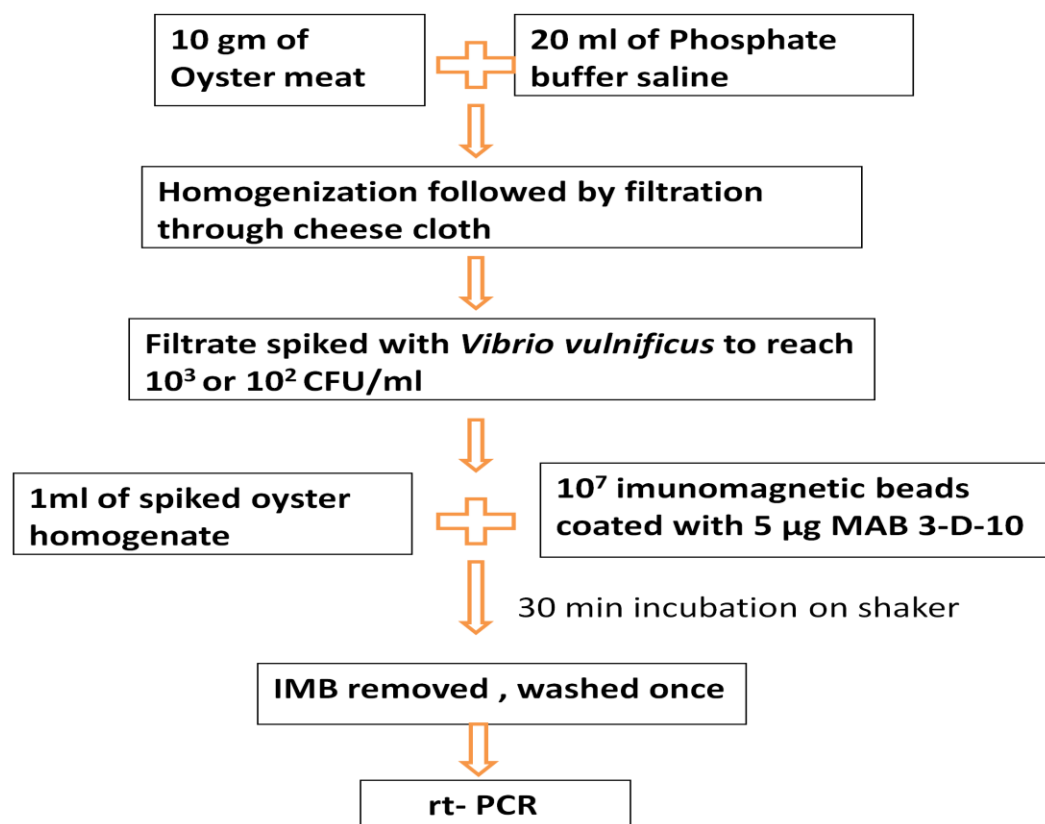


Figure 4.1: Schematic representation of Immunomagnetic separation coupled with rt-PCR protocol for the detection of pathogenic *V. vulnificus* from oyster homogenate

4.3 Results and Discussion

4.3.1. Standard Curve

Standard curve was prepared to establish a relationship between log cell concentration and Crossing Cycle threshold (Ct) values. Eight different concentrations of *V. vulnificus* ATCC 27562 (10^0 to 10^7) were prepared and the highest concentration gave a Ct value of 16. With the decreasing bacterial concentration in the samples, the number of cycles required to produce noticeable fluorescent signal increased and the standard curve demonstrated a linear relationship between bacterial counts and Ct values with a correlation coefficient of $r^2=0.99$. The standard curve was able to detect bacterial numbers as low as 10 at the end of 40 cycles (Fig 4.2).

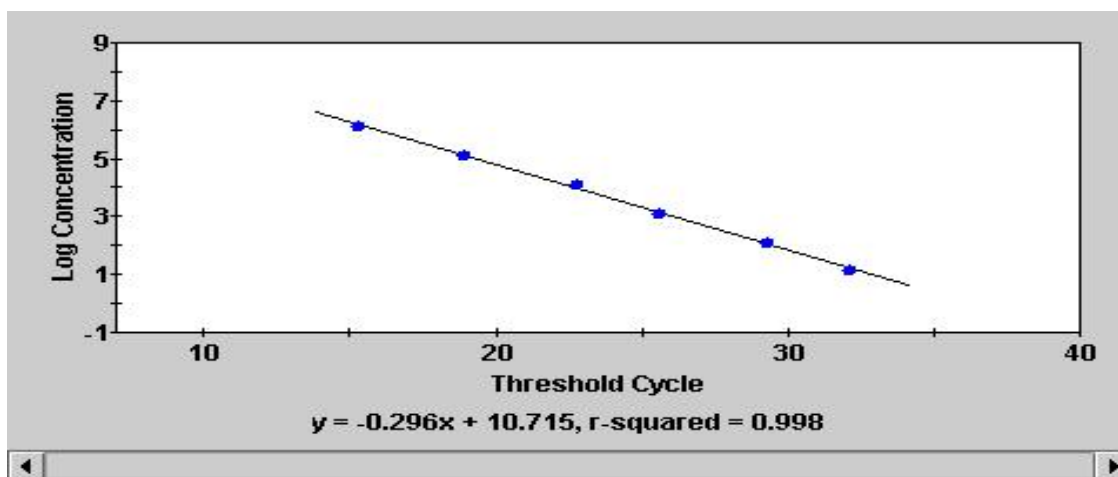


Fig 4.2: Standard curve showing the log cell number plotted vs. the rt-PCR cycle threshold for serial 10 fold dilutions of *V. vulnificus* culture.

4.3.2 Specificity of IMS rt -PCR

The specificity of IMS rt- PCR was determined by testing 11 Vibrio strains and it was found that our method specifically identified all *V.vulnificus* strains and did not react with other Vibrio strains tested. Specificity of this test is a combined function of IMS and rt-PCR steps.

Anti - H monoclonal antibodies specifically target *V. vulnificus* as they are very specific (Jadeja et al, 2010 and Simonson et al, 1988) while rt-PCR targets species specific *vvh A* gene of *V. vulnificus* hence reduces the chance of false positive results. In a similar type of study Fu et al., (2005) also found that the IMS-rt PCR combination exhibited better specificity than IMS or rt-PCR alone.

4.3.3 IMS rt-PCR Assay from Seeded Oyster Homogenate

Sensitivity of the assay was tested with oyster homogenate spiked with *V. vulnificus* at two different concentrations 10^2 and 10^3 . One important objective of this study was to improve the sensitivity of rt-PCR detection by removing the PCR inhibitors present in oyster homogenate. It was found that when rt-PCR was not combined with IMS the sensitivity of detection was compromised by 1-2 logs (data not shown) because of PCR inhibitors found in oyster homogenate. A similar observation was made by Kaufman et al (2002) who reported that oyster tissue homogenate decreased sensitivity of rt-PCR detection of *V. parahaemolyticus* up to 2 logs. In contradiction to these findings Panicker and Bej., (2004) found that the sensitivity of the rt-PCR assay was not affected by oyster homogenate, but they also reported that in their study the enrichment step significantly diluted oyster homogenate which possibly reduced the concentration of PCR inhibitors present in oyster homogenate.

Our results with IMS reagent containing 3-D-10 MAB exhibited 45.1% and 48.1% (Table 4.1) average binding in oyster homogenate spiked with 10^3 *V. vulnificus* ATCC 27562 and 1007 respectively. At lower *V. vulnificus* concentration (10^2 C.F.U./ml) of our assay decreased to 26.7% and 28% for *V. vulnificus* ATCC 27562 and 1007 respectively. As the bacterial concentration decreased in oyster homogenate the sensitivity of assay also decreased.

Similar results were observed by Skjerve et al., (1990) and Fratamico et al., (1992) in their respective studies. One possible explanation for this decrease might be that bacteria have less chance of interacting with IMS reagents.

During the IMB washing step as we found that on each washing step a large percentage of bound bacteria were detached from the beads and washed away. Skjerve et al., (1990) also found that during the IMS process each washing step dramatically reduced bacterial recovery.

Table 4.2. IMS rt-PCR Assay of Oyster Homogenate Spiked with *V. vulnificus*

MAB	Strain of <i>V. vulnificus</i>	Inoculum	Number of <i>V. vulnificus</i> detected by IMS rt-PCR	%Binding[*]
3-D-10	ATCC 27562	3. 1X10³	1.4 X10³	45.1^a
		8.6 X10²	2.3 X10²	26.7^b
	1007	2.9 X10³	1.4 X10³	48.7^a
		7.5X10²	2.1X10²	28.0^b

*means within column with different letters are significantly different ($P < 0.05$)

Initially we have used three repeated washing steps in order to remove PCR inhibitors and other bound meat particles, but it was found that with three washing steps recovery rate of bacteria dropped to undetectable levels in some cases (approximately 50 to 60% loss of bound bacteria at each wash.-data not shown) hence, in this study we have used single washing steps. Hudson et al., (2001) had a similar type of reduction with *L. monocytogenes* which was reduced by approximately 50%.

4.3.4 Enrichment Study

In the summer months oysters commonly have very high levels of bacterial counts (up to 10^5 CFU/gm) and *V. vulnificus* may comprise up to 50% of these microflora and can decrease rapidly and sometimes they may become non-detectable by various standard methods of pathogen detection. However, Whitesides and Oliver (1997) found that culturability may be regained with a temperature upshift.

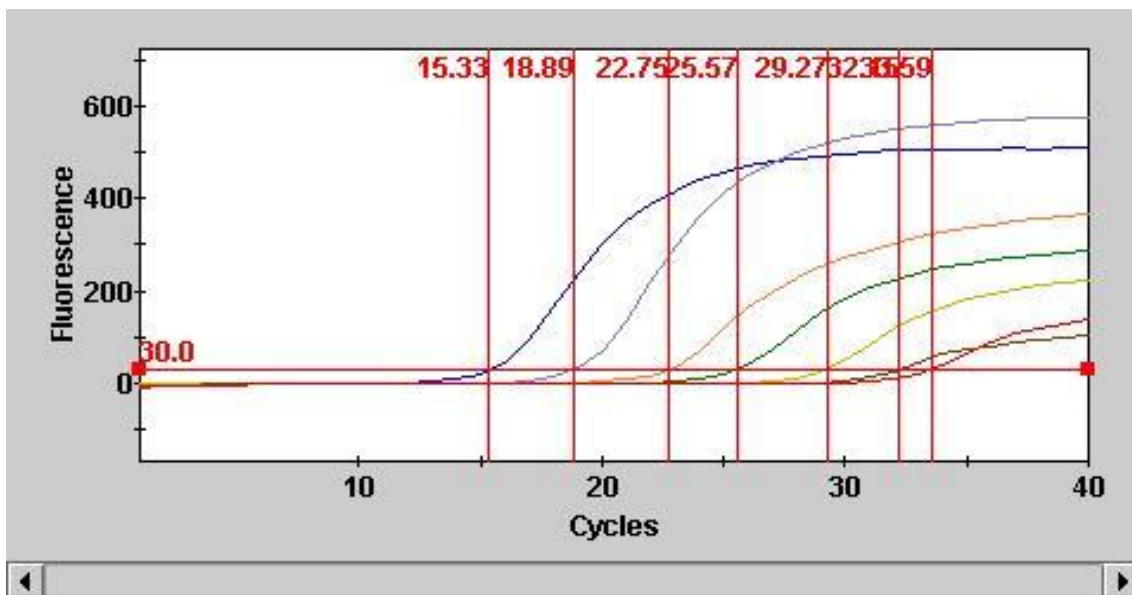


Fig 4.3. Representation of optic graph for the number of cycles versus the number of fluorescence units for each sample used to calculate the *ct* value

In such cases a short enrichment step could be an approach to improve sensitivity of various detection methods. In this study we found that the sensitivity of IMS rt-PCR assays was 10^2 CFU/ml, but when combined with a short 5 hr enrichment step our assay was successfully able to identify <10 cells/g oyster tissue. Panicker and Bej (2005) successfully combined a 5 h enrichment step with rt-PCR to obtain a detection limit of 1CFU/gm *V. vulnificus*.

Our IMS rt-PCR assay was specifically able to identify *V. vulnificus* from oyster homogenate at concentrations as low as 10^2 CFU/ml within 3 h. When combined with a short enrichment step the sensitivity of the assay further increased to <10 CFU/ml. The assay described here could be a rapid and sensitive alternative to various conventional culture based *V. vulnificus* identification methods.

4.4 References

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CHAPTER 5
LATERALFLOWCHROMATOGRAPHY (DIP STICK) ASSAY FOR
VIBRIO VULNIFICUS

5.1. Introduction

V. vulnificus is halophilic and inhabits coastal warm waters around the world (Vickery et al., 2007). In the U.S. *V. vulnificus* is abundantly found in the Gulf of Mexico during the warmer months of the year (Panicker et al., 2005). *V. vulnificus* is considered to be the most invasive Vibrio in the U.S. causing high fatalities especially in immunocompromised individuals (Desenclos et al., 1991; Oliver, 1989). The primary source of transmission of *V. vulnificus* infection is through consumption of raw or undercooked shellfish particularly in raw oysters, In addition to this skin cuts or wounds exposed to *V. vulnificus* in marine water are also a well documented route of infection (Patel et al., 2002).

The conventional culture methods for *V. vulnificus* detection often rely on long and laborious processes of enrichment and plating on specific media, including a series of biochemical tests as confirmatory steps. The rapid detection of *V. vulnificus* is necessary and recommended not only in a situation where a person is suspected to have been infected with *V. vulnificus* but also for monitoring this pathogen in the environment and in seafood as a pro-active measure to decrease the *V. vulnificus* related infections and outbreaks. In recent years various rapid DNA based methods such as real-time PCR and DNA hybridization have been developed and utilized for rapid detection of *V. vulnificus* but the complex nature of these tests and high level of skills required to run these test make them unsuitable for onsite screening. One approach to overcome these problems is to use a device which requires minimum user dependent steps such as a lateralflow device (dip stick/ test strip). The dip stick is a very versatile test, and has been successfully developed for many bacterial and viral analytes such as shrimp white spot syndrome virus and *Vibrio harvei* (Sithigorngul et al., 2007). The dip stick test targets specific antibodies to detect analyte and when present it develops a visible signal. In recent years the

dip stick device gained popularity for its point of care screening capabilities and other benefits such as compact size and rapid detection of target analyte. The dip stick for detection of *V. vulnificus* from oysters could be an answer to seafood industries rapid screening needs. A successfully developed dip stick would not only provide rapid screening of pathogens but also could be utilized for a standalone detection method when combined with short pre-enrichment of pathogens. For the development of a sensitive and specific dipstick for *V. vulnificus*, utilization of species specific antibody is essential. It is well documented that *V. vulnificus* possess species specific H antigen and armed with this knowledge Jadeja et al (2010) utilized *V. vulnificus* anti H monoclonal antibodies to develop Immunomagnetic separation technique for *V. vulnificus*. The purpose of this study is to develop a rapid, user friendly and compact screening device which can detect the presence of *V. vulnificus* from oyster homogenate within 5 min.

5.2. Materials and Methods

5.2.1. Bacterial Strains

A total of 8 *Vibrio* strains including *V. parahaemolyticus* ATCC 17802, *V. vulnificus* ATCC 27562, *V. cholerae* ATCC 14035, *V. mimicus* ATCC 33653, *V. fluvialis* ATCC 33809, *V. harveyi*, ATCC 14126, *V. campbellii* ATCC 25920, *V. damsela* ATCC 35083 and Two different *V. vulnificus* isolates 1007 and C7184 were used in this study. All strains were preserved in Tryptic soy broth (TSB) supplemented with 30% glycerol and stored at -80 °C. Bacteria were grown in TSB with 2% or 3% NaCl depending on the requirements of each organism.

5.2.2. Anti -H MAB Production

Species specific Anti –H monoclonal antibodies were prepared by the method previously described by Jadeja et al. (2010). Briefly, purified flagellar core isolated from the motile strain of *V. vulnificus* ATCC 27562 was used to immunize 8 week old BALB/c mice for 4 to 8 weeks.

Mice with elevated anti-H flocculation titers were boosted and sacrificed to collect spleen cells after 3 days. Hybridoms were produced by mixing isolated spleen cells with myeloma cells at 4:1 ratio and fused with 50% polyethylene glycol. The hybridomas were screened for desired antibody production by ELISA utilizing the purified flagellar core protein as an antigen. Hybridomas producing target antibodies were cloned twice utilizing a limiting dilution method. Finally antibodies were purified by affinity chromatography.

5.2.3. Preparation of Test Strip

The test strips were prepared by Arista Biotech Co. Ltd, (PA, USA). Species specific Anti *V. vulnificus* -H monoclonal antibody 3-D-10 was conjugated to 40 nm colloidal gold particles at a ratio of 10 µg/ml colloid. Resultant antibody conjugate was dispensed onto the membrane (15 µ pore size) at 0.645mg/ml concentration at a rate of 1µl/cm using ISO Flow (Imagene Technologies, USA). The control line was prepared by using Goat anti Mouse IgG at 1mg/ml concentration and sprayed on the membrane at the rate of 1.5µl/cm. After an adequate drying period test strips were assembled as demonstrated in (Fig-1) and secured in plastic housing. Prepared dipsticks were individually packed and stored in a desiccated bag.

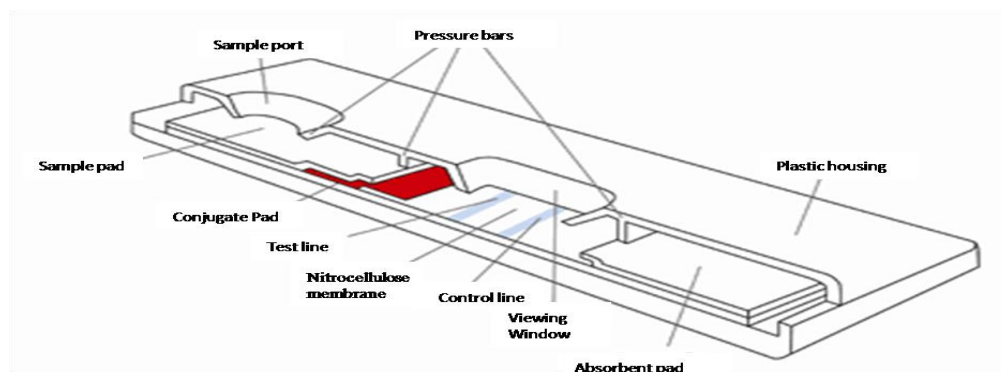


Figure5.1. Schematic representation of test strip device.
Source: <http://www.azonano.com/details.asp?ArticleID=1898>

5.2.4. Specificity Testing

Specificity of the lateralflow device was tested against pure cultures of 8 vibrio strains: *V. parahaemolyticus* ATCC 17802, *V. vulnificus* ATCC 27562, *V. vulnificus* 1007, *V. vulnificus* C7182, *V. cholerae* ATCC 14035, *V. mimicus* ATCC 33653, *V. fluvialis* ATCC 33809, *V. harveyi*, ATCC 14126, *V. campbellii* ATCC 25920 and *V. damsela* ATCC 35083. All bacterial cultures were grown overnight on TSB +2% or 3% NaCl depending on the nutritional need of individual organisms. These overnight grown cultures were serially diluted to obtain a bacterial concentration of 10^4 CFU/ml and applied on test strips.

5.2.5. Sensitivity Testing

Sensitivity of the dip stick device was tested against *V. vulnificus* ATCC 27562 and two clinical isolates, *V. vulnificus* 1007 and C7184. Overnight grown culture of bacterium was serially diluted with PBS to reach 10^6 to 10^1 cells per ml. Each dilution was tested again on three dip stick devices. The last dilution that produced visible results was noted and bacterial counts of the same sample were determined by the plate count method.

5.2.6. Sensitivity Testing of Pre-enriched Samples

Oyster meat homogenate was prepared by stomaching 10 gm of oyster meat with 20 ml of APW. The oyster homogenate was then filtered through cheesecloth to remove large particles and spiked with *V. vulnificus* ATCC 27562 to reach concentrations from 1 to 10^6 CFU/ml. One ml from each sample was collected at every hour for 8 hours and immediately tested with the dipstick device. For lateral flow testing 3 drops (aprox. 150 μ l) of spiked oyster homogenate was applied to the device and for each sample three test strips were tested.

5.2.7. Storage Stability Testing

Dip stick device strips were stored at three different temperatures 4⁰ C, 25⁰ C and 40⁰ C for two weeks. Each week lateral flow devices stored at different temperatures were tested with *V. vulnificus* ATCC 27562 at 10⁵ and 10⁴ CFU/ml to determine the effect of storage temperature on the performance of the device.

5.3. Results and Discussion

5.3.1. Anti- H Monoclonal Antibody Production

Six different stable hybridomas producing IgG were isolated. From the cell culture fluid antibodies were purified and MAB 3-D-10 which exhibited the highest anti H ELISA titer were used to develop the dip stick device.

5.3.2. Specificity Testing

Prepared dip stick devices were tested with 8 different *Vibrio* spp along with two clinical isolates of *V. vulnificus* 1007 and 8264. All vibrio strains except for *V. vulnificus* ATCC 27562 along with two other clinical isolates failed to produce visible signal on the device at tested range of bacterial concentration (10⁶ to 10³). Anti *V. vulnificus* H monoclonal antibodies are the most crucial elements of the dip stick device which provided very high specificity. In earlier studies by Simonson and Siebeling (1988) demonstrated that anti *V. vulnificus* H monoclonal antibodies are highly specific and in their study where they tested 31 vibrio species with anti *V. vulnificus* H monoclonal antibodies and MAB specifically identified *V. vulnificus* and did not react with any other *Vibrio* strains tested. The findings of this study are also in agreement with our previous study Jadeja et al., (2010) where we tested anti *V. vulnificus* H MAB against 11 different *Vibrio* strains and 40 different *V. parahaemolyticus* isolates and found that MAB only

reacted with *V. vulnificus*. So this high specificity of MAB makes them suitable for use in development of immunochromatographic technique which distinctively identifies *V. vulnificus* from complex environmental samples.



Fig. 5.2. Test strips. *V. vulnificus* ATCC 27562 bacterial suspension 10^4 CFU/ml (A) and (B) *V. parahaemolyticus* were applied to the test strips. T = test line; C= control line.

5.3.3. Storage Stability Testing

Storage stability of the strips was tested by storing strips at 4°C , 25°C and 40°C for two weeks. The reason behind selecting these specific temperatures was that we want to determine the effect of two possible extreme temperatures where these assays might be used for onsite pathogen testing. At the end of 1 and 2 weeks storage, samples were tested against two different concentrations of *V. vulnificus* 10^5 and 10^4 CFU/ml to determine the effect of temperatures on the sensitivity of test. All dip stick devices were successfully able to identify the organism, hence we found that different storage temperatures did not affect or alter the sensitivity of the dip stick device. In a similar study Sithigorngul et al (2007) developed a dip stick assay for *V.*

harveyi and in their 60 day long thermal stability testing at 60⁰ C they did not find the effect of temperature abuse on testing capability of the device.

5.3.4. Pre-enrichment Sensitivity

At the end of the first hour of the pre-enrichment study oyster homogenate inoculated with 10³ *V. vulnificus* was able to produce a clear visible signal on the dip stick device. After 4 hours of enrichment, the oyster homogenate inoculated with 10² CFU/ml *V. vulnificus* exhibited strong immunoreactions with the dip stick device however, oyster homogenate inoculated with 10¹ CFU/ml *V. vulnificus* failed to produce a visible signal on the dip stick device until 6 1/2 hr incubation. Hence, pre-enrichment of an oyster homogenate in APW for 6 ½ h increases the sensitivity of the dip stick device to the level where it can detect the bacterial concentration at 10¹ CFU/ml. Kawatsu et al (2006) developed an immunochromatographic assay device to detect *V. parahaemolyticus* Thermostable direct hemolysin (TDH) which can identify 2X10¹ CFU/ml after 16 h enrichment in APW. Our results are also comparable with the study conducted by Sithigorngul et al (2007) where they were able to identify 10¹ CFU/ml *V. harveyi* after 6h incubation. As our dipstick device was able to detect *V. vulnificus* from oyster homogenate within 5min, analysis of any oyster sample for presence of *V. vulnificus* could be completed within 6 1/2 h including enrichment period, this is significantly less time required than conventional culture based methods.

5.3.5. Dipstick Sensitivity Test

The lowest concentration of *V. vulnificus* ATCC 27162 that produced positive test strip results without any enrichment process was approximately 10⁴ CFU/ml (Table 5.1). In a similar type of study Sithigorngul et al (2007) developed a dipstick device for detection of *V. harveyi*

and reported the sensitivity of 10^6 CFU/ml. A study by Yan et al (2006) who used a slightly different approach -Up- converting phosphor technology, to develop a quantitative detection device for *Yersinia pestis* reported a detection limit of 10^4 CFU/ml.

Table 5.1. Pre-enrichment Time Required to Detect Various *V. vulnificus* Concentrations in Oyster Homogenate.

No.	<i>V. vulnificus</i> ATCC 27562 in oyster homogenate	Pre-enrichment period for dipstick detection
1	10^6 CFU/ml	0 h
2	10^5 CFU/ml	0 h
3	10^4 CFU/ml	0 h
4	10^3 CFU/ml	1 h
5	10^2 CFU/ml	4 h
6	10^1 CFU/ml	6 ½ h

So the dipstick for *V. vulnificus* has better or comparable sensitivity to other devices of its kind. The capability of the device to detect high to moderate levels of pathogens from oyster meat make them suitable for onsite screening of oysters without any enrichment process.

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CHAPTER 6
CONCLUSION

In accordance with our objectives we have successfully produced and utilized anti *V. vulnificus* -H monoclonal antibodies to develop various serological detection techniques for *V. vulnificus* from oysters.

The satisfactory development of an immunological separation (IMS) assay and optimization of the same for *V. vulnificus* detection from oyster homogenate fulfilled our primary objective. This has enabled us to develop a rapid detection method, in which IMS coupled with rt-PCR has resulted in an assay which was able to identify 10^2 CFU/ml of *V. vulnificus* from oyster homogenate within 3h.

The study focused on the further utilization of our species specific monoclonal antibodies to develop a dip stick assay, which exhibited a sensitivity of 10^4 CFU/ml of *V. vulnificus* from oyster homogenate within 5 min. and when combined with 6 ½ h enrichment period dip stick was able to identify < 10 C.F.U/ ml *V.vulnificus* cells. Finally, in our study we found that IMS rt-PCR and dip stick assays could be use as rapid and sensitive identification methods for *V. vulnificus* .

APPENDIX 1

LIST OF DIFFERENT *VIBRIO* SPECIES USED

Species	Culture Number	Type of Strain	Source ^a
<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>Vibrio 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>	ATCC 3132 y25084	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

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

APPENDIX 2

LIST OF MEDIAS AND REAGENTS USED

1. Phosphate Saline buffer (PBS)- (NaCl-7.65g, Na₂HPO₄, anhydrous-0.724 g, KH₂PO₄-0.210 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 121⁰ C for 15 min.
TCBS Agar – Made according to manufacturer’s instructions (Troy Biologicals Inc. Troy, MIL)
3. Tryptic Soy Broth and agar with 2% Nacl (TSB) – Made according to Manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO 63103).
4. Nutrient Agar with 2% NaCl- Made according to manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO 63103)
5. Vibrio Maintenance medium- Tryptone-8g, NaCl- 20 g, Nutrient Broth- 4g, MgCl₂- 4g, KCl- 4g, Agar- 4 g, d.water- 1000 ml)

APPENDIX 3

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