APPLICATION OF MOLECULAR DETECTION METHODS TO MOST PROBABLE NUMBER (MPN) ENUMERATION OF *Vibrio vulnificus* IN OYSTERS

By

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by

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I dedicate this work to Sergio, Arlette, Chae, Eddy, and all of my friends; and to my parents, who have always believed in me and provided me with unconditional love and guidance throughout my life. This work would not have been possible without their love and support.
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APPLICATION OF MOLECULAR DETECTION METHODS TO MOST PROBABLE NUMBER (MPN) ENUMERATION OF *Vibrio vulnificus* IN OYSTERS

By

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Major Department: Food Science and Human Nutrition

*Vibrio vulnificus* illness presents a severe problem for the shellfish industry and government organizations that monitor shellfish quality. This virulent bacterium is associated with primary septicemia, gastroenteritis, and wound infections. The source of infection has been frequently traced back to the consumption of raw oysters. Individuals with the highest risk for *V. vulnificus* infection are those with liver dysfunctions, hemochromatosis, diabetes, alcoholism or immunocompromise. Although the number of cases of *V. vulnificus* primary septicemia is low, the fatality rate is high and can exceed 50%. This study enumerated *V. vulnificus* from oyster samples by VVAP plate count of colony blot hybridization using a nonradioactive *V. vulnificus* alkaline phosphatase-labeled gene probe (VVAP) in order to evaluate novel detection methods for most probable number (MPN) enumeration. These methods included VVAP probe confirmation of MPN (VVAP MPN), a microtiter-based assay (Microtiter VVAP MPN), or real-time PCR confirmation of MPN (R-PCR MPN) to rapidly enumerate *V. vulnificus*
in oyster homogenates. PCR assay was also performed in combination with two different extraction methods, using two different methods of PCR detection (SYBR Green I and TaqMan®).

Media comparison studies examined the recovery of indigenous and artificially inoculated *V. vulnificus* on nonselective, semi-selective, and selective agars. Recovery of *V. vulnificus* was always higher with the nonselective than with selective media (TCBS and MCPC). Semi-selective agar with 3% NaCl was found to inhibit the recovery of indigenous but not seeded *V. vulnificus*. These studies suggest that indigenous *V. vulnificus* strains may be more sensitive to higher salt concentrations than seeded *V. vulnificus* strains.

Results comparing different molecular methods for confirmation of MPN indicated that MPN enumeration may underestimate the numbers of *V. vulnificus*, as determined by VVAP plate counts. MPN numbers confirmed by DNA probe in either the tube or microtiter format were significantly lower than *V. vulnificus* plate counts at both high and low levels of *V. vulnificus*. In contrast, R-PCR confirmation produced MPN numbers that were comparable to VVAP plate counts for samples with higher levels of *V. vulnificus* contamination; however, at lower levels there was significantly decreased sensitivity with the R-PCR MPN assay, warranting further investigation. Examination of different methods of DNA template preparations or detection methods did not reveal any significant differences in results. Advantages of R-PCR confirmation of MPN include increased sensitivity and more rapid detection (2 to 8 hours) of hundreds of samples while VVAP analysis requires a few days to analyze. Rapid and accurate assessment of seafood product safety is desirable for consumer confidence and for regulatory concerns.
CHAPTER 1
INTRODUCTION

Microbiology of Oysters

_Crassostrea virginica_ and _C. gigas_ are among the most commercially important bivalves (oysters) in the world (Keithley and Roberts 1988). Oyster meat provides great nutritional value. For example, 100-grams of uncooked oyster meats are a rich source of proteins (8.4%), minerals (potassium, iron, and sodium), and only 1.8% of fats (Campbell 2001). According to the Interstate Shellfish Sanitation Conference (ISSC 1998), the quality of oysters is assessed in several ways. First, shellstock oysters should close quickly when tapped; and if the shell does not close tightly or the oyster meat is dry when the shell is open, the product should be discarded. Second, if the oyster’s shell is broken or damaged, consumption is not recommended. Lastly, shucked oysters should have a creamy tan color and fleshy meat; and should smell fresh.

According to Prieur et al. (1990), oysters are filter feeders that capture suspended food particles from the ocean. Oysters are also capable of trapping bacteria and other microorganisms in the water; and serve as carriers for human pathogens (especially members of the family _Vibrionaceae_). During oyster feeding, microorganisms (especially bacteria) attach to oyster tissues, including the hemolymph, gills, adductor muscle, and the mantle, to take advantage of the flow created by the oyster to obtain nutrients (Tamplin and Capers 1992). _Vibrio vulnificus_ and other vibrios are indigenous to shellfish (such as clams, mussels, and especially oysters (DePaola et al. 1994, Oliver et al. 1983, Wright et al. 1996). Contamination of oysters with the bacterium _V. vulnificus_
has had a negative financial impact on the oyster industry because the infections caused by this pathogen may lead to serious illness and death in susceptible individuals.

Human illness associated with *V. vulnificus* remains a major problem to the shellfish industry and consumer safety. Recently, outbreaks involving *Vibrio* spp. (particularly, *V. parahaemolyticus*) posed a serious threat to the seafood industry (MMWR 1998). Conventional methods currently available to detect and enumerate these organisms are generally time-consuming, expensive, and unreliable; and most require further confirmation by additional assays, such as biochemical and immunological (FDA approved) techniques. Currently, the FDA recommends the use of post harvest treatment of oysters to lower the numbers of *V. vulnificus*.

Clearly, methods for rapid assessment of this bacterium are needed to better regulate and improve seafood safety. Separate studies have shown that PCR offers improved detection with more specificity and higher sensitivity (Blackstone et al. 2003, Higuchi et al. 1993, Hill et al. 1991). Problems inherent in traditional PCR assays include the inability to detect presumably dead cells and the use of post PCR processing, which is time-consuming and results in low resolution and low sensitivity. Campbell (2003) was able to overcome this problem by using real-time PCR.

**General Characteristics of Vibrio Species**

Members of the family *Vibrionaceae* are characterized as Gram-negative rods, facultatively anaerobic, and generally halophilic bacteria. Major pathogens within the genus *Vibrio* include *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae*. *Vibrio vulnificus* is very similar to *V. parahaemolyticus*, except that *V. vulnificus* is able to ferment lactose and is less salt tolerant than *V. parahaemolyticus* (Kelly 1982). *Vibrio*
*cholerae* can be discriminated from the other two because it can ferment sucrose; and because it does not require NaCl.

*Vibrio vulnificus*, a virulent pathogen associated with primary septicemia, gastroenteritis, and wound infections, was first described in by Hollis et al. (1976). This foodborne pathogen is found throughout coastal waters of the United States. Contamination of an open wound with salt water harboring the organism is the main cause of wound infections. Gastroenteritis is rare and may occur in healthy individuals after ingesting raw or undercooked shellfish contaminated with *V. vulnificus*. Primary septicemia results primarily from consuming raw oysters (and occurs in immunocompromised patients; or those with liver disease, cancer, diabetes, or hemochromatosis). Although the infectious dose of *V. vulnificus* is unknown, previous studies have shown that even one organism is enough to cause disease in animal models with iron overload (Starks et al. 2000, Wright et al. 1981). This bacterium can be isolated from stool, blood, and wound samples of infected persons. Patients suffering from primary septicemia have a higher mortality rate (> 50%) than those suffering from wound infections (about 20-25%). The importance of *V. vulnificus* to the seafood industry is primarily a consequence of the high mortality rate, and *V. vulnificus* disease is the leading cause of seafood-related deaths in this country (Shapiro et al. 1998, Tacket et al. 1984).

*Vibrio parahaemolyticus* is another *Vibrio* species commonly found in marine and estuarine environments throughout the world. Illness caused by this pathogen consists of gastroenteritis after ingesting contaminated shellfish and fish. Diarrhea, abdominal cramps, nausea, vomiting, headache, fever, and chills characterize disease caused by *V. parahaemolyticus* (Joseph et al. 1983). Illness is generally self-limiting; and few cases
require hospitalization and/or antibiotic treatment. The infectious dose of this bacterium is extremely high (about 1 million organisms) except for individuals who over consume antacids (FDA/CFSAN 2003). Furthermore, virulence factors remain undefined in this pathogen, although virulent strains of *V. parahaemolyticus* produce the thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) genes that have been implicated in foodborne diseases.

*Vibrio cholerae* strains have spread in epidemic forms throughout history and across the world, especially in India, Asian countries, and South America. Disease caused by *V. cholerae* leads from mild, watery diarrhea to profuse, watery diarrhea, lasting from hours to days. Other symptoms include abdominal cramps, nausea, vomiting, dehydration, shock, and if not treated promptly, death from fluid and electrolyte loss. The infectious dose of *V. cholerae* is similar to that of *V. parahaemolyticus*. After *V. cholerae* has been ingested, it attaches to the small intestine using the toxin-coregulated pilus (TcpA) and symptoms are the result of the production of the cholera toxin (CT). Cholera toxin consists of A and B components that bind the cell membrane (B) and generate a toxic activity (A). Cholera toxin modifies the Gs protein, increasing cyclic AMP (which in turn blocks sodium absorption, elevates chloride secretion, and causes diarrheal stool) (Keusch and Acheson 1999). Typically, this bacterium is found in contaminated water; and has been recovered from stool samples. Although cholera can be rapidly fatal and produce large numbers of deaths during epidemics, early medical treatment to prevent dehydration (loss of electrolytes) avoids all complications and death, and tetracycline reduces infection symptoms.
Recent Outbreaks Involving *Vibrio* spp.

*Vibrio* spp., especially *V. cholerae*, have been studied extensively and are implicated in major epidemics and pandemics throughout history. A recent outbreak caused by *V. cholerae* was reported in South-America (Peru); and quickly grew to epidemic proportions and spread to other South-American and Central-American countries, including Mexico. Over a million cases and 10,453 deaths in the Western Hemisphere between 1991 and 1995 were attributed to this pathogen (Morbidity and Mortality Weekly Report (MMWR) 1991). *Vibrio cholerae* outbreaks are frequently associated with contaminated water; whereas other *Vibrio* outbreaks are most often a result of contamination from food handlers and/or ingestion of raw or undercooked shellfish, especially oysters (DePaola et al. 1994, Kelly 1982, Tamplin et al. 1982).

Recently, outbreaks involving *V. parahaemolyticus* have startled the seafood industry, as well as consumers (MMWR 1998). All of these outbreaks have involved the consumption of raw or undercooked shellfish, especially oysters, clams, and mussels. *Vibrio parahaemolyticus*, outbreaks lead to gastro-intestinal disease. Virulent *V. parahaemolyticus* generally produce hemolysin genes, which encode TDH or TRH (Kim et al. 1999, Kishishita et al. 1992). DePaola and colleagues (2000) described the first confirmed oyster-associated outbreak in the Pacific Northwest caused by *V. parahaemolyticus* in 1997. This outbreak resulted in 209 confirmed cases mainly from the Washington and British Columbia area. *Vibrio parahaemolyticus* O3:K6 has been associated with outbreaks (416 cases and 98 culture confirmed) from the ingestion of raw oysters. This new O3:K6 serotype was also found in Taiwan, Laos, Japan, Thailand, and Korea (Hara-Kudo et al. 2001). Investigators indicated that high water temperatures may have played a role in this outbreak. Moreover, in 1991, raw and grilled oysters and clams...
were the common food source associated with gastroenteritis infections in Hawaii (MMWR 1991). All the oysters implicated in this outbreak were purchased from a Virginia distributor while the clams were purchased from Massachusetts dealers.

*Vibrio vulnificus*, on the other hand, is not generally implicated in outbreak disease but instead involves isolated cases caused by the consumption of raw oysters (MMWR 1993). One study showed that in three separate cases, the patients died within two to three days after eating raw oysters, emphasizing the virulent and threatening nature of this organism (MMWR 1996). Other reported *V. vulnificus* case also demonstrated the patients died within two days after consuming raw oysters (MMWR 1993; MMWR 1996). Currently, there is no standard vibrio surveillance available. However, Gulf Coast states and the CDC are now evaluating *V. vulnificus* illness. The reports examined by the CDC revealed that about 50% of the reported cases were related to the consumption of raw oysters whereas about 40% were wound infections, and 11% were from unknown sources (MMWR 1993, MMWR 1996). However, it should be noted that the vast majority of *V. vulnificus* cases are isolated infections and not outbreaks of disease.

**Distribution and Prevalence of *V. vulnificus* in Shellfish**

The infections caused by *V. vulnificus* are correlated with the distribution of this organism in estuarine environments. The main source of implicated cases is due to raw oyster consumption coming from *C. virginica* harvested from Gulf Coast estuaries (Shapiro et al. 1998), although this organism has also been recovered from the Atlantic Coast (Motes et al. 1998 and Shapiro et al. 1998) and Pacific Coast sites (Kaysner et al. 1989). Bacterial concentration of this organism in Gulf Coast estuaries can range from $10^3$ to $10^6$ organisms per gram of oyster tissue (Tamplin et al. 1982). Atlantic Coast sites, characterized by high salinity, showed reduced numbers of *V. vulnificus* although the
temperatures throughout the warm months were comparable to Gulf Coast temperatures (Motes et al. 1998). Another study, however, found numbers in Chesapeake Bay estuaries comparable to those from the Gulf Coast (Wright et al. 1996).

The growth kinetics of *Vibrio* species are influenced by several factors, including pH, temperature, salinity, competitors, exposure to light, and nutrient deprivation (FDA 1998, Kasper and Tamplin 1993). The optimum growth temperature of *V. vulnificus* ranges between 13 to 22°C (Kasper and Tamplin 1993, Motes et al. 1998). This organism is usually isolated at moderately low salinities ranging from 7 to 16% while areas with high salinities typically have lower numbers of *V. vulnificus* in oysters (Kelly 1982, Motes et al. 1998). Previous studies have also confirmed that *V. vulnificus* is able to tolerate and survive at an optimal salinity between 1.0 to 2.0% NaCl (Kelly 1982, Vanoy et al. 1992). Infections are usually higher during the warmer months of April through October and appear to be associated with higher seawater temperatures and lower salinity (Kelly 1982, Shapiro et al. 1998, Vanoy et al. 1992).

Studies have also evaluated the impact of temperature on bacterial contamination during storage of shellstock oysters. Freezing, as well as irradiation and other post harvest treatments, can effectively reduce bacterial levels but will kill the oysters. Storage of oysters at 10°C decreased the multiplication of *V. vulnificus* but did not eradicate the organism (Cook and Ruple 1989, Cook 1994). In a separate study conducted by Cook and Ruple (1992), storage of oysters for 12 weeks at minus 20°C also did not decrease the levels of *V. vulnificus*. Calero et al. (unpublished data) described the evaluation of ice immersion for rapid chilling as a post harvest treatment. *Vibrio vulnificus*, fecal coliforms, and total bacterial numbers were monitored and this study showed that rapid
chilling on ice may lower the numbers of *V. vulnificus* in some samples, but did not provide consistent reductions throughout the study. Also, this treatment may increase in total bacterial count, and fecal coliform content exceed recommended limits (230 MPN g\(^{-1}\)) in some samples.

**Epidemiology and Pathogenesis of *V. vulnificus***

Although the number of cases of *V. vulnificus* primary septicemia is low, the fatality rate is high and can exceed 50% (Blake et al. 1979). Wound infections are responsible for 20-25% fatality rate and usually require surgical removal of affected tissue or amputation. Individuals who are immunocompromised or who have high serum iron levels (from hemochromatosis) or chronic liver damage are at the highest risks for infection by this organism (Blake et al. 1979). Chills, nausea, fevers, and skin lesions typically characterize disease caused by this pathogen. Development of the sepsis is frequently quick and may lead to more complications such as septic shock, which has an extremely high lethality rate (Kumamoto and Vukich 1998). Virulence factors such as the polysaccharide capsule of *V. vulnificus*, and host factors such as high iron levels, evidently increase the mortality of the disease caused by this bacterium in animal models (Powell et al. 1997; Simpson and Oliver 1987a; Wright et al. 1981; 1990). It is estimated that the infectious dose of *V. vulnificus* is approximately 1.0 x 10\(^6\) cells in mice that have a normal iron load; but, disease and even death may occur at very low doses in mice that have high iron levels and receive encapsulated strains (Wright et al. 1981). However, human lethal dose (LD\(_{50}\)) remains unknown.

Three biotypes have been described for *V. vulnificus* subtyping (Bisharat et al. 1999). Biotype 2 strains, which only exhibit one O-polysaccharide chain on the lipopolysaccharide (LPS), are pathogenic to eels and differ phenotypically from biotype
1, which have heterogeneous O-polysaccharide chains. Biotype 3 has recently been identified from a new strain of *V. vulnificus* that has been implicated with wound infection and bacteremia outbreaks in Israel. Biotype 1 is commonly associated with human infections caused by *V. vulnificus*; therefore, it is widely studied throughout the world. For this reason, this study will concentrate mainly on *V. vulnificus* biotype 1.

Approximately 50 *V. vulnificus* foodborne cases involving hospitalization per year are reported in the U.S., and these infections hold one of the highest death rates (>50%) among foodborne pathogens (Blake et al. 1979, Hlady and Klontz 1996, Klontz et al. 1988, Linkous and Oliver 1999, Shapiro et al. 1998, Tacket et al. 1984). The majority of reported cases are from the Gulf Coast areas, and the prevalence of infections may be due to the fact that oyster production is concentrated in this area (Motes et al. 1998, Shapiro et al. 1998). Other countries including Sweden, Germany, Holland, South Korea, and Denmark have also been linked to infections related to this organism from contaminated oysters and seawater (Dalsgaard et al. 1996, Linkous and Oliver 1999). The lethality of this bacterium, *V. vulnificus*, is emphasized from cases where death occurred within hours or a day after hospitalization.

Individuals who are at the highest risk for infection are mostly males over the age of 50 years, who have liver disease, diabetics, hemochromatosis, hepatitis, or alcoholism, and those with compromised immune systems (Hlady and Klontz 1996, Linkous and Oliver 1999, Strom and Paranjpye 2000). Major differences in the disease and mortality rates between males and females have been observed is case studies of human disease, as males account for 85% of cases. In a previous study, male and female rats were injected with a high dosage of purified lipopolysaccharide (LPS). Most of the male rats (82%)
died after being injected, but only 21% of the female rats died. However, females with depleted estrogen levels had twice the mortality (75%) of females with an excess of estrogen (Merkel et al. 2001).

Clinical symptoms with primary septicemia include sudden onset of fever, vomiting, chills, diarrhea, abdominal pain, pain in the extremities, and cutaneous lesions, which may become necrotic and require supportive care, debridement, and/or amputation (Borenstein and Kerdel 2003, Strom and Paranjpye 2000). Patients may also acquire the disease from wound infections and suffer similar symptoms (Hlady and Klontz 1996). Wound infections arise when individuals sustain a cut/wound while coming in contact with seawater and shellfish. Early recognition of the clinical symptoms is crucial for the proper treatment of wound and blood infections (Kumamoto and Vukich 1998, Strom and Paranjpye 2000). The recommended treatment involves two antibiotics (doxycycline and ceftazidime) that are administered intravenously and have proven to reduce fatalities (Kumamoto and Vukich 1998).

The endotoxic activity of the LPS is generally attributed as the cause of lethality associated with bacterial septicemia. This putative virulence factor is thought to produce lethality as a result of an accumulation of tumor necrosis factor (TNF), which can lead to an overload of nitric oxide synthase in response to LPS. Supporting this hypothesis was the fact that administration of nitric oxide synthase inhibitor simultaneously with LPS suppressed the lethality of *V. vulnificus* LPS (McPherson et al. 1991, Merkel et al. 2001). Conversely, Powell and colleagues (1997) showed that LPS was relatively inert in mouse models of infection. In their study, animals were challenged with encapsulated MO6-24/O strain and unencapsulated CVD752 strain. The results showed that the animals were
able to remove the unencapsulated CVD752 strain, thus eliminating any association amongst TNF-alpha with or without capsular polysaccharide (CPS) (Powell et al. 1997). Mice injected with purified CPS were slightly more active inducers of TNF-alpha than were mice injected with LPS.

Several studies have shown that there is a correlation between CPS expression and virulence (Kreger and Shirley 1981, Simpson et al. 1987b, Wright et al. 1990, 1999, 2001, Yamamoto et al. 1990). CPS provides *V. vulnificus* with resistance to bactericidal activities of macrophages and complement (Amako et al. 1984, Kreger and Shirley 1981, Starks et al. 2000, Tamplin et al. 1985). Studies showed that *V. vulnificus* mutants that were deficient in CPS expression demonstrated decreased virulence when compared to the wild type in an overloaded iron mouse model (Wright et al. 1990, 1999, 2001). Encapsulated virulent strains are characterized by opaque colony morphology, and it is important to note that not all *V. vulnificus* strains are virulent. Strains with translucent morphology show reduced CPS expression and are less virulent or avirulent (Simpson et al. 1987b).

Iron availability in the host has been implicated in the pathogenicity of numerous bacterial infections (Weinberg 1978, Wright et al. 1981, Simpson and Oliver 1987a). Wright et al. (1981) evaluated the role of iron in *V. vulnificus* infections by injecting mice with iron. The results of this study revealed a decrease in the LD$_{50}$ as well as a decrease in the time of death post-treatment (after injecting the mice with iron). Thus, iron appeared to be a limiting factor in the ability of this bacterium to survive and develop in the host. The growth of *V. vulnificus* is apparently facilitated by the production of siderophores (hydroxymate and phenolate iron chelators) that acquire iron from the host’s
iron-binding proteins, transferrin and lactoferrin (Litwin et al. 1996; Morris et al. 1987b; Simpson and Oliver 1987a; Stelma et al. 1992; Wright et al. 1986, 1990). Furthermore, nonpathogenic strains of *V. vulnificus* possess lower levels of siderophores under high iron conditions when compared to virulent and moderately virulent strains (Stelma et al. 1992). Individuals with liver disease and those with hemochromatosis may be at the highest risk (Blake at al. 1979, Tacket et al. 1984) of acquiring infections caused by *V. vulnificus* as a consequence of their iron status. Hemochromatosis is a disease resulting from an excess absorption of iron in body tissues, and liver disease may also release iron stores, resulting in saturation of iron-binding proteins. Thus, underlying disease and conditions are required for the serious and fatal health problems associated with this bacterium.

**Viable but Nonculturable cells (VBNC)**

*Vibrio* spp. may enter a viable but nonculturable (VBNC) state when nutrients have been depleted and when other physical stresses such as cold temperatures (5-10°C) are present. Studies have documented that the VBNC state refers to cells that are unable to support cell division on agars that normally sustain the growth of that organism (Bryan et al. 1999, Colwell et al. 1985, Jiang and Chai 1996, Oliver 1991, Stelma et al. 1992, Whiteside and Oliver 1997,). For these reasons, traditional culture techniques that help isolate this organism from contaminated waters and seafood samples may fail to detect VBNC cells.

Several investigators have shown that the VBNC state is reversible (Whiteside and Oliver 1997) and have developed PCR and DNA probe assays to detect *Vibrio* spp. Jiang and Chai (1996) also investigated the possible resuscitation of nonculturable cells during nutrient deprivation and low temperatures. After starving *V. parahaemolyticus* strains
over a wide range of temperatures, these strains changed colony morphology to rippled type. The *V. parahaemolyticus* cells that were starved at low temperatures went into the VBNC state after about 50-80 days at 3.5°C. These cells were resuscitated once the temperature was increased (room temperature); thus, surviving cells may have become injured because of the low temperatures rather than by the depletion of nutrients.

Post harvest storage of oysters invariably involves refrigeration or freezing. These cold conditions could induce VBNC state of *V. vulnificus* cells that would be non detectible in standard assays. Thus, evaluation of post harvest treatment may underestimate cell number and the potential contamination problem. These treatments have also shown that *V. vulnificus* numbers are lowered but not eliminated (Kasper and Tamplin 1993, Cook 1994, Cook 1997). Other postharvest treatments such as irradiation and low temperature pasteurization have further demonstrated a reduction of *V. vulnificus* cells, but these treatments will also kill the oysters (Ama et al. 1994, Andrew et al. 2000, Dixon and Rodrick 1998).

**Traditional Microbiological Media for Isolating Vibrio spp.**

Enrichment protocols and a variety of different plating media have been examined for isolation, enumeration, and detection of *V. vulnificus*. Typically, this pathogen has been recovered by inoculation of samples to enrichment broth, such as alkaline peptone water (APW). However, APW broth allows not only growth of the target organism, but also overgrowth of other competitive flora that may be present in the oyster or water samples. Therefore, selective media are required for isolation. Enumeration of *Vibrio* spp. is traditionally performed by the most probable number (MPN) method, which involves end point titration of sample for growth in APW, followed by presumptive isolation of selective media and further confirmatory assays (FDA 1998).
The first selective/differential agar medium used for *V. vulnificus* isolation was thiosulfate citrate bile salts-sucrose (TCBS), which offers moderate sensitivity but low specificity and was designed to detect and discriminate among different *Vibrio* spp. (Lotz et al. 1983, Nicholls et al. 1976). TCBS (pH 8.6) consists of 0.5% yeast extract, 1% sodium chloride, 1% sodium thiosulfate, 1% sodium citrate, and 2% sucrose. The addition of oxgall and sodium cholate bile salt was found to inhibit gram-positive bacteria. Nonetheless, the specificity of this agar has been questioned as various non vibrio microorganisms (about 50%) are able to grow on TCBS agar after enrichment (Donovan and van Netten 1995). Also, nonpathogenic *Vibrio* species are capable of growing on this agar making it impossible to differentiate *Vibrio* pathogens (Lotz et al. 1983). Generally, *V. parahaemolyticus*, *V. vulnificus*, and *V. mimicus* appear as blue/green colonies while *V. alginolyticus*, *V. fluvialis*, and *V. cholerae* appear as larger yellow colonies from sucrose fermentation (FDA 1998).

Due to problems with TCBS agar, other plating media with better selectivity and discrimination were developed to increase the accuracy of detection/isolation of *V. vulnificus*. One example of a selective medium that is more discriminatory for *V. vulnificus* is the *V. vulnificus* (VV) agar (pH 8.6), which uses 2% salicin as a source of carbohydrate, 0.0005% potassium tellurite, 0.00015% crystal violet, and 0.8% oxgall to inhibit growth of both gram-positive and gram-negative bacteria other than *V. vulnificus* (Brayton et al. 1983). Non *vibrio* strains that grow on VV agar are unable to process tellurite and contain smaller colonies. Thus, the recovery of *V. vulnificus* (gray colonies with black centers) on VV agar is considerably higher than the rate recorded for TCBS agar (Brayton et al. 1983, Miceli et al. 1993).
Another medium used for enumeration and isolation is the *V. vulnificus* enumeration medium (VVE agar), consisting of 1% tryptose, 0.5% cellobiose, 0.1% of oxgall as well as sodium cholate, sodium taurocholate and potassium tellurite. This golden tan agar turns *V. vulnificus* colonies to greenish blue in color from the hydrolysis of X-Gal after an overnight incubation at 35°C. The VVE agar provides reliable identification of *V. vulnificus* in oysters followed by DNA probe (Morris et al. 1987a) test for the enumeration of this organism. This agar also provides rapid results when compared to other procedures and provides a high level (92%) of recovery (Miceli et al. 1993).

Isolation of *V. vulnificus* from shellfish was described by Massad and Oliver (1987), using cellobiose-polymyxin B-colistin (CPC). This selective agar was developed to identify/differentiate *V. vulnificus* and *V. cholerae* and consists of 1% bacto-peptone, 0.5% beef extract, 2% NaCl, bromothymol blue, and cresol red. Various vibrio and non vibrio strains were examined, but only *V. vulnificus* and *V. cholerae* are able to grow on this medium. The specificity of this agar was evaluated by increasing the temperature to 40°C instead of 37°C to avoid the growth of other vibrio strains. Typical *V. vulnificus* colonies appear yellow in color while *V. cholerae* colonies appear purple in color. Furthermore, non-target bacteria are unable to grow on this medium except for one *Vibrio parahaemolyticus* strain. When compared to other selective agars such as TCBS, VV, and VVE, CPC agar shows better specificity and better recovery (Høi et al. 1998, Kaysner et al. 1989, Massad and Oliver 1987, Sloan et al. 1992). Other studies confirmed these findings by comparing TCBS and CPC agar. In one study, over 100 colonies were selected from these two agars and confirmed by PCR assay. This study showed that a higher recovery is
obtained with CPC (7.8%) than with TCBS (3.7%). Another study by Sloan et al. (1992) compared five different selective enrichment broths (APW, glucose-salt teepol broth (GSTB), Marine broth (MB), Horie’s arabinose-ethyl violet broth (HAE), and Monsur’s broth (MNS) and two selective agars (CPC and SDS agars) for the recovery and enumeration of *V. vulnificus* in oysters. This study showed *Vibrio* species are unable to grow to significant levels in media other than APW enrichment broth and CPC agar, which provide the highest recovery of *V. vulnificus* cells from oysters.

Currently, the FDA Bacteriological Analytical Manual (BAM) uses a modification of CPC agar (MCPC) for the isolation of *V. vulnificus* (Tamplin et al. 1991). This modification consists of a reduced concentration of colistin (400,000 U/liter) and no change to the polymyxin B concentration. *Vibrio vulnificus* from APW enrichment of seawater, sediment, and oysters were correctly identified using MCPC, followed by confirmation with a species-specific monoclonal antibody utilizing an immunoassay described below and biochemical assays (Tamplin et al. 1991).

Finally, Cerda-Cuellar et al. (2000) recently designed the selective *V. vulnificus* medium (VVM) to detect this bacterium from estuarine environments. VVM contains cellobiose as a carbon source, electrolytes to promote bacterial growth, and polymyxin B and colistin to suppress the growth of non target organisms. This agar differs from MCPC medium in that a *V. vulnificus* digoxygenin-labelled oligonucleotide probe (V3VV) is used in combination with VVM agar while the MCPC uses a species-specific monoclonal antibody (FRBT37) to confirm the identity of *V. vulnificus* in an EIA (Tamplin et al. 1991). Typical *V. vulnificus* colonies appear as bright yellow colonies with yellow halo, and the other *Vibrio* spp. either do not grow or produce greenish blue colonies.
Confirmation of *V. vulnificus* isolates is obtained with a specific probe (V3VV), and no hybridization occurs with non-target isolates.

**Detection and Enumeration Methods**

**Bacteriological Analytical Manual (BAM) Most Probable Number (MPN)**

The most probable number (MPN) assay is based on the dilution of a sample for an endpoint titration. Each dilution, which may or may not contain viable organisms, is then inoculated into 3 or 5 tubes of enrichment broths. According to the FDA (1998), the results obtained from the MPN enumeration reveal the amount of inocula (bacteria) producing growth at each dilution to provide an estimate of the original undiluted concentration of bacteria in the sample. Thus, serial dilutions of several tubes are used to attain estimates over a wide range of possible concentrations at each dilution. Finally, MPN tables are used to compute the correct combination of positive tubes that are turbid.

MPN values are usually based on three decimal dilutions at 0.1, 0.01, and 0.001 g inocula for 5 tubes each. *Vibrio vulnificus* positive tubes are based on the isolation of colonies on MCPC agar and TCBS agar plates, with confirmation using immunological or molecular assays described below. FDA BAM MPN is an estimate of colony-forming units (CFUs) but not a direct count of individual bacteria cells. This method is especially useful for low concentrations of organisms (<100/g), which are usually found in milk and water, where those foods that may interfere with accurate colony counts (FDA 1998). Although this method is very useful, it is laborious and requires large quantities of enrichment tubes, and that multiple agar media be streaked for isolation for each tube.

Interestingly, Hartel and Hagedorn (1983) developed a microtiter MPN for the enumeration of fecal coliforms from soil as an alternative to the multiple tube assay. Briefly, they utilized a 96-well microtiter plate (12 dilutions and 8 replicates per dilution)
instead of the standard tube FDA BAM MPN method. In this study, soil samples were
diluted by adding 10 g of soil in 90 mL of 0.1% peptone water. The wells were then filled
with 0.1 mL of lactose broth and mixed. Serial dilutions were performed in the microtiter
plate along with the appropriate control. The microtiter plates were incubated for 9 h at
35°C. Next a sterile 48 prong multipoint inoculator (Laboratory Essentials, Dublin, OH)
was used to inoculate the sample in each well to a T1N1 agar plate. The plates were
incubated, positive colonies were counted, and a three-digit code (last three dilutions of
sample) was converted to MPN values. The advantage of using this method over the FDA
BAM MPN method is that it will reduce the volumes of reagents and media used as well
as being more efficient and cheaper. It may be possible to adapt this method for
applications to MPN enumeration of *V. vulnificus* or other *Vibrio* spp.

**Biochemical Assays**

Biochemical assays have been used to confirm and identify *V. vulnificus* and
include API 20E system (bioMerieux, Inc., Hazelwood, MO), API®, and BIOLOG
(BIOLOG Inc., Hayward, CA) plates. API 20E system (Fig. 1-1) is a commercial
biochemical assay that rapidly detects gram-negative and enteric bacteria. Basically, a
plastic strip holding several mini-tubes is inoculated with a saline solution of pure
bacterial culture with a sterile pipette. This inoculation aids in the rehydration of the dry
media inside the mini-tubes (compartments). Following incubation at 37°C for 18 hours,
a color reaction is recorded, and three test reactions are added together and converted to a
7-digit number, which can then be looked up in a codebook (Lindquist 2001). This
codebook provides the identification of either the genus and/or species. Juang and
Morgan (2001) found that although the API 20E system works well for the identification
of gram-negative bacteria at the genus level; thus, additional methods must be used to
further identify at the species level with the use of molecular techniques (Juang and
Morgan 2001). The BIOLOG technique (sole-carbon source) was initially designed to
detect microbial isolates based on a most probable number method. BIOLOG plates use a
multivariate profile of color production caused by the utilization of sole carbon sources
and concurrent reduction of tetrazolium dye. After incubation, the number of positive
wells (those that utilized the substrate and grew at high rates) are counted and recorded.
This assay is useful because of its quickness and simplicity for results showing distinct
color development (Gamo and Shoji 1999). However, the availability, flexibility, and
cost of these biochemical tests may reduce its application.

**Enzyme Immunoassay (EIA)**

Tamplin et al. (1991) was able to identify *V. vulnificus* by utilizing a
species-specific monoclonal antibody FRBT37 (used in the FDA BAM for confirmation
of *V. vulnificus* colonies). The effectiveness of this monoclonal antibody was evaluated
by testing seawater, sediment, and oysters with several *V. vulnificus* strains and 72 non *V.
vulnificus*. This assay was capable of identifying about 99% of the *V. vulnificus*
confirmed isolates (347 of 348). Furthermore, there was no significant difference
between EIA and the biochemical analysis for the confirmation of *V. vulnificus*. Using
direct detection from enrichment broth showed that the lowest level of detection was 2.0
x 10^3 cells while the highest level of detection was about 1.0 x 10^6 cells (Tamplin et al.
1991). The recovery of this bacterium was rapidly obtained in less than 24 hours and the
need for MCPC agar to streak for isolation was eliminated. However, problems with
overgrowth of competing organisms in enrichment medium have been reported.
Currently, the FDA BAM recommends using gene probes for the detection of *Vibrio* spp.
Figure 1-1. Example of API 20E system. API 20E was inoculated with target bacterium and incubated at 37°C for 18 hours. Color reactions were recorded, and three test reactions were added together and converted to a 7-digit code. This code was looked up in a codebook, which provides the identification of either the genus and/or species (with permission from Lindquist 2001).

as well as the use of post harvest treatment of oysters to lower *V. vulnificus* numbers.

Additionally, one problem with the EIA method is that although it is quite rapid and specific for the detection of *V. vulnificus*, the monoclonal antibody is no longer commercially available.

*Vibrio vulnificus* Alkaline Phosphatase-Labeled DNA Probe (VVAP) Plate Counts Using Colony Blot Hybridization

In 1993, Wright et al. developed a more rapid and sensitive assay to study and enumerate *V. vulnificus* (Wright et al. 1993). In this study, a 24 base pair nonradioactive alkaline phosphatase (enzyme that removes 5’ phosphate group from DNA, RNA, nucleotides, proteins, etc.) VVAP DNA probe was used, which was acquired from a segment of the cytolysin gene (*vvhA*). The cytolysin gene encodes a 56 kDa, heat labile enzyme that lyases red blood cells and contains cytotoxic activity in several mammalian cell lines. The VVAP probe was demonstrated to be highly specific and sensitive for
enumerating and identifying *V. vulnificus* from both clinical and environmental isolates. Nonselective media was used to perform colony lifts. In 1996, this method was used to monitor *V. vulnificus* in the Chesapeake Bay, and this study found that water temperature, salinity and depth influence the distribution of *V. vulnificus* (Wright et al. 1996).

The probe contains an alkaline phosphatase label for probing colonies grown on Luria-Broth agar (LA). The colonies are then lifted from the LA plates to a sterile filter paper that is prepared for hybridization with a DNA oligonucleotide that is specific for the target bacterium. Once hybridized, the filters are washed a few times to prevent nonspecific binding of the DNA. Lastly, the filters are then developed with an alkaline phosphatase substrate (NBT/BCIP) for about 1-2 hours followed by additional washes with water. This substrate reacts further after the dephosphorylation of the 5’ phosphate group and intensifies the color reaction as well as increases the sensitivity of the assay. The filters are allowed to completely dry and then 25-300 positive colonies, which are brownish/blue in color are counted. Negative colonies are beige/yellow in color. There are some problems with the probe, which include fading of the probe (partial coloration), flaking of colonies (Fig. 1-2), and the halo effect on enrichment filters (Porter 2002); therefore, an alternative confirmation assay such as the polymerase chain reaction (PCR) may need to be implemented. The FDA is currently testing the DNA probe for the enumeration of *V. vulnificus* and adoption as a standard method, and the probe has been used for confirmation of MCPC positive colonies from FDA MPN enumeration (A. Depaola, personal communication).

**Polymerase Chain Reaction (PCR)**

PCR is a powerful amplification technique that detects and identifies many pathogens at low levels and may be useful in detection of VBNC cells as well
Figure 1-2. Partial signaling of filter. This filter was processed for colony blot hybridization using the VVAP probe (species-specific for *V. vulnificus*). The positive colonies appear as brownish/blue in color whereas the negative colonies are beige/yellow in color. The arrows show the flaking effect of one of the colonies, which emphasizes one of the problems with the probe. (Aono et al. 1997, Bej et al. 1991, Higuchi et al. 1993, Hill et al. 1991). PCR is a highly sensitive assay that amplifies regions of DNA by annealing specific primers to single stranded DNA (ssDNA) and reforming the double stranded DNA (dsDNA) using a thermostable Taq DNA polymerase enzyme from *Thermus aquaticus*. PCR reactions contain a mixture dinucleotide triphosphate (dNTPs), buffer, forward and reverse primers, Taq polymerase, and the DNA template. Also, to guarantee that reliable PCR results are achieved, negative controls and a positive control, using PCR water, *V. vulnificus* DNA, and uninoculated APW broth are essential. After the first denaturation, DNA at 92-95°C, the PCR reaction goes through 25 to 40 cycles of denaturation, annealing, and extension. Denaturing the dsDNA permits the unwinding to ssDNA so that annealing of primers can occur as the temperature is lowered, usually between 50-60°C, as single stranded primers attach to the ssDNA at their specific sites. The extension occurs at approximately 72°C where the polymerase enzyme combines with the primers
and the ssDNA and reforms dsDNA molecule by incorporating the dNTPs. During the cycling process, the number of dsDNA copies multiplies exponentially \(2^n\) with each cycle. Ultimately, a final extension step occurs to allow the completion of any incomplete reactions. After the PCR reaction is complete, the amplified DNA product will have produced over a million copies. Although, this technique is highly sensitive and specific, the PCR product must be analyzed using agarose gel electrophoresis. Post PCR processing can result in low resolution, poor precision, are size-based discrimination only, and are not quantitative.

**PCR for the Detection of *Vibrio* spp.**

PCR assays for the identification of *V. vulnificus* generally target and amplify the cytolysin-hemolysin gene (*vvhA*) (Hill et al. 1991). One of the first PCR assays was described by Hill et al. in 1991, and this study demonstrated that PCR was capable of amplifying *V. vulnificus* DNA from artificially inoculated oyster samples. Also, this study compared different DNA purification methods to determine which method would be most effective with the PCR. The results demonstrated that the DNA extracted with the GITC-chloroform extraction method was the most efficient for use as a PCR template. Aono et al. (1997) reported that PCR was extremely useful in the identification of 3,703 *vibrio* isolates. Their study evaluated a PCR assay to determine if PCR would isolate *vibrio* isolates from marine environments followed by confirmation of this organism by DNA-DNA hybridization and biochemical assays (API 20E). The results showed that the PCR assay was useful for the identification of *V. vulnificus*.

Arias et al. (1998) investigated PCR for the isolation and recovery of *Vibrio* spp. from marine samples including salt water and shellfish. Depending on the extraction method used, the detection limits ranged from approximately \(10^1\)-\(10^3\) CFU/mL of *Vibrio*
vulnificus cells in the samples. Also, the plating efficiency of CPC and TCBS was evaluated and determined to be better than PCR. This may be due to the small sample volume that was examined. PCR has some advantages over culture methods. These advantages include rapid results obtained within hours and identification of atypical vibrio strains.

In a study by Kaufman et al. (2002), a multiplex PCR was developed to detect Vibrio parahaemolyticus isolated from patients who had consumed contaminated seafood. DNA purification involved the use of the boiling method (boil for 10 min) and a 5 µL aliquot that was used as the crude DNA template for multiplex PCR amplification. The multiplex PCR was able to detect the presence of both the thermostable direct hemolysin (tdh) and the thermostable direct hemolysin related (trh) genes found in pathogenic V. parahaemolyticus strains. Recently, another PCR assay was effectively used for the identification of a newly emerged V. parahaemolyticus O3:K6 strain in pure cultures and artificially inoculated waters from the Gulf of Mexico. Detection limits for V. parahaemolyticus O3:K6 in purified cultures were $10^2$ CFU/100 mL and $10^3$ CFU/100 mL for the inoculated water samples (Myers et al. 2003).

Molecular techniques may provide rapid, sensitive analysis of these pathogenic strains. Nonetheless, there are major problems presented with PCR assays, including limited sample size, false positive amplification of DNA derived from dead cells, as well as false negative reaction resulting from inhibitors present in the food matrix. DNA purification and subsequent concentration may remove inhibitors and increase sample size, but is time-consuming and not cost effective for large numbers of samples. Problems with sample size and dead cells may be addressed by combining molecular
strategies with standard microbiology methods such as MPN, which require cell growth and increase sample size. Additionally, the PCR methods described above for the detection of *V. vulnificus* are not quantitative, so the employment of techniques such as real-time PCR will be needed to provide assays that are able to both detect and enumerate this species in oysters.

**Real-Time PCR**

Real-time polymerase chain reaction (R-PCR) has proven essential in the detection and enumeration of bacteria by quantifying the amplified products as they formed (Higuchi et al. 1993). One of the available systems for R-PCR uses a fluorescently tagged probe (TaqMan®), which acts as a signal for real-time detection of PCR products as they form. Quantitation with this method is centered on the fact that the rate of product formation is proportional to the amount of specific DNA template in the sample. The TaqMan® probe contains an oligonucleotide labeled with both a 5’ reporter dye 6-carboxy-fluorescein (FAM) and a 3’ quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA). The TaqMan® probe hybridizes to the target sequence within the PCR Product. When the probe is cleaved by the 5’ nuclease activity of the Taq DNA polymerase, the reporter and quencher dyes are separated, causing the reporter dye fluorescence to increase. The fluorescence signal is produced only if target DNA is present in the sample because the separation of the reporter dye from the quencher dye occurs during the amplification. Also, non-specific amplification is not detected (Blackstone et al, 2003, Higuchi et al. 1993, Kaufmann et al. 1997, Kim et al. 1999, Lyon 2000). The increase in reporter fluorescence (target sequence) can then be recorded using detection systems such as the GeneAmp® 5700 or, ABI PRISM® 7700, or the iCycler iQ™ Sequence Detection System, which include a computer software for data analysis.
An amplification plot is displayed once the fluorescent signal of the reporter increases to detectable levels. Figure 1-3 is an example of an amplification plot of cycle-threshold (Ct) versus reporter value (Rn). Ct is where the sample crosses the threshold (level of detection or point at which a reaction reaches a fluorescent intensity above the background), and the Rn is a measure of the reporter signal.

Real-time PCR detection can also employ SYBR® Green I dye as a more cost effective alternative to TaqMan® probes. R-PCR is monitored by the increase in fluorescence when SYBR® Green I dye, a strain that is stable at extreme temperatures, binds to the minor groove of the dsDNA and thus can accurately calculate the amount of double-stranded product made in the presence of single-stranded oligonucleotide primers.

Specificity can be determined by the fact that the double-stranded DNA with no base mismatches will show a higher melting temperature than the nonspecific templates that contain mismatches. An advantage of SYBR® Green I dye is that it is ideal for the use in target identification or when only a small number of reactions are required for a given assay (Higuchi et al. 1993, Mouillesseaux et al. 2003, De Medici et al. 2003). Also, the SYBR® Green I double-stranded DNA binding dye does not require a probe and offers additional experimental flexibility. It is flexible because no target-specific probes are required; however, a major problem with this stain is that both specific and non-specific products such as primer-dimers will generate a signal. This problem has been overcome by DNA melting/dissociation curves and the development of the TaqMan® probe.

Real-Time PCR of Vibrio spp.

Blackstone et al (2003) developed and evaluated a real-time PCR assay to identify the presence of pathogenic *V. parahaemolyticus* strains. Real-time PCR was only able to
amplify *tdh* positive *V. parahaemolyticus* strains while no amplification was observed with other *Vibrio* spp. and non-*vibrio* spp. Furthermore, this molecular assay was compared to a modification of the FDA BAM streak plate/probe procedure using a DNA probe that targets the *tdh* gene. This study showed that R-PCR is highly specific, consistent, and rapid for the detection of *V. parahaemolyticus* strains containing the *tdh* gene in oyster samples whereas the DNA probe method had a much lower detection level.

A quantitative PCR assay was also developed for the detection of culturable and VBNC *V. cholerae* O1, non-O1, and non-O139 strains in pure cultures, oyster samples, and synthetic seawater. Results showed that only *V. cholerae* strains were positively amplified while non-target strains were not amplified. The ability of *Vibrio* spp. to enter a VBNC state at low temperatures may contribute to the failure of conventional
enumeration methods to identify/enumerate this pathogen from seawater and seafood. In this study, Lyon (2000) used DNase to eliminate the extracellular DNA isolated from dead cells that enter the VBNC state. The DNase treatment led to a 1-log reduction in numbers detected, presumably from contribution of DNA derived from dead *Vibrio* cells. Thus, with this treatment, TaqMan® PCR could be used to target the hemolysin (*hly*/*A*) gene of intact cells only. Spiked oyster samples had a sensitivity of 6-8 CFU/g and 10 CFU/mL in seawater samples. This study also confirmed that the TaqMan® PCR technique is a powerful tool that rapidly detects and enumerates *Vibrio* spp. as an alternative to conventional culture methods.

Recently, Campbell (2003) described a real-time PCR assay that targeted the *Vibrio vulnificus* hemolysin gene (*vvh*/*A*). The real-time PCR assay was used to accurately enumerate *V. vulnificus* from oysters and to examine the VBNC state. Their results demonstrated that *V. vulnificus* cells sustained viability even at cold temperatures throughout the study as determined by the BacLight™ method; however, nonselective agars were unable to detect *V. vulnificus* after 28 days. Moreover, TaqMan® PCR was able to quantitate *V. vulnificus* cells VBNC cells throughout this experiment. Their results also showed that the bacterium could be accurately enumerated in both seeded and uninoculated oysters, and that numbers obtained by the real-time PCR.DNA probe method were not significantly different from those obtained by plate counts using DNA probe colony hybridization.

In summary, contamination of shellfish by *V. vulnificus* has had devastating economical consequences for the seafood industry. The lack of appropriate rapid and quantitative methods for detection, isolation, and enumeration has hindered efforts to
examine environmental survival, host range, resistance to post harvest treatments, and virulence distribution of *V. vulnificus* strains. It is important to point out that much of the current research involves the study of *V. parahaemolyticus* strains while fewer studies on different molecular detection methods for *V. vulnificus* have been published. Although the numbers of *V. vulnificus* cases are relatively low, infections caused by this bacterium progress rapidly and result in a high fatality rate. My hypothesis is that the use of molecular methods for detection and confirmation of this organism in standard MPN methods may provide more rapid, economical, and accurate enumeration of *V. vulnificus*. Therefore, the implementation of improved methods for the enumeration of this bacterium may aid in evaluating strategies for the control and elimination of this organism from seafood. As the primary impact of *V. vulnificus* on the seafood industry is from oyster contamination, my studies used oysters as a model to assess *V. vulnificus* detection and enumeration methods in shellfish.

The specific aims of this study were as follows:

- To compare numbers of *V. vulnificus* in oysters, as determined by VVAP plate counts, to numbers obtained using molecular DNA probe confirmation methods and modified microtiter or test tube assays for most probable number (MPN) determinations.
- To compare DNA probe vs. real-time PCR for the confirmation of MPN enumeration.
- To improve detection and enumeration techniques of *V. vulnificus*. 
CHAPTER 2
MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

*Vibrio vulnificus* MO6-24/O, a virulent, capsulated strain, was used as a positive control for all experiments. *Vibrio parahaemolyticus* strains, both TDH negative and TDH positive strains were used as the negative controls. These strains included *V. parahaemolyticus* NY 3483 (TDH positive), NY 3547 (TDH negative), TX 2103 (TDH positive), 10290 (TDH positive), 17802 (TDH negative), and 43996 (TDH positive). All the strains were grown on Luria-Bertani broth (LB) with 1.0% tryptone, 0.5% yeast extract, and 1.0% NaCl and/or Heart-Infusion (HI) broth with 1% casein digest peptone, 0.3% yeast extract, 0.5% NaCl, 0.5% brain heart infusion. All strains were stored at minus 70°C in LB with 50% glycerol. *Vibrio vulnificus* and *Vibrio parahaemolyticus* strains were incubated at 37°C with vigorous agitation in the incubator (New Brunswick Scientific, Edison, N.J.) overnight. Unless stated otherwise, all media were purchased from Difco (Detroit, MI), and reagents were purchased from Sigma Chemicals (St. Louis, MO).

Media Comparison Studies

Recovery of Indigenous *V. vulnificus* from Oyster Homogenates

Fresh oysters were purchased from a local market during the winter months. Oysters (approximately 5 oysters) were scrubbed, shucked, and mixed with an equal weight (1:1) of PBS. This mixture was blended for 90 seconds in sterile blenders. After blending, the oyster homogenates were further diluted in PBS buffer for direct plate
analysis. One milliliter of oyster homogenate was placed in tubes containing 9 mL of sterile phosphate buffered saline (PBS). The tubes were serially diluted (1:10) with 1 mL of sample. Then 100 µL of sample from each dilution was spread-plated onto seven different media, which included Luria Broth Agar (LA), prepared using LB and 1% agar; LA prepared with 3% agar (LA+3%); agar prepared with 1% tryptone, 1% NaCl, 1% agar (T1N1); agar prepared with 11% tryptone, 3% NaCl, 1% agar (T1N3); LA prepared with 1.5% artificial seawater (Mentor, OH; ASW); thiosulfate-citrate-bile salts-sucrose agar (TCBS); and modified cellobiose polymyxin colistin (MCPC) (Tamplin et al. 1991). The plates were then incubated overnight at 35-37°C. After incubation, colony blot hybridization was performed as described below (Wright et al. 1992; 1993; 1996). For the selective TCBS and the MCPC agar plates, single colonies were picked and plated on LA in order to lift the colonies for identification by DNA probing as well.

**Seeding Study of Oyster Homogenates**

Ten-milliliter aliquots of the oyster homogenates were seeded with dilutions of overnight cultures (1 mL) of *V. vulnificus* MO6-24/O to achieve inocula ranging from ca. 10⁴ to 10⁷ CFU g⁻¹. The concentration of the initial inocula of washed cells was determined by optical density (A₆₀₀nm) and plate counts. Unseeded homogenate was utilized as the negative control for the presence of indigenous vibrios. Aliquots (100 µl) of inoculated homogenates were plated on the same media listed above in replicates of four. Plates were incubated for 24 hours at 35-37°C, and colonies were transferred to filters for enumeration by DNA colony blot hybridization as described below. *Vibrio vulnificus* colonies were also enumerated on selective media by transferring typical single colonies from TCBS or MCPC agars to nonselective LA or T1N1 and subsequently DNA probing (VVAP colony blot hybridization) for identification as described below.
Enumeration Methods Comparison Studies

Oyster Collection and Handling

Fresh oysters were collected monthly from May, 2003 through July, 2003 by Leavin’s Seafood oyster processing plant, Apalachicola Bay, Fl. The freshly harvested oysters were washed and refrigerated overnight at 4°C. For purposes of examining sensitivity of enumeration assays, some of the oysters received freezing treatment using nitrogen tunnel freezing for 5 min. The oysters were packed frozen on ice (24 oysters per box) and transported to Gainesville, Fl. and aliquots were analyzed within 2 hours to determine levels of *V. vulnificus*. The remaining oysters were stored in a freezer at –20°C and then analyzed at 14, 21, and 28 days post freezing treatment. Ten to twelve oysters (or equivalent of 100 g of oyster meat) were thawed at room temperature 15 min prior to processing. Oysters were scrubbed, shucked, and mixed with an equal weight (1:1) of PBS. This mixture was blended for 90 seconds in sterile blenders. After blending, the oyster homogenates were further diluted in PBS buffer for direct plate analysis.

*Vibrio vulnificus* enumeration by VVAP plate counts using Colony Blot Hybridization

For enumeration of *V. vulnificus* by DNA probe of colonies from spread plate, the oysters were processed as described above. Oyster homogenates (1 mL) were used serial diluted 10 fold using PBS buffer. Appropriate dilutions were spread plated on T1N1 agar, and duplicate plates were incubated overnight at 35-37°C. For the first dilution, an aliquot of 200µl was spread plated into T1N1 agar and for the remaining dilutions (10^{-2} and 10^{-3}) a 100µl aliquot was spread plated into T1N1 agar. Colonies from these plates were used for colony blot hybridization to determine the numbers of *V. vulnificus* in oyster samples.
Colony blot hybridization is a technique that uses species-specific DNA probe (VVAP) that targets the \textit{vvhA} gene for the detection and enumeration of \textit{V. vulnificus} (Morris et al. 1987a, Wright et al. 1993). Briefly, bacterial colonies grown overnight on agar plates were lifted to sterile filter papers (85 mm Whatman #541) to transfer the colonies. The filters were microwaved in lysis solution (0.5 M NaOH and 1.5 M NaCl) for 1 to 6 min and transferred to a clean container of ammonium acetate neutralization buffer followed by two washes with standard saline citrate buffer (SSC). Following drying, filters were treated with proteinase K (20 µg/mL) to remove background enzymatic activity. Filters were pre-hybridized under stringent conditions (56°C) for 30 min in buffer containing SSC with 0.5% bovine serum albumin, 1% sodium dodecyl sulfate (SDS), 0.5% polyvinylpyrrolidoe (PVP). The buffer was discarded, and pre-warmed, fresh hybridization buffer with nonradioactive alkaline phosphatase labeled DNA probe (10 nM) was then added to the filters and incubated at 56°C for 1 hour. Filters were rinsed in 1X SSC/1%SDS at 56°C followed by additional rinsing with 1X SSC at room temperature. Nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) substrate (Roche Diagnostics, Indianapolis, IN) was used for the development of the alkaline phosphatase labeled filters in the dark. Placing the filters into a new container with de-ionized water for 10 min stopped the development. The positive and negative control strains were also applied to filters and developed simultaneously as described above. Filters were enumerated by counting the probe-positive colonies, which change from a yellow/beige color to a blue/brown color. Negative controls remain yellow/beige in color. The plates containing colonies in the countable range (25-250)
were counted, and results from duplicate or more plates were averaged yielding one count of colony forming units (CFU) per gram for each sampling.

**Vibrio vulnificus Alkaline Phosphate-Labeled DNA probe (VVAP) MPN Method**

A modification to the Most Probable Number (MPN) method (FDA *Bacteriological Analytical Manual*) was used to estimate numbers of *V. vulnificus* with the species-specific DNA probe described above as an alternative to biochemical assays. The VVAP MPN method differs from the standard MPN, in that growth from enrichment tubes are assayed directly by DNA probe without the streaking of colonies for isolation onto selective media (MCPC) and subsequent confirmation of species that is required for the standard method. For the VVAP MPN method, oyster homogenates were aseptically prepared, dilutions of homogenate (1 mL) inoculated into APW enrichment broth (9 mL) for MPN analysis (5 tubes/dilution), and incubated for 16 to 18 hours at 35-37°C. An aliquot (about 10 µl) from each APW dilution tube was applied to a nonselective medium (T1N1) using an inoculation loop. Plates were incubated overnight at 35-37°C followed by colony blot hybridization analysis for confirmation of positive tubes to determine MPN enumeration by using the 5-tube MPN calculator (Lindquist 2002).

**Microtiter VVAP MPN Method for Vibrio spp.**

A microtiter VVAP MPN method was implemented to enumerate *V. vulnificus* to determine whether a smaller volume of homogenate (0.2 mL) could be used to inoculate enrichment broths (1.8 mL) in a microtiter assay would provide the same sensitivity as 1 mL of sample used in the standard protocol and with the tube VVAP MPN described above. Briefly, oyster meat was aseptically prepared and homogenized as described above. APW broth was added to the 96 well plates (2.2 mL well volume, polypropylene, round-bottom plate (Fisher Scientific) with a multi-channel micropipette (Hartel and
Hagedorn 1983), and then inoculated with serial dilutions (1:10) of oyster homogenates. Each 96-well plate had a *V. vulnificus* positive control, *V. parahaemolyticus* strain negative control, and an uninoculated APW broth as an additional negative control. The 96-well plates were incubated for 16 h at 37°C. After incubation, the samples were transferred to T1N1 agar using a sterile 48-prong microplate replicator (Laboratory Essentials, Dublin, OH). Plates were incubated overnight at 37°C, and DNA probing was performed as described above. One milliliter of the remaining microtiter MPN aliquots was transferred to 1.5 mL microcentrifuge tubes for DNA extraction for real-time PCR analysis as described below while the other remainder was frozen at –20°C without extracting. The positive MPN wells determined from DNA probing were converted to MPN values as described above.

**Figure 2-1. Schematic of microtiter VVAP MPN.** The microtiter MPN sample was prepared for analysis by real-time PCR and DNA colony blot hybridization. Oyster homogenates (OH) were serially diluted 10 fold (1:10, 1:100, 1:1000) and inoculated into 5 well MPN series in a microtiter plate.

**Real-Time PCR MPN Analysis for enumerating *Vibrio vulnificus***

A real-time PCR assay for *V. vulnificus* was previously described by Campbell (2003) and used Primer Express software from Applied Biosystems (Foster City, CA) to
design oligonucleotide primers and probe targeting the \textit{V. vulnificus} cytolsin gene, \textit{vvhA} (GenBank Accession Number M34670). The specificity and the sensitivity of the primer sets and probe were confirmed with 28 isolates of \textit{V. vulnificus}, which showed positive amplification while no amplification was observed with the 22 non \textit{V. vulnificus} species. The sequence for the \textit{V. vulnificus} MO6-24/O forward primer was 5’–TGT TTA TGG TGA GAA CGG TGA CA -3’ and the sequence for the Vv reverse primer was 5’ – TTC TTT ATC TAG GCC CCA AAC TTG -3’ (Campbell 2003). The sequence of the TaqMan® probe was 5’–FAM-CCG TTA ACC GAA CCA CCC GCA A-TAMRA-3’.

Both the primers and the fluorogenic probe were synthesized by GENOSYS (The Woodlands, TX). Upon receipt, both primers were resuspended in 10% TE buffer (1.0 mM Tris-Cl, 0.1 mM EDTA pH 8.0) and stored at –20ºC.

The previous work used TaqMan® probes and purified DNA templates. While TaqMan® offers the advantage of being rapid and specific for the target organism, a disadvantage is the expensive cost of this probe, and this assay can be influenced by Taq polymerase inhibitors, competing nucleic acids, or the inefficiency of cell lysis (Johnson et al. 1984). Therefore in the present study, alternative detection with SYBR green dye and the use of two different extraction methods were examined.

For DNA extractions bacterial growth in APW enrichment (1 mL) from the microtiter VVAP MPN assay described above was transferred to a clean, sterile 1.5 mL microcentrifuge tube and centrifuged for 10 min at 5,109 x g. The supernatant was discarded, and the resulting pellet was re-suspended in 180 µL buffer ATL supplied with QIAamp DNA Mini Kit (Qiagen®, Valencia, CA). DNA was extracted using QIAamp®
DNA Protocol for Tissues as per product literature. The extracted DNA template was stored at –20°C until real-time PCR was performed.

A boiling lysis method was also implemented and compared to the results using QIAamp DNA Mini extraction kit. The remaining one-milliliter aliquot from the VVAP MPN described above was transferred to a clean, sterile 1.5 mL microcentrifuge tube and centrifuged for 10 min at 5,109 x g. Supernatant was discarded, and the resulting pellet was re-suspended in 400µl of PBS. Microcentrifuge tubes were then placed in a dry-heat microcentrifuge tube incubator (Fisher Scientific, Pittsburgh, PA), at 100°C for 7 min. After boiling, the samples were centrifuged at 15,000 x g for 1 minute, and the supernatants were used as the DNA template. All samples were stored at -20°C.

Real-time PCR amplification using the TaqMan® probe assay (Applied Biosystems, Inc) was run in 0.2 µL microcentrifuge tubes with a total reaction volume of 25 µL using reagent concentration as recommended by the manufacturer and DNA templates (2 µl) from either Qiagen extracted or boiled templates. Each sample was performed in duplicate. Real-time PCR thermal cycling was run using the GeneAmp® 5700 sequence detection system, the ABI PRISM® 7700 Sequence Detection System, and the iCycler iQ™ detection system thermal cycler (BioRad Laboratories, Hercules, CA) using the following conditions: holding samples at 50°C for 2 min, denaturing at 95°C for 10 min, followed by 40 cycles of amplification consisting of melting at 95°C for 15 seconds, and a combined annealing/elongating step at 60°C for 1 minute. Fluorescent measurements were recorded using the IBM computer software that was connected to all the sequence detection systems. A positive control of *V. vulnificus* template DNA and
two negative controls (de-ionized water and uninoculated APW broth) were prepared with each PCR master mix.

PCR was also performed as described above but with the addition of SYBR Green I dye (Applied Biosystems, Inc) for PCR product detection and without the TaqMan® probe. The specificity of the amplified product using SYBR Green I dye was determined by examining the melting peak of the product in a dissociation curve using first derivative plot of the fluorescence (SYBR Green I dye) versus temperature (De Medici et al. 2003, Mouillesseaux et al. 2003).

**Statistical Analysis**

All results were analyzed using analysis of variance on SAS Statistical Software (Version 8.2). Data were sorted by samples source and by method treatments separately. Samples with higher numbers of *V. vulnificus* were analyzed independently of samples with lower numbers of bacteria. Significance of differences in numbers obtained from different detection methods was derived from the mean log MPN g-1 of each sample method and its standard deviation was determined for all samples. P values of < 0.05 were considered significant. All the results from DNA probing methods were recorded as either positive or negative for detection based on a color change and then the three lowest dilutions from MPN analysis were used for the 3-digit MPN code in the MPN calculator. Positive detection by R-PCR methods resulted from amplification above the Ct value threshold of 0.1. Any Ct value greater than or equal to 34-35 cycles was considered a negative result or as no amplification.
CHAPTER 3
RESULTS

These studies consisted of two stages of research. The first stage included preliminary studies assessing the plating efficiency of different media for indigenous and artificially seeded *V. vulnificus* in oyster homogenates using VVAP plate count enumeration by colony blot hybridization with DNA probe. The second stage of research compared three different detection methods for confirmation of enumeration of *V. vulnificus* by most probable number (MPN) analysis in order to determine which method would provide the greatest accuracy, sensitivity, and specificity. Enumeration from the oyster samples was performed by conventional method of VVAP plate count by colony blot hybridization, and these numbers were compared to results obtained using modifications of FDA BAM MPN using either VVAP MPN, microtiter VVAP MPN, or real-time PCR (R-PCR) MPN were enumerated, as described in the materials and methods. In addition, comparisons of two different DNA extraction methods, as well as two different R-PCR (SYBR Green I dye vs. TaqMan® probe) detection systems, were utilized for *V. vulnificus* enumeration.

Several methods have been used to enumerate and confirm *Vibrio* spp. Unfortunately, these methods have not proven to be adequate for the accurate enumeration and reliable identification of this organism. For example, some of the biochemical assays described in the introduction present problems, including isolation of nontypable strains of *Vibrio* spp. that result in unreliable confirmation. Also, the antibody
for EIA is no longer commercially available. All of the assays are relatively expensive. Therefore, this study compared alternative methods enumeration and identification of *V. vulnificus* in oyster samples.

**Media Comparison Studies**

**Enumeration of Indigenous *V. vulnificus* from Oyster Homogenates using Different Media**

In this study, the efficiency of different media for the recovery and enumeration of *V. vulnificus* MO6-24/O was examined in uninoculated oysters obtained from local markets. *Vibrio vulnificus* were enumerated by VVAP colony blot hybridization using alkaline phosphatase probe as described in the materials and methods. The numbers of indigenous *V. vulnificus* in these oysters ranged from non detectable levels to $5.3 \times 10^3$ CFU g$^{-1}$ (Table 3-1). The recovery of this organism from nonselective media (LA, T1N1, 3%LA) or from media that were semi-selective as a result of increased NaCl concentration (ASW, T1N3) ranged from 10 to 100 fold higher than that of the differential/selective media (TCBS and MCPC); thus, using selective media may not provide the accurate enumeration of *V. vulnificus*. In addition, no major differences in plating efficiency were observed between T1N1 agar and L-Agar. However, T1N3 agar exhibited approximately 10 fold lower recovery than the other nonselective or semi-selective media. This may be due to the higher NaCl concentration (3%) in this medium, when compared to the other agars. Also, studies have documented that *V. vulnificus* prevalence is greater at lower salinities (Kelly 1982, Shapiro et al. 1998, Vanoy et al. 1992), and increased NaCl probably accounts for reduced recovery of *V. vulnificus* on T1N3 agar.
Comparison of Plating Efficiency on Different Media for *V. vulnificus* from Artificially Inoculated Oyster Samples

The oyster samples were collected and processed as described in the materials and methods. Oyster homogenates were seeded with concentrations of pure culture of *Vibrio vulnificus* MO6-24/O ranging from $10^1$ to $10^7$ CFU g$^{-1}$ to evaluate the plating efficiency of the seven different agars described in material and methods. No particular nonselective or semi-selective medium greatly increased the recovery of *V. vulnificus* (Table 3-2). However, the plating efficiency was generally higher on nonselective or semi-selective media than on the selective/differential media (TCBS and MCPC), which usually did not show any growth of *V. vulnificus* at the lower dilutions ($10^1$ to $10^3$).

### Table 3-1. Recovery and enumeration of indigenous *V. vulnificus* in oyster homogenates.

<table>
<thead>
<tr>
<th>Media</th>
<th>Study 1 (CFU g$^{-1}$)</th>
<th>Study 2 (CFU g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Agar</td>
<td>$4.2 \times 10^3$</td>
<td>ND$^b$</td>
</tr>
<tr>
<td>T1N1</td>
<td>$5.3 \times 10^3$</td>
<td>$5.0 \times 10^1$</td>
</tr>
<tr>
<td>T1N3</td>
<td>$7.0 \times 10^2$</td>
<td>ND</td>
</tr>
<tr>
<td>3%LA</td>
<td>$1.6 \times 10^3$</td>
<td>$7.5 \times 10^1$</td>
</tr>
<tr>
<td>ASW</td>
<td>Sp$^c$</td>
<td>$8.5 \times 10^1$</td>
</tr>
<tr>
<td>TCBS</td>
<td>$2.0 \times 10^2$</td>
<td>ND</td>
</tr>
<tr>
<td>MCPC</td>
<td>$1.0 \times 10^2$</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$Number of *V. vulnificus* was enumerated from oyster homogenates on nonselective (LA, T1N1, 3%LA) or semi-selective (T1N3, ASW) media as determined by colony blot hybridization with VVAP probe method. Enumeration on TCBS and MCPC was determined by transferring all single colonies to LA for subsequent identification by colony blot hybridization as described in the text.

$^b$None detected.

$^c$Overgrowth of spreading colonies (Sp) prevented the enumeration of individual colonies.
Enumeration Methods Comparison Studies

Comparison of Enumeration Methods for *V. vulnificus* Using VVAP Plate Counts vs. either VVAP MPN, Microtiter MPN, or TaqMan® PCR MPN

The purpose of this study was to compare numbers obtained from three different confirmation methods for MPN enumeration. Oyster samples from Apalachicola Bay, FL were collected and analyzed in May and July, 2003. The CFU g\(^{-1}\) of indigenous *V. vulnificus* in oysters was determined by VVAP plate counts, using colony blot

Table 3-2. Recovery and enumeration of *V. vulnificus* from inoculated oyster homogenates using selective and nonselective media.

<table>
<thead>
<tr>
<th>Media</th>
<th>10(^7)</th>
<th>10(^6)</th>
<th>10(^5)</th>
<th>10(^4)</th>
<th>10(^3)</th>
<th>10(^2)</th>
<th>10(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA</td>
<td>9.2 x 10(^6)</td>
<td>1.2 x 10(^6)</td>
<td>9.7 x 10(^5)</td>
<td>9.7 x 10(^4)</td>
<td>1.7 x 10(^3)</td>
<td>3.0 x 10(^2)</td>
<td>1.7 x 10(^1)</td>
</tr>
<tr>
<td>T1N1</td>
<td>9.7 x 10(^6)</td>
<td>1.7 x 10(^6)</td>
<td>8.4 x 10(^5)</td>
<td>1.2 x 10(^4)</td>
<td>2.6 x 10(^3)</td>
<td>2.3 x 10(^2)</td>
<td>1.7 x 10(^1)</td>
</tr>
<tr>
<td>T1N3</td>
<td>1.5 x 10(^7)</td>
<td>3.3 x 10(^6)</td>
<td>1.1 x 10(^4)</td>
<td>1.5 x 10(^4)</td>
<td>4.8 x 10(^3)</td>
<td>2.0 x 10(^2)</td>
<td>2.3 x 10(^1)</td>
</tr>
<tr>
<td>3%LA</td>
<td>1.1 x 10(^7)</td>
<td>1.5 x 10(^6)</td>
<td>1.1 x 10(^4)</td>
<td>1.5 x 10(^4)</td>
<td>1.2 x 10(^3)</td>
<td>1.0 x 10(^2)</td>
<td>4.7 x 10(^1)</td>
</tr>
<tr>
<td>ASW</td>
<td>1.0 x 10(^7)</td>
<td>1.9 x 10(^6)</td>
<td>1.2 x 10(^4)</td>
<td>1.1 x 10(^4)</td>
<td>4.4 x 10(^3)</td>
<td>9.7 x 10(^1)</td>
<td>7.7 x 10(^1)</td>
</tr>
<tr>
<td>TCBS</td>
<td>6.8 x 10(^5)</td>
<td>ND(^b)</td>
<td>1.3 x 10(^2)</td>
<td>2.5 x 10(^4)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MCPC</td>
<td>5.8 x 10(^5)</td>
<td>3.8 x 10(^5)</td>
<td>ND</td>
<td>2.0 x 10(^3)</td>
<td>1.6 x 10(^3)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\)Number of *V. vulnificus* was enumerated from seeded oyster homogenates on nonselective (LA, T1N1, 3%LA) or semi-selective (T1N3, ASW) media as determined by colony blot hybridization with VVAP probe method. Enumeration on TCBS and MCPC was verified by transferring all single colonies to LA for subsequent identification by colony blot hybridization as described in the text.

\(^b\)None detected.

hybridization with the DNA probe that targets the *vvhA* gene (Wright et al. 1993). The average log CFU g\(^{-1}\) of *V. vulnificus* isolates, as determined by VVAP plate counts in all five samples, was found to range from 2.1 log CFU g\(^{-1}\) to 4.2 log CFU g\(^{-1}\) (Table 3.3).

Some of the samples received post-harvest freezing treatment, and the purpose of using treated samples was to lower the *V. vulnificus* contamination levels in order to test the limits of sensitivity for these assays. Samples 1 and 2 were from day zero pre- and post-freezing treatment and were analyzed independently of samples 3, 4, and 5 from days 14,
21, and 28 post treatment for statistical purposes, as they reflected high versus low numbers of *V. vulnificus* contamination. Thus, samples 1 and 2 contained about $10^4$ CFU g$^{-1}$ and were grouped together, while samples 3, 4, and 5 contained approximately $10^2$ CFU g$^{-1}$ and served as the lower level *V. vulnificus* contamination group.

Statistical comparisons using analysis of variance (SAS program) between VVAP plate counts and the three different methods for detection of MPN analysis indicated that these assays varied in their sensitivity and accuracy. As shown in Table 3-3, significant differences in the enumeration of *V. vulnificus* were observed between the standard VVAP plate counts and both the VVAP MPN and the microtiter VVAP MPN enumeration for all samples at both lower and higher levels of *V. vulnificus* contamination (p<0.0001). However, no significant differences were noted between the numbers obtained by VVAP MPN and the microtiter VVAP MPN for any samples. Comparison of the VVAP plate counts to the TaqMan® R-PCR MPN, showed no significant difference at the higher levels of *V. vulnificus* contamination. However, at the lower levels, there were significant differences between *V. vulnificus* numbers obtained using the VVAP plate counts vs. the TaqMan® R-PCR (p=0.0005). Further, results from the microtiter VVAP MPN method were not significantly different than those seen with the TaqMan® R-PCR MPN for samples 3-5, but there were significant differences for samples 1 and 2 at the higher levels of *V. vulnificus* contamination (p<0.0001). Also, there was a significant difference between VVAP MPN and TaqMan® R-PCR MPN at both the higher and lower levels (p=0.02) of *V. vulnificus* contamination.
Comparison of SYBR Green I dye vs. TaqMan® R-PCR Detection and Different Template Preparations for *V. vulnificus* MPN Analysis

Campbell (2003) designed primers and probe that targeted the *vvhA* gene for development of a R-PCR assay using the TaqMan® detection system and DNA template extracted by the Qiagen kits. Their study demonstrated species-specificity, and confirmed that amplification of the *vvhA* gene yielded a specific fluorescent signal for accurate enumeration by R-PCR assay. These primer sets were used in the present study to compare SYBR Green I dye detection of PCR product with TaqMan® R-PCR analysis of enrichment broths in the microtiter MPN. In addition, two different extraction methods, a boiling lysis and a Qiagen® DNA extraction/purification kit, were examined to determine which of these methods was the most effective with R-PCR MPN. Thus, TaqMan® R-PCR was compared to SYBR Green I real-time PCR MPN using both the boiling and Qiagen extraction kit.

As shown in Table 3-4, no significant differences were observed between the MPN enumeration results obtained by use of the two extraction methods in combination with either SYBR Green I dye or TaqMan® R-PCR detection. Furthermore, Figure 3-1 shows melting peaks obtained from replicate samples after amplification of *V. vulnificus* DNA using SYBR Green detection with the boiling method, and the single peak is indicative of the specificity of this assay.
Table 3-3. Comparison of methods for enumerating *V. vulnificus* from oyster samples.

<table>
<thead>
<tr>
<th>Oyster Samples&lt;sup&gt;b&lt;/sup&gt;</th>
<th>VVAP Plate Counts (LogCFU g&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>VVAP MPN (LogMPN g&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Microtiter VVAP MPN (LogMPN g&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>TaqMan® R-RPC MPN (LogMPN g&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Untreated</td>
<td>4.2 ± 0.2</td>
<td>2.9 ± 0.4&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>2.6 ± 0.2&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td>2) Day 0</td>
<td>4.0 ± 0.6</td>
<td>3.6 ± 0.8&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>2.9 ± 0.3&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>4.4 ± 0.8</td>
</tr>
<tr>
<td>3) Day 14</td>
<td>2.5 ± 0.2</td>
<td>1.1 ± 0.1&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>1.6 ± 0.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.9 ± 0.5&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>4) Day 21</td>
<td>2.6 ± 0.3</td>
<td>1.1 ± 0.4&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>1.4 ± 0.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.8 ± 0.2&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>5) Day 28</td>
<td>2.1 ± 0.1</td>
<td>0.7 ± 0.3&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>1.2 ± 0.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.8 ± 0.7&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean log CFU or MPN g<sup>-1</sup> of replicate samples (n=3) ± Standard deviation (SD) of *V. vulnificus* MO6-24/O enumerated by VVAP plate counts using DNA probe of colony blot hybridization, by VVAP MPN from enrichment tubes, by microtiter plate VVAP MPN from, or by TaqMan® R-PCR confirmation of MPN as described in the materials and methods.

<sup>b</sup>Oyster samples were collected on May, 2003 and July, 2003 from Apalachicola Bay, FL and were either refrigerated 24 h prior to analysis (untreated) or received nitrogen freezing post harvest treatment and were analyzed at Days 0, 14, 21, or 28 post-treatment.

<sup>c</sup>Indicates significant differences between VVAP plate counts and either VVAP MPN, microtiter VVAP MPN, or TaqMan® (p<0.0001).

<sup>d</sup>Indicates significant differences between TaqMan® MPN and either VVAP MPN or microtiter VVAP MPN (p=0.02).
Table 3-4. Comparison of MPN enumeration using R-PCR with either SYBR Green I or TaqMan® in combination with two different DNA extraction methods.

<table>
<thead>
<tr>
<th>Sample&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Numbers of <em>V. vulnificus</em> by R-PCR MPN Methods (Log MPN g⁻¹± SD)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TaqMan® R-PCR</th>
<th>SYBR Green I R-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extracted</td>
<td>Boiled</td>
<td>Extracted</td>
</tr>
<tr>
<td>1) Untreated</td>
<td>4.5 ± 0.1</td>
<td>4.6 ± 0.2</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>2) Day 0</td>
<td>4.4 ± 0.8</td>
<td>4.2 ± 0.9</td>
<td>4.2 ± 0.9</td>
</tr>
<tr>
<td>3) Day 14</td>
<td>1.9 ± 0.5</td>
<td>1.8 ± 0.5</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>4) Day 21</td>
<td>1.8 ± 0.2</td>
<td>1.3 ± 1.2</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>5) Day 28</td>
<td>0.8 ± 0.7</td>
<td>0.9 ± 0.8</td>
<td>0.8 ± 0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean log MPN g⁻¹ of replicate samples (n=3) ± Standard deviation (SD) using R-PCR confirmation of positive samples with either Qiagen extracted or boiled lysate DNA and SYBR Green I dye or TaqMan® probe detection as described in the materials and methods.

<sup>b</sup>Oyster samples were collected May, 2003 and July, 2003 from Apalachicola Bay, FL and were either refrigerated 24 h prior to analysis (untreated) or received nitrogen freezing post-harvest treatment and were analyzed at Days 0, 14, 21, or 28 post-treatment.
Figure 3-1. Melting curve and melting peaks. Melting curve/dissociation curve and melting peaks for different amplification products (oyster homogenate and its serial dilutions). A melting curve was obtained after 40 cycles of amplification with *V. vulnificus* primer sets and compared by using the boiling extraction method.
In recent years, infections caused by *V. vulnificus* have increased despite intensive educational and control efforts by state and federal agencies (MMWR 1993). Illnesses associated with this foodborne pathogens result from the contamination of seawater and shellfish and is independent of fecal indicators commonly used for seafood safety analysis. Thus, the need for accurate assessment has led researchers to develop pathogen-specific detection and enumeration methods. Previous studies have shown that *V. vulnificus* is ubiquitous in estuarine environments, and the occurrence of this bacterium is greatly influenced by temperature and salinity (Kelly 1982, Motes et al. 1998, Shapiro et al. 1998). In this study, the numbers of *V. vulnificus* as determined by the standard VVAP plate counts, were compared MPN g⁻¹ obtained by three different MPN methods (VVAP MPN, microtiter VVAP MPN, and R-PCR MPN for the identification and enumeration of *V. vulnificus* in oysters. Also, two different DNA template preparation methods for R-PCR (QIAamp® DNA Mini Kit versus boiling method) were compared to determine which extraction method was more efficient in combination with the R-PCR MPN. Finally, two different real-time detection systems were evaluated for sensitivity in R-PCR analysis.

The current FDA BAM MPN procedure uses alkaline peptone water (APW) enrichment that allows not only the growth of the target organism, but also the growth of other bacteria present in the sample. Because of this overgrowth, the use of APW enrichment broth can reduce the sensitivity of the assay, especially if high numbers of
competitive organisms are present. Recovery and misidentification of non-target organisms may also lead to over estimations of bacterial concentration with the MPN method. In the current FDA BAM method, positive turbid MPN tubes are streaked for isolation onto selective agars (MCPC and TCBS). A few (usually 3-5) of the typical colonies grown on these agars are selected and examined using biochemical assays or the DNA probe procedure (FDA 1998). If colonies hybridized with the DNA probe, the individual tubes are considered positive for MPN calculations (FDA 1998). Although this method is useful for the enumeration and estimation of *V. vulnificus*, this procedure depends on time-consuming biochemical assays for the identification, and confirmation of suspect colonies requires large quantities of media and is labor-intensive. Further, selective media biochemical test kits required for the FDA MPN are expensive and inaccurate (Dalsgaard et al. 1996, Arias et al. 1998). Currently confirmation by monoclonal antibody is not possible due to lack of availability. For this reason, a modification of the FDA BAM MPN procedure was made using either VVAP DNA probe (Wright et al. 1993) or R-PCR (Campbell 2003), instead of biochemical assays for *V. vulnificus* confirmation, thereby eliminating the long processing time and reducing expenses.

In the present studies, VVAP plate counts were used as the standard for enumeration of *V. vulnificus* as this method is an FDA BAM standard for *V. parahaemolyticus* enumeration, has been used extensively for *V. vulnificus* enumeration, and is currently under review for acceptance as an FDA approved method for enumeration of *V. vulnificus* (A. Depaola, personal communication). Previously, Gooch et al. (2001) confirmed that direct plating methods involving a nonradioactive DNA
probe were better than the BAM MPN method for the enumeration of \textit{V. parahaemolyticus}. This study also showed that the DNA probe methods were completed within 1 to 2 days while the MPN assay required about 4 days. Moreover, DePaola et al. (1997) found that the direct plate methods used to enumerate \textit{V. vulnificus} were more precise than the MPN analysis, especially when combined with immunological assays. To the contrary, Kaysner et al. (1989) reported that the MPN procedure had a higher recovery level of \textit{V. vulnificus} than with direct plating methods utilizing TCBS and CPC agars. However, in light of other problems inherent in the traditional MPN analysis and the fact that a monoclonal antibody specific for \textit{V. vulnificus} confirmation in FDA BAM assay is no longer commercially available, the DNA probe VVAP plate count was used in this study as the standard for \textit{V. vulnificus} enumeration as it is more sensitive than the traditional MPN method for detection in oyster samples.

In the media comparison studies, indigenous \textit{V. vulnificus} derived from uninoculated Apalachicola, Fl oyster homogenates were examined to evaluate the plating efficiency of seven different selective (TCBS, MCPC), semi-selective (T1N3, ASW with higher salt concentrations) and nonselective media (LA, T1N1, 3\%LA). These media were selected because they are easy to prepare, inexpensive, and have been used in numerous studies for the isolation and detection of \textit{Vibrio} spp. The results from this study demonstrated that recovery on selective agars used in the FDA MPN protocol was about 10 to 100 fold lower than the recovery demonstrated for nonselective or semi-selective media. These results indicated that selective agars such as MCPC and TCBS may not offer accurate enumeration for this bacterium. T1N3 is a semi-selective agar that contains a higher NaCl concentration than nonselective agars. Although this medium is mainly
used for *V. parahaemolyticus* enumeration, this agar was utilized in media studies in an attempt to decrease background microorganisms; however, the use of T1N3 plates also produced lower recovery of *V. vulnificus* in comparison to non selective agars.

The plating efficiency of the media listed above was also evaluated in oysters that were artificially seeded with *V. vulnificus*. The results obtained from this study did not show that any particular nonselective or semi-selective medium was better than the others in terms of plating efficiency. On the other hand, TCBS and MCPC selective/differential agars again generated low recovery levels (10 fold lower) of *V. vulnificus* for some samples, and no detection was observed at the lowest dilutions. These results were supported by Kaysner et al. (1989) who found that the plating efficiency of selective media greatly reduced the recovery of *V. vulnificus* in comparison to numbers obtained by colony blot hybridization using the VVAP DNA probe detection on non selective agar as described by Wright et al. (1993).

One of the problems with enumeration by VVAP plate count is that target colonies may be overgrown by spreading of background colonies, which may at times cover the entire plate and prevent transfer of colonies to filters for analysis. Nonselective agars used in this assay do not contain the inhibitors found in TCBS and MCPC, which not only make these media selective but also apparently inhibit spreading colonies. Thus, increased background growth in the DNA plate count method, which utilizes nonselective media, may lower the recovery and sensitivity to underestimate enumeration (Porter 2002). The current alternative to DNA probe plate counts is the FDA MPN analysis using a biochemical confirmation. As this method is time-consuming and expensive, this study explored other methods for confirmation of positive MPN samples. One method
examined the use of DNA probe to directly evaluate growth from enrichment tubes that were spotted to agar plates. However, application of the VVAP probe as a direct confirmation method for MPN analysis may result in similar problems with overgrowth, especially since the samples are spotted instead of spread-plated. For this reason, the use of R-PCR as a confirmation method was also examined as a possible way to avoid this problem.

Previous studies have demonstrated the efficacy of real-time R-PCR to enumerate and detect *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* (Blackstone et al. 2003, Campbell 2003, Lyon 2000). The detection rate of the R-PCR assay was investigated for either direct enumeration of bacteria in oyster homogenates or MPN enrichment. Previous reports by Lyon (2000) showed that R-PCR could monitor *V. cholerae* as it entered a viable but nonculturable (VBNC) state at low temperatures. Their study revealed the failure of traditional enumeration methods to identify/isolate *V. cholerae* from seawater and seafood. It is important to note that in their study, DNase was used to lower the extracellular DNA isolated from dead cells and distinguish them from cells in the VBNC state. The DNase treatment led to a 1-log reduction in total cell number of bacteria detected by R-PCR, presumably due to the elimination of DNA contribution by dead cells. Thus, TaqMan® R-PCR was designed to target the *V. cholerae* hemolysin (*hlyA*) gene of intact cells only (Lyon 2000).

Recently, Campbell (2003) described a real-time PCR assay that targeted the hemolysin gene (*vvhA*) of *V. vulnificus*. The R-PCR assay was used to accurately enumerate *V. vulnificus* from oysters and to examine the VBNC state. TaqMan® R-PCR was able to quantitate *V. vulnificus* cells in either seeded or uninoculated oyster samples.
These results showed that, for the seeded oysters, numbers of *V. vulnificus* enumerated with the DNA probe method were not significantly different than the numbers obtained by R-PCR. They also found that this assay may not be as sensitive for the detection of indigenous bacteria, as further purification steps were required to obtain comparable numbers. Moreover, their results demonstrated that *V. vulnificus* cells sustained viability even at cold temperatures throughout the study using the *BacLight™* method (Molecular Probes, Eugene, OR) of live/dead discrimination; however, nonselective agars were unable to detect *V. vulnificus* growth after 28 days. These VNBC cells were also detected and accurately enumerated by R-PCR. These studies confirmed that the TaqMan® PCR technique is a powerful tool that rapidly detects and identifies *Vibrio* spp. rather than using the conventional microbiological (MPN) methods, which are less sensitive.

Comparison studies of MPN enumeration using a variety of confirmation methods demonstrated that these assays varied in sensitivity and precision. Significant differences were observed between the standard VVAP plate counts and both the VVAP MPN and the microtiter VVAP MPN for samples either at lower or higher levels of *V. vulnificus* contamination (p<0.0001), indicating that these assays lack the sensitivity of VVAP plate counts. However, when VVAP MPN using standard enrichment tubes and microtiter VVAP MPN were compared to each other, no significant difference was observed for any of the samples. These results indicate that the more convenient microtiter assay may be used to replace the standard VVAP tube MPN. Microtiter VVAP MPN method was not significantly different than the TaqMan® R-PCR MPN for samples 3-5, but there was a significant difference for samples 1 and 2 at the higher levels of *V. vulnificus* contamination (p<0.0001). Also, there was a significant difference between VVAP MPN
and TaqMan® R-PCR MPN at both the higher and lower levels (p=0.02), suggesting the R-PCR analysis may offer a more sensitive method of confirmation of *V. vulnificus* MPN.

Results from oysters with high levels of *V. vulnificus* (samples 1 and 2) demonstrated no significant differences between the numbers obtained by the VVAP plate counts and the R-PCR MPN assay. However, at lower levels of *V. vulnificus* contamination (samples 3-5), there were significant differences (p<0.0005) between results for VVAP plate counts and R-PCR MPN using the TaqMan® probe with the Qiagen extracted DNA. These results suggest that R-PCR may provide more accurate detection than the other methods but that problems with sensitivity of either the MPN assay itself or the detection method are still a problem at lower concentrations of target organism. Similar results were obtained by Campbell (2003) who compared R-PCR to direct plate enumeration using the VVAP probe. Their results showed that R-PCR was found to present some difficulty at the lower limits of detection, but no significant overall differences between TaqMan® R-PCR enumeration and the direct plate counts using the VVAP probe. In this study, problems with competitive inhibitors and other background organisms were eliminated by Qiagen extraction; however, our studies did not find any differences in the sensitivity between samples derived from boiled lysates vs. those that were purified by DNA extraction. Thus, our results indicate the differences in sensitivity between the DNA probe plate count and R-PCR MPN may lie with problems inherent to the MPN analysis. For example, overgrowth of other organisms in enrichment broths may inhibit growth of *V. vulnificus* be lethal. Some of the analysis, non-numerical results, and these short-comings are generally improved with the application of R-PCR.
Problems of traditional PCR assays include poor precision, low resolution, non-automated. In addition, R-PCR has shown to be effective when working with a large number of oyster samples as well as detecting viable but nonculturable cells (VBNC). Results obtained in the present study support these findings since large numbers of oyster samples were analyzed rapidly and efficiently. Another problem with traditional PCR assays is the inability to distinguish between living and dead cells, and this problem is not eliminated with R-PCR as Campbell (2003) did find that both viable and non-viable cells may be detected prior to cultures becoming completely nonculturable. The amplified DNA from dead organisms can overestimate the numbers of living organisms. A solution to this problem is the detection of mRNA, using reverse transcription PCR (RT-PCR) as an indicator of live cells. The decreased stability of bacterial RNA in comparison to DNA generally precludes the detection of non-viable cells. Sheridan and colleagues (1998) described a study where RT-PCR was used as an indicator of live cells in *Escherichia coli* isolates that were treated by heat or ethanol. Their findings demonstrated that the difference in detection times post heat or ethanol treatment might be linked to the effects of the treatment on the stability of RNA derived from dead cells. Also, they showed mRNA was an indicator of cell viability (Sheridan et al. 1998). Another study also used RT-PCR for the detection of viable *Listeria monocytogenes* by targeting the *iap* gene product. Their results showed that *iap* mRNA was specific for *L. monocytogenes*, and that *L. monocytogenes* was also detected in artificially inoculated beef (Klein and Juneja 1997). Bej et al. (1996) also developed a RT-PCR assay to detect *V. cholerae* by targeting mRNA of the cholera toxin gene (*ctx*). Their study showed that the RNA, which was
purified and treated with DNase I, was detected in *V. cholerae* viable cells but only with a sensitivity of about $10^3$ cells.

It should be noted that the samples analyzed using R-PCR at the lower levels (Days 14-28) of *V. vulnificus* contamination had a significant difference than the standard VVAP plate counts. This difference may be due to a few factors in the processing of the oysters. First, the samples were all extracted after blending and freezing the oyster homogenates overnight. Not extracting the oyster samples immediately after blending may have inactivated nucleases through the freezing treatment. Another explanation as to why the TaqMan® R-PCR MPN was lower than the VVAP plate counts may be due to the fact that the oyster homogenates were analyzed approximately 15 min after being blended. Therefore, the present study further demonstrates that extra care must be taken in the way oyster samples are stored for future R-PCR amplification.

In the present study, TaqMan® R-PCR detection of MPN enrichment samples was also compared to SYBR Green I analysis using both the boiling and Qiagen extraction kit. The results showed no significant differences between the results obtained with the two extraction methods in combination with either SYBR Green I dye or TaqMan® R-PCR MPN enumeration. These results indicate that the more cost effective SYBR detection might be used in place of TaqMan® probes without any decrease in sensitivity. These findings are in agreement with an earlier report by DeMedici et al. (2003) who evaluated four different extraction methods in combination with SYBR Green I R-PCR to detect *Salmonella* strains. Similarly, their study found that the SYBR Green I R-PCR and the boiling extraction method were the most effective and simple assays for detecting
Salmonella strains. Blackstone and colleagues (2003) also support these results by using a simple boiling method in combination with R-PCR for the detection of V. parahaemolyticus in oyster samples. The boiling extraction method may reduce time and labor when compared to the Qiagen® DNA mini extraction kit, particularly when processing large quantities of oyster samples. Based on these results and those obtained in our studies, future studies should use the combination of SYBR Green I dye and the boiling method since these two methods are simple, rapid, and less expensive than the TaqMan® probe and Qiagen® DNA mini extraction kit.

Conclusions. Although previous studies have looked at different enumeration methods, few investigations have systematically compared assays to determine the best method for the detection and enumeration of V. vulnificus in oyster homogenates. The purpose of the present study was to compare the different available recovery media and to evaluate three different MPN methods using VVAP plate counts as the standard for the enumeration of V. vulnificus. The recovery of V. vulnificus from uninoculated and artificially seeded oyster homogenates showed that selective agars (TCBS and MCPC) may lower recovery rate, indicating that the current use of these media in the BAM MPN may also serve to underestimate the numbers of V. vulnificus. Further, none of the seven media greatly increased the plating efficiency and even small increases in salt concentrations may decrease recovery. Data reported in the present study demonstrated that the R-PCR assay could be as sensitive as VVAP plate counts and was more sensitive than MPN methods using DNA probe enumeration of V. vulnificus in oysters. VVAP MPN (using enrichment broth in tubes) and the microtiter VVAP MPN frequently underestimated the numbers of V. vulnificus in oyster homogenates as determined by
VVAP plate count. This decreased sensitivity may be due to biological and/or organic inhibitors and/or competitors found within the oyster homogenate that either inhibit the growth of *V. vulnificus* or contribute to overgrowth by other contaminating bacteria in enrichment broths. These findings support the use of R-PCR for confirmation of *V. vulnificus*, as this assay also provides a more rapid molecular analysis. The use of real-time R-PCR in combination with MPN described in this study may eliminate post PCR processing such as gel electrophoresis, which is labor intensive, not quantitative, provides low resolution and sensitivity, and uses ethidium bromide (Singer et al. 1999).

These findings could pose great significance to the public health and the shellfish industry, as the high throughput of R-PCR assays may replace the recommended protocols currently in use by the FDA, permitting practical application of rapid, quantitative evaluation of pathogens in shellfish. The approach to methods comparisons, as described is this study, may be used to validate the R-PCR assays. These methods, when combined with other molecular methods, could greatly improve standard environmental monitoring of *V. vulnificus* in shellfish and seawater. Finally, future research should investigate alternative enumeration assays to standard MPN methods in order to increase sensitivity and also compare other PCR systems for the detection and enumeration of *V. vulnificus* in oysters. Improved PCR assays may further provide more rapid, less expensive analysis of post harvest treatments and better monitoring of shellfish and seawater.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Ana Gabriela Calero was born in Managua, Nicaragua in 1977. In 1989, her family immigrated to America settling in Miami, Florida, and in 1996 Ana graduated from Braddock Senior High School and became an American citizen. Ana then attended the University of Florida and received her B.S. in microbiology and cell science in 2000. In 2001, Ana began her master’s studies at the University of Florida (in the laboratory of Anita C. Wright), performing several molecular techniques.

During her graduate career, Ana was an active member of the International Gourmet Association (IGA), where she was involved with cooking, cultural exchange, and interaction with faculty and international students; and gained knowledge of food safety. Elected the IGA president, Ana obtained sponsors for the club from Kraft Foods and helped set up cooking workshops. In addition, Ana has served as a conversational partner for foreign students to improve their English-speaking skills; and has taught three laboratory courses to undergraduate food scientists.

After completing her master’s program, Ana intends to move to Tampa, Florida, to work for the Florida Department of Health Bureau of Laboratories. Ana also plans to pursue a Ph.D. in epidemiology at the University of South Florida, with a focus on foodborne pathogens.