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Application of Monoclonal Antibodies to Develop Rapid Immunoassays for *Vibrio vulnificus*: A Mini Review

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Abstract
Vibrio vulnificus (*V. vulnificus*) infection is a disease with serious health implications. Notably there are only about 50 confirmed cases of *V. vulnificus* per year but, the severity of the disease that is caused by this organism makes it the most pathogenic *Vibrio*. 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. In the case of *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, rapid and sensitive detection methods are required. Application of species-specific monoclonal antibody (MAB) could increase the sensitivity and speed of *V. vulnificus* detection by eliminating enrichment steps. This review discusses the recent advances in the area of antibody based *V. vulnificus* detection strategies.

1. Introduction

Vibrios are responsible for numerous deaths worldwide and members of this genus can cause infections in various forms ranging from gastroentitis, septic shock to soft tissue necrosis.

According to the CDC in 2007, 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. are recognized as human pathogens. The most common pathogenic *Vibrio* species include *V. cholerae, V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. fluvialus, V. furnissii, V. hillisae, V. metschnikovii, V. damsla 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% (Linkous and Oliver, 1999). A close look at the CDC data suggests that *V. vulnificus* was responsible for 31 out of 36 vibrio related deaths in 2007, this mortality rate shows 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 when temperature dropped below 15°C, levels of *V. vulnificus* decreased to undetectable levels (Pfeffer et al., 2003). Salinity also has a significant effect on *V. vulnificus* survival, this pathogen prefers a low to intermediate salinity (5-25%). (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 10CFU/g of Gulf Coast harvested oysters, but bacterial count increases with temperature, by the end of April *V. vulnificus* density usually exceeded 10^3 CFU/g (Motes et al., 1998). An average of 10^4 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. vulnficus* multiply rapidly in oysters and hence, levels at market can be >1 log greater than at harvest (Cook et al., 2002). Beside shellfish *V. vulnificus* is also found in costal 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 10^4 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. vulnficus* 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. *V. vulnificus* Infection and Detection

2.1. Virulence Factors

Factors such as low pH survival, polysaccharide capsule, lipopolysaccharide (LPS), extracellular virulence factors, iron acquisition, attachment and adhesion protein expression contributes to the pathogenicity of *V. vulnificus* (Levin, 2005).

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. vulnficus*, breakdown lysine to produce cadaverine, which not only provide protection against low pH but also acts 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 intensively studied virulence factors (Tamplin et al., 1983; Tamplin et al., 1985). It is believed that the capsule protects the organism from host's defense mechanism and provides resistance to opsonization by complement and therefore, preventing 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 uncapsulated and capsulated *V. vulnificus* found that the capsulated strain significantly reduced the LD 50 value in the experiments indicating it was more virulant (Write et al., 1981).

Lipopoly scaccharide (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 decrees 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 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* pathogenecity and hence explained the high infection and mortality rate among individuals with elevated serum iron levels. Typically, in human 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(Webster and Litwin, 2000).

The catechol siderophore is the major siderophore which scavenge iron from transferring 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. Kim et al., (2006) reported that without pili the bacteria was unable to attachment to epithelial cells and resulted in significant increased LD 50 value in mice. 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) 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 study 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.

V. vulnificus produces various extracellular factors which contribute in the pathogenecity 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* pathogenecity are protease, collagenase, elastase, lipase, mucinase, RTX toxins and hyaluronidase.

2.2. *V. vulnificus* Infection

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

2.2.1. Gastroenteritis

This is the least sever 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 involves in this infection development includes chronic alcoholism and routine antacid use (Johnson et al., 1984).

2.2.2. Wound Infection

This type of *V. vulnificus* infections has fatality rates up to 25% (Oliver 1989). The bacterium can infect the pre-existing 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. Savior wound infection often requires amputation of the limbs or at least surgical removal of affected tissue.

2.2.3. 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 undercook oysters. 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 Cost. 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.

2.3. Detection of *V. vulnificus*

2.3.1. 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 second suggested method include direct plating and DNA hybridization (BAM). But, it has been well documented that both of these traditional methods are very time consuming and take up to 5 days to conform the presence of *V. vulnificus*.

2.3.2 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

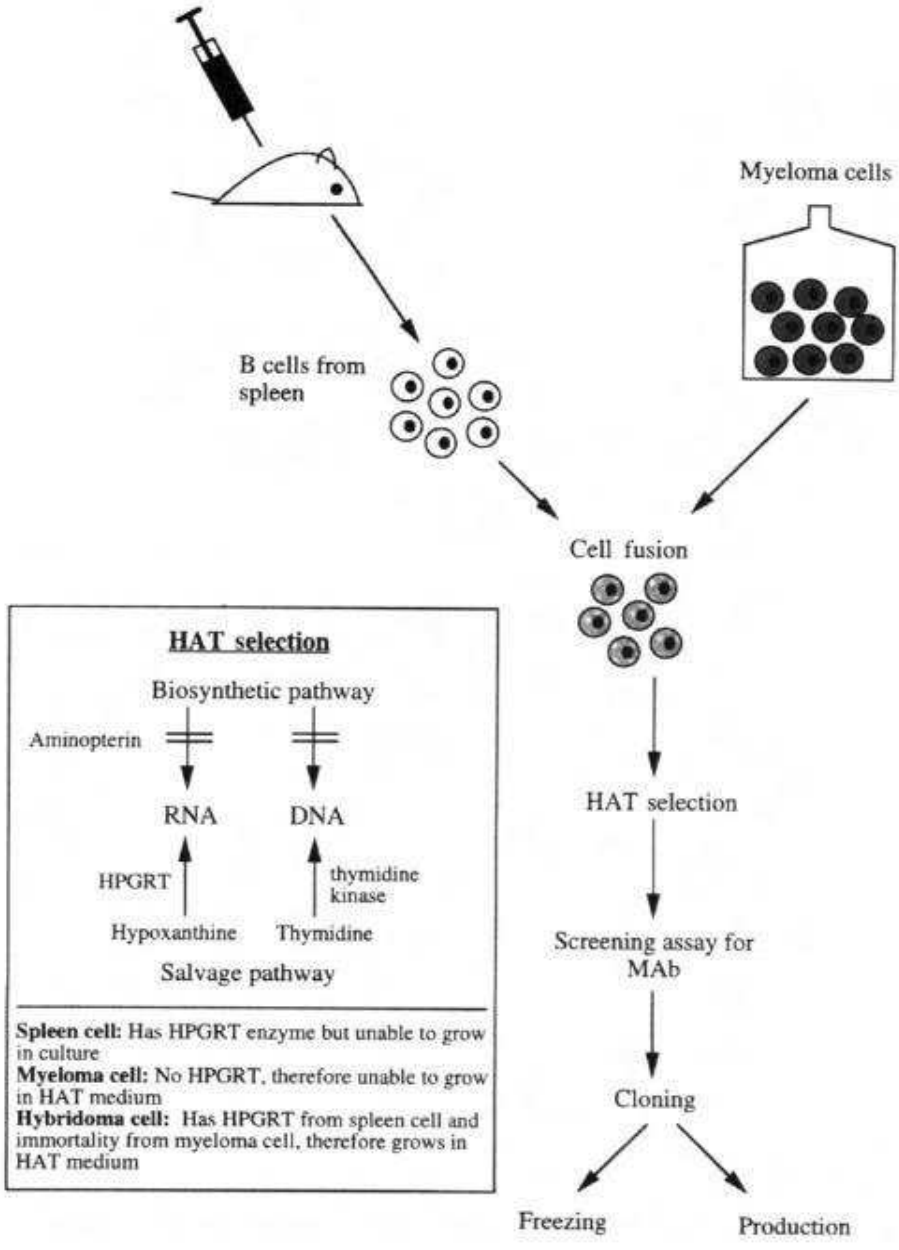
coaglutination assay for

V. vulnificus with about 99% detection rate. In order to improve sensitivity of the coagulation test Simonson and Siebeling (1986) used anti-flagellar monoclonal antibody which successfully identified all *V. vulnficus* 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.*

vulnficus but they were not as specific as anti-flagellar monoclonal antibody and had about a 1% false positive result (Simonson and Siebeling, 1986). Because of high specificity and sensitivity of anti-flagellar monoclonal antibody it could be used in development of various immunoassays for *V. vulnificus* such as immunomagnetic separation and lateral flow immunochromatography.

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).



Source: (Atbitar 2003)

Figure 1. *Monoclonal antibody production process.*

The general scheme for MAbs production is shown in figure 1. 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).

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 hypoxanthine phosphoribosyl ltranferase deficient myeloma cells are used for hybidoma 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). Jadeja et al., (2010) successfully developed several Anti-H monoclonal antibodies specific to *Vibrio vulnificus*. In the same study they also observed that the species specificity of the selected antibodies, which positively identified and coagglutinated 70 isolates identified genetically as *V. vulnificus* and did not react with 40 Vibrio parahaemolyticus isolates or nine other Vibrio species tested.

2.3.3. Lateral Flow Chromatography Test (Dipstick Test)

Dipstick test is very popular technique among all membrane based Immunoassays and widely utilized in 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.

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

Table 1. *Some of the recently developed dipstick assays for variety of analytes.*

Classification of analytes	Analyte	Assay type	References
Bacteria	Vibrio harveyi	Non- competitive	Sithigorngul et al.,
	Legionella pneumophila	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.,

Vibrio harvei and White spot syndrome virus (Sithigorngul el al 2007; Sithigorngul et al., 2006). Table 1 lists recently developed dipstick assays for various analytes. 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, assay is very simple and requires minimal user dependent steps, test is also shelf-stable
hence, suitable for many field use applications and finally, relatively low cost and short assay development time (O' Farrell, 2009). Jadeja et al, (2015) successfully developed lateral flow detection devices for detection of *V. vulnificus* from oyster using the anti-H monoclonal antibodies. Their lateral flow device was able to detect the presence of V. vulnificus at levels of approximately 4 log CFU/ml from phosphate buffered saline and oyster homogenate without any pre-enrichment process. When combined with 6.66 h enrichment period the assay was able to detect pathogen counts as low as 10 CFU/ml from oyster homogenate.

2.3.4. 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. 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. 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 inhibiters present in the sample (Fu et al., 2005). Jadeja et al, (2010) optimized the IMS protocol for detection of different strains of *V. vulnificus*. from spiked oyster homogenate and observed the *V. vulnificus* recovery levels between 25 to 57% with IMS.

3. Conclusions

V. vulnificus infection poses a considerable risk to human health. Recent advances in the field of *V. vulnifucs* detection made a great impact on the human health and also the sea-food industry. Though there are significant advances made in the detection and diagnostic of this pathogen, there is still a dire need to develop and optimized more rapid and sensitive *V. vulnificus* detection methods.

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