new england fisheries development association, inc.

FINAL REPORT

Contaminant Standards for Fish and Shellfish in the Northeast Region

Saltonstall-Kennedy Grant
#NA-89-EA-D-SK005

Federal Funds:	$420,000
Matching Funds:	$280,000
Total Project Funds:	$700,000

Project Duration: November 1, 1988 - June 30, 1991

submitted to:

DOC/NOAA Fisheries
Northeast Region
One Blackburn Drive
Gloucester, MA 01930

submitted by:

New England Fisheries Development Association, Inc.
309 World Trade Center
Boston, MA 02210-2001
(617) 439-5480

note: The views expressed herein are those of the New England Fisheries Development Association and do not necessarily reflect the views of NOAA or any of its subagencies

PROJECT FINAL REPORT to NEFDA

Rapid Screening Methods for Microbial Contaminants in Seafood

Drs. Aaron Margolin and Stephen Jones

University of New Hampshire

I. Executive Summary

The objective of this project was to develop rapid screening methods for detecting microbial pathogens in shellfish. The work focused on enteric viruses and the bacterial pathogen, *Vibrio vulnificus*. The goal was to develop both a non-radioactive gene probe assay for the rapid detection of low concentrations of enteric viruses and a method based on the polymerase chain reaction (PCR) for identifying and quantifying *V. vulnificus* in shellfish.

Previous gene probe assays, though sensitive for viral detection, utilized radiation and were labor intensive, making them unattractive for routine viral monitoring by the shellfish industry. The advantages of gene probes are specificity, the ability to screen numerous sample quickly, and the detection of viruses which are difficult to cultivate or cannot be grown at all. Current assays which use non-radioactive labels lack the needed sensitivity for viral detection. Alternative assays which first amplify the target genome for subsequent detection, such as the polymerase chain reaction, require the use of an enzyme to covert viral RNA to DNA. This enzyme is very sensitive and cannot be used in shellfish extracts or homogenates.

One of the key problems for detecting bacterial pathogens is that many are gram negative and can become non-culturable, while remaining viable and potentially virulent, upon exposure to adverse environmental conditions. Most present methods for detecting *V. vulnificus* require initial cultivation prior to detection steps. Thus, the goal for the *V. vulnificus* assay was to optimize the PCR method for detecting low levels of *V. vulnificus* in shellfish without initial cultivation.

For the viral work, we proposed the development of a new assay, using poliovirus as a model virus, which also amplifies a specified segment of DNA, but does not first require the conversion of viral RNA to DNA. The assay employs the use of a capture probe to which the viral RNA binds. The capture probe developed for our assay consisted of two segments of the poliovirus cDNA, which were isolated by gel electrophoreses and then denatured to produce single stranded segments. The single strands of DNA were then used to coat hybridization membranes which were later used for the immobilization of viral RNA. In subsequent hybridizations, a second probe would be allowed to bind to the immobilized viral RNA. This second probe was constructed to have three distinctive sections: 1) a viral binding site; 2) a T-7 polymerase promoter region; and 3) a detection sequence. Amplification of the desired DNA segment occurs after the addition of the T-7 polymerase. Four segments of synthetically manufactured oligo-DNA, which was either complimentary to the poliovirus genome or contained the T-7 polymerase promoting area, were obtained from a DNA synthesizer. The appropriate segments were hybridized to their compliment to form double stranded segments. These were then ligated together to form the first

two parts of the new probe. A 434 base pair segment of our poliovirus cDNA was then purified, cut and ligated to the first two sections to form the completed probe. Completion of this part of the project enables production of RNA transcripts which can be detected by non-radioactive probes.

Work on the *V. vulnificus* assay focused on optimizing PCR conditions, testing different primers, developing the best method for preparing target DNA in shellfish tissue for amplification, and screening different environmental isolates with the developed assay. Pure cultures were amplified directly without lysis, and all hemolysin positive environmental strains from the Great Bay Estuary were detectable with the PCR assay. However, only 5 of 7 hemolysin negative strains were detectable. The limit of detection of the assay using a new set of primers was approximately 2 cells. Optimal detection of *V. vulnificus* in shellfish tissue involved direct digestion of seeded, homogenized tissue with guanidine isothiocyanate (GITC), without prior cultivation of bacterial cells, followed by chloroform extraction and ethanol precipitation prior to amplification by PCR. The success of this project provides a firm basis for further modifications that will allow for development of rapid, inexpensive methods for routine monitoring of seafood for the presence of *V. vulnificus*.

Future work includes evaluation of the newly constructed enteric virus probe when used in conjunction with the capture probe in water and with seeded shellfish extracts and homogenates. In addition, the sensitivity of both the viral and the bacterial assays must be determined as compared to traditional culture methods and use of ^{32}P or otherwise labeled gene probes. The assays must also be tested with shellfish samples obtained from contaminated and uncontaminated shellfish-growing waters to determine the reliability and reproducibility of the assays. Finally, these assays need to be used in ecological studies to gain a better understanding of the fate of microbial pathogens in estuarine water and shellfish.

II. Introduction

A causal relationship between shellfish and outbreaks of enteric infections were reported as early as 1895, when a third of the typhoid fever cases in Brighton, England, were estimated to result from consumption of sewage polluted oysters or mussels. Since then, shellfish associated epidemics of infectious hepatitis serve as reminders that pathogenic viruses of human origin find their way into estuary waters. Even though enteric bacterial infections are less frequent than in the past, diseases caused by indigenous bacterial pathogens like *Vibrio vulnificus* continue to pose a significant public health risk for shellfish consumers.

Edible bivalve mollusks of the class Pelecypoda (oysters, mussels, clams) are the only

molluskan shellfish of commercial importance for which sanitary controls are required. These shellfish, characterized by their two shell valves hinged together at one end and closed by a large adductor muscle, are some of the few shellfish which are commonly eaten raw. The United States presently leads the world in the harvesting of bivalve mollusks.

The partial control of enteric bacterial diseases spread by shellfish has resulted from the establishment of bacteriological criteria, using indicator organisms such as total and fecal coliforms, as the standards for shellfish sanitation programs. Unfortunately, the continued occurrence of shellfish transmitted viral gastroenteritis, hepatitis A virus, and indigenous pathogens like *V. vulnificus* has not been prevented. The value of these coliform tests as the principle microbiological criteria for assessing the sanitary quality of shellfish and shellfish growing waters is one part of a more general controversy over the validity of such tests as indicators of the biological safety of waters and shellfish. A new approach for assessing the sanitary quality of seafood and growing water that involves new detection methods for specific microbial pathogens is needed to better protect shellfish consumers.

III. Purpose

A. Current methods which are available for monitoring virus (animal cell culture, radioimmuno assay, fluorescent antibody, enzyme-linked-immunosorbent assay, and radioimmunofoci assay) and *V. vulnificus* (traditional culture methods, enzyme immunoassay) contamination in shellfish have several drawbacks which make routine monitoring for these pathogens impractical. Hence, the sanitary quality of shellfish has been assessed solely using bacterial indicators of fecal pollution. As previously mentioned, this practice is not adequate for indicating viral contamination or the presence of indigenous estuarine bacterial pathogens in shellfish and growing waters.

An alternative to current viral detection methods, known as nucleic acid hybridization, is a sensitive technique for the detection and identification of a viral genome. It is being used increasingly for the rapid detection of viruses that are difficult to cultivate. Jiang et al. reported detection of Hepatitis A virus in seeded estuarine samples by hybridization with a RNA probe. Recently, Dr. Margolin's laboratory has been able to detect enteric viruses in ground and surface waters using a gene probe to poliovirus and hepatitis A virus. They have also adapted their system for the detection of poliovirus in shellfish from Narragansett Bay, the Great Bay of New Hampshire and Maine, from shellfish suspected of causing outbreaks of gastroenteritis, and in freshwater shellfish from the Colorado River.

Although gene probes also provide an alternative technique for the detection of enteric viruses in shellfish, there are several problems with the design of the assay. For example, the current assay uses a ^{32}P isotope for the detection of enteric viruses. This limits the use of the assay to laboratories which are licensed for radioactive compounds. Additionally, the half life of the isotope, two weeks, requires constant production and labelling of the probe in order for the probe to have the required sensitivity for detection of low levels of virus. A second problem arises from the large amount of proteins found in a homogenized shellfish sample. It becomes necessary to first remove these proteins from the sample prior to assaying, so the sensitivity of the gene probe assay will not be impaired. This requires extensive phenol/chloroform extractions followed by chloroform extractions and finally ether extractions. This procedure is very labor intense and potentially exposes individuals to harmful organic compounds.

The ideal gene probe assay to be used for routine monitoring of virus contamination in shellfish should minimize, if not completely remove, protein extractions. Also, the label used with the gene probe should be non-radiative so it can be widely used by all laboratories. Amplification of the viral genome would allow both of these objectives to be accomplished.

The polymerase chain reaction, or PCR, is a reaction which was developed to amplify DNA so it could be detected with greater sensitivity. This system, however, was developed for use with DNA and not RNA. The genomes of enteric viruses are single stranded and composed of RNA. Current protocol requires the use of reverse transcriptase, an enzyme which is able to convert the single strand of RNA into a strand of DNA. Though this enzyme works well for purified material, it does not work efficiently in shellfish extracts that contain low levels of viral RNA and high levels of environmental contaminants. Hence, we have not been able to utilize the polymerase chain reaction in our assay.

One of the key problems for detecting bacterial pathogens in the environment is that many are gram negative and can become non-culturable, while remaining viable and potentially virulent, upon exposure to adverse environmental conditions. Most present methods for detecting bacterial pathogens require initial cultivation prior to detection steps, and thus may not allow for detection of viable, non-culturable cells. Even published methods using PCR, which has been used successfully in the detection of a variety of different bacterial pathogens, often depend on cultivation of the target bacteria for amplification prior to PCR amplification.

The presence of *V. vulnificus* in shellfish is a major public health concern because of the severity of the disease symptoms experienced by infected, susceptible individuals. The shortcomings of traditional detection methods has been a major limitation to understanding the ecology, shellfish colonization, and fate in harvested shellfish of this organism. The purpose of

this project was to use the emerging PCR technology to develop a rapid, sensitive assay, that did not include any culture steps, for the detection of *V. vulnificus* in shellfish.

B. Objectives:

Development of a non-radioactive gene probe assay using an enzymatic amplification method for the detection of poliovirus and hepatitis A virus, and a PCR method for detection of *V. vulnificus* in contaminated shellfish.

Specific objectives:

1. Develop a capture probe for the immobilization of the viral RNA.

2. Develop a second probe which contains the homologous viral sequences ligated to the T7 polymerase sequence which is additionally ligated to the necessary detection sequences.

3. Acquisition of preliminary data demonstrating the efficacy of using non-radioactive labels for virus detection.

4. Evaluation of the sensitivity of this assay compared to a gene probe assay using a ^{32}P labeled probe and to traditional tissue culture methods.

5. Adaptation of the polymerase chain reaction for the detection of the cytotoxin-hemolysin gene of *Vibrio vulnificus* in shellfish.

6. Determine the cost of sample analysis using the newly developed assays for a large seafood facility with an in-house laboratory and the cost of testing for a small seafood facility which contracts with an outside laboratory.

IV. Approach/Findings

A. Viral assay

The approach used in construction of the T-7 polymerase chain reaction is listed below. Probe development was dependent on isolation of part of our existing poliovirus cDNA probe which would then be ligated to the synthetically prepared double stranded segments.

1. Poliovirus cDNA plasmid isolation:

Poliovirus cDNA (bp 115 - 7440) cloned into the PST 1 site of the plasmid pBR322 and transformed into E.coli HB-101 was used for the large scale plasmid preparation. The steps in this procedure are as follows: 1) A single colony of E.coli HB-101 was isolated from LB agar to which tetracycline was added at 12.5 µg/ml of agar; 2) The colony was inoculated into 3 ml of LB broth with tetracycline (same concentration) and incubated for 18 hrs at 37° C; 3) 200 ml of LB broth with tetracycline was subsequently inoculated with 1 ml of the broth culture and incubated for an additional 18 hrs at 37° C with agitation; 4) 100 ml of this culture was used as the inoculum into 1 L of LB broth with tetracycline and incubated 18 hrs at 37° C with agitation; 5) bacterial cells were harvested by centrifugation at 10,000 x g for 10 minutes; 6) supernatant was decanted and the cells were suspended in 10 ml of TE (Tris-HCl, EDTA); 7) cell suspensions were placed into 50 ml tubes (20 ml/tube) and centrifuged 10,000 x g for 10 minutes; 8) supernatant was then decanted and cells were resuspended in 6 ml of TE; 9) 0.6 ml of lysozyme (10 mg / ml) was added and cells were incubated on ice for 10 minutes; 10) 1.5 ml of 0.25 M EDTA solution was then added and the solution was returned to ice for an additional 10 minutes; 11) after 10 minutes incubation, 5 ml of 10% SDS was added the sample was incubated at 37° C until the cells lysed; 12) high molecular weight chromosomal DNA was removed by centrifugation for one hour at 15,000 x g; 13) supernate was carefully decanted with the aid of wooden applicator sticks and to it was added 1 g of cesium chloride per ml of supernate, the mixture was incubated at 37° C until all salt was dissolved; 14) ethidium bromide (10 mg/ml) was added at a ratio of 1 ml per 10 ml of sample; 15) proteins were removed from the sample by centrifugation at 10,000 x g for 10 minutes. The sample was now ready for ultracentrifugation.

Plasmid cDNA was isolated by density gradient centrifugation in a Beckman L8-70 ultracentrifuge at 45k for 36 hrs using a Beckman Ti 80 rotor. Once banded, the plasmid was recovered by puncturing the side wall of the tube, just below the cDNA band, with a 5 ml syringe and a 21 gauge needle. The band was withdrawn and the ethidium bromide was removed using n-butanol extractions. To remove cesium chloride, the plasmid was dialyzed for 36 hrs at 4° C

against TE buffer in a 2 L flask, changing the buffer 3 X in a 24 hour period. Recovered cDNA was stored in ethanol containing 0.3 M sodium acetate at -20° C. Agarose gel electrophoresis was used to determine the purity of the plasmid obtained and approximate concentrations (Figure 1).

2. Isolation of poliovirus cDNA:

Isolation of the poliovirus cDNA from the plasmid vector was done by a restriction digest using the restriction endonuclease known as PST 1. The steps utilized to accomplish this are as: 1) DNA stored in ethanol from the large scale plasmid prep procedure was pelleted using microcentrifuge tubes and a Beckman microcentrifuge at 4° C; 2) the supernatant was poured off and the pellet was allowed to dry; 3) the dried pellet was then suspended in 1-10 µl of sterilized Milli-Q-Water (Millipore Co.); to this was added a medium salt restriction buffer and 10 U of the PST 1 enzyme; 4) the mixture was incubated for 1 hr at 37° C; 5) to visualize the DNA it was electrophoreses on a 1% agarose gel using a Tris-Borate-EDTA buffer, the gel was then stained with ethidium bromide and the DNA bands were then detected with a UV light source. Digestion of the cDNA and subsequent visualization by gel electrophoresis yielded four bands (Figure 2). The 434, 1174 and 1689 base pair fragments of the poliovirus cDNA were identified by comparison with a DNA molecular weight marker, cut from the gel with a razor and placed in individual tubes. The poliovirus cDNA was the isolated from the agarose by phenol/chloroform followed by chloroform extractions. All of the recovered cDNA was stored in ethanol at -20° C.

3. Probe development:

The probe was designed to have a purification sequence, viral recognition sequence, T-7 polymerase promoting sequence and a detection sequence (Figure 1). Two recognition sites, one for PST 1 and one for SAL 1 restriction endonucleases were also included in the probe. An explanation of the individual probe components is given below, the sequence of the synthesized probe is shown in attachment 1.

A) The purification sequence is a series of adenines (5' AAA AAA AAA AAA AAA 3') attached to the viral recognition sequence. Once the probe is assembled and amplified, the purification sequence will be used to separate the two strands of DNA. The strands will be separated by passing the denatured DNA through a oligo-dt column, the oligo-dt will bind the polyadenylated strand. The polyadenylated strand can then be eluted off the column and hybridized to a complementary sequence consisting of the T-7 promotor sequence and detection sequence, producing a probe with a single and double stranded region.

B) The viral recognition site (5' CAT ACT ACG TAG AGA GTC GGG ACG 3') is composed of 24 bases which are complementary to bases 6664 - 6687 of the poliovirus genome. This area of the probe will hybridize to poliovirus RNA. At the opposite end of the poly A region is a SAL 1 cohesive terminus which will facilitate its' ligation to the promotor sequence.

C) The T-7 polymerase promoting sequence (5' AAT TTA ATA CGA CTC ACT CAC TAT AGG GA 3') is next in series after the recognition sequence. The promotor was designed to contain both the T-7 RNA polymerase binding site and also a preferred transcription initiation site. Cohesive termini were added to each end, SAL 1 for ligation to the recognition sequence and PST 1 for ligation to the detection sequence.

D) The detection sequence will consist of the 400 bp fragment isolated from the poliovirus cDNA. Due to the low recovery rate of this fragment by gel electrophoresis, it may have to be amplified by PCR prior to ligation to the synthesized portion of the probe.

E) The restriction sites were incorporated into the probe to facilitate ligation of the probe components and also to allow for the amplification of the promotor/detection sequence independent of the entire probe. As discussed earlier, the separate amplifications are necessary to ultimately obtain a probe with a single and double stranded region.

4. Preparation and quantification of ssDNA:

The ssDNA was received in lyophilized form. The DNA was rehydrated with 1 ml of TE (10 mM Tris-Hcl,1 mM EDTA), aliquotted and stored at -70° C. Spectrophotometric readings were performed on each of the strands using a Beckman DU model 2400 spectrophotometer. Absorbance readings at A_{260} were taken, and the values obtained were extrapolated from a previously prepared dsDNA standard curve. The values were then divided by 1.37 since 1 absorbance unit of ssDNA is equal to 37 µg whereas 1 absorbance unit of dsDNA is equal to 50 µg of DNA. The amounts of DNA obtained were in the range of 160 to 381 µg of total DNA per single stranded fragment.

These numbers were used to prepare equal molar amounts of each complementary fragment for subsequent hybridization reactions. The values obtained by the spectrophotometric readings are not an indication of the quality of the DNA, it was later determined that one of the fragments obtained was degraded. The degradation was most likely the result of nuclease activity. This

breakdown of DNA results in an erroneous spectrophotometric reading due to hyperchromicity. Therefore the quality of the DNA strands were determined by gel electrophoresis which is discussed in the next section.

5. Hybridization and electrophoresis:

This part of the procedure involved the hybridization of each of the two complementary pairs for the purification/recognition and T-7 promoter sequences. The hybridization temperature was set at 25° C below the T_m (temperature in which 50% of the strands are hybridized), which would allow for the maximum rate of association. The T_m was determined using the following equation:

$$T_m = 16.6 (\log [Na^+]) + 0.41 (\% GC) + 81.5 - 675/N$$

N= probe length

The hybridization temperature of the purification / recognition sequence was found to be 40.2° C and the T-7 promoter sequence was found to be 34.8° C.

Initially the ionic strength used in the hybridization reaction was defined by using a 0.18 M concentration of Na^+ (1X SSC). This concentration has been described in the literature as being the minimum concentration that should be utilized under standard conditions. The ionic strength is an important aspect of hybridization since the Na^+ concentration is necessary to neutralize interstrand electrostatic repulsive forces between the negatively charged phosphates, thereby stabilizing the double stranded molecule. The hybridization reaction was performed using 5 mg of each of the complementary fragments in a 100 µl reaction volume. Prior to incubation, at the appropriate hybridization temperatures, the mixtures were incubated at 65° C for 10 minutes in order to disrupt any random intrastrand hydrogen bonds or secondary structures which may exist and decrease the hybridization rate.

Initially, visualization of the single strand and hybridized DNA fragments were attempted by electrophoresis on a 3% agarose gel. The agarose gel was found to give inadequate resolution of small DNA fragments. A non-denaturing polyacrylamide gel was therefore used for separation and identification of the DNA molecules. Besides enabling a high degree of resolution upon visualization of the DNA, there are also several other benefits in using a polyacrylamide gel: 1) separation of DNA molecules whose lengths differ by as little as 0.2% (1 base in 500); 2) accommodation of much larger quantities of DNA than agarose gels; and 3) recovered DNA from a polyacrylamide gel is extremely pure upon comparison to agarose gels. The acrylamide to bisacrylamide ratio used was 29:1. The percent acrylamide used in casting the gels was 12%,

which has an effective separation range of 40 to 200 base pairs. The gels were run in tris-borate-EDTA (TBE) buffer and stained with ethidium bromide, and visualized with the aid of a UV light.

6. Ligation:

The next step in the construction of the DNA probe is the ligation of the two previously hybridized double stranded molecules. Once the two hybridized fragments are combined, the sticky ends of the two fragments hybridize and the single strand breaks must then be covalently sealed. The sealing of the breaks was performed with DNA ligase. The ligase used was T-4 ligase, which is derived from the T-4 bacteriophage. This enzyme catalyzes the formation of a phosphodiester bond between the 3' hydroxyl and 5' phosphate of two adjacent nucleotides, thereby establishing the structural continuity of the DNA strand. The ligation reaction is dependent on several parameters which include temperature, ionic concentration, the nature of the DNA ends (cohesive "sticky" or blunt) and the concentration of the DNA fragments. In the assembly of the probe for this project the two fragments to be ligated have cohesive complementary ends. The primary parameters that can effect the reaction for this study are the temperature and ionic concentration of the reaction mixture.

In our first attempted ligation the two hybridized fragments were first dialyzed against TE in order to remove any excess salts. Equal molar concentrations of the two fragments were then combined with the addition of T-4 ligase and buffer. Incubation was at 37° C for 2 hours. Polyacrylamide gel results did reveal one possible ligation. A successful ligation would is viewed as a band with a lesser degree of electrophoretic mobility than the two fragments which it is composed of. However, due to the degradation of one of the oligo bands, this ligation was thought to consist of a single strand ligated to a double stranded piece. A number of other ligations were attempted, each time varying the total reaction volume and keeping the incubation temperature and time constant, no ligation occurred in any of these attempts.

One of the potential causes for this was that the hybridized fragments also contained a considerable amount of extraneous DNA of a higher molecular weight than the hybridized fragments. This excess DNA was seen as a nonspecific trailing area when viewing the polyacrylamide gels. In order to eliminate this excess DNA, the bands which represented the hybridized fragments were cut from the gel and purified by using an electro-separation system. This electro-separation system allows for the isolation of DNA fragments from gels under the driving force of an electric field, the dsDNA migrates out of the gel and is trapped between a set of membranes. This system also provided for the removal of salts and other contaminating substances without denaturation or significant loss of sample material. Both of the hybridized

fragments were purified in this manner.

The electroeluted fragments were then prepared for ligation. Ligation was done at 37° C for 2 hours. Unfortunately, results of the ligation were not successful, as seen by gel electrophoresis.

After a number of unsuccessful ligations at 37° C, which is the temperature in which the ligase enzyme has optimal activity, it was determined that the T_m of the tetra nucleotide which was to be ligated was around 15° C. At a reaction temperature of 37° C the hydrogen bonding between the four complementary bases is not strong enough to stabilize and prevent separation of the fragments. In order to optimize ligation, the reaction should take place under conditions that allow the annealing of the termini. However the temperature optimum for DNA ligase activity is 37° C. Therefore, the optimum temperature for the ligation of cohesive termini is a compromise between the T_m of the cohesive ends and the temperature optimum for the DNA ligase. The reaction temperature that was next employed was 16° C. Ligations were incubated for a period of approximately 18 hours.

To alleviate the possibility of extraneous DNA from nuclease degradation interfering with the ligation, electroeluted fragments which had been previously concentrated by ethanol precipitation and further washed with 70% ethanol were used. The reaction was done at 16° C for 18 hrs. No ligation was apparent on the polyacrylamide gel. This was possibly due to the potential disruption of the sticky ends by the electroelution and precipitation procedures.

As an alternative approach, a ligation protocol was designed to minimize the number of DNA manipulations after the hybridization reaction, thereby maintaining its integrity for ligation. Two reactions, one utilizing 0.5X SSC and the second utilizing 0.1X SSC were prepared. Post hybridization, the sample was immediately ligated without any further manipulations, such as dialysis, electroelution or ethanol precipitation. The ligation was performed at 16° C for 18 hours. Results from both reactions produced a band in gel electrophoresis that was less mobile than either of the two individual hybridized fragments. It is thought that this band could potentially be the product of the two ligated fragments, however, further analysis and characterization is now being done to confirm this.

7. Ligation control:

To ensure that the difficulty in getting the DNA to ligate was not due to the ligase enzyme, a ligation control was set up. Lambda DNA Eco R1, Hind III Digest was used as the control DNA. This DNA consists of 13 fragments of various molecular weights, each fragment contains sticky ends. These sticky ends when incubated in the presence of ligase will recombine and yield higher

molecular weight fragments if the ligase is active. Results had shown that the enzyme efficiently ligated the fragments of the control DNA.

For the bacterial assay, initial work focused on optimizing the PCR assay for pure cultures of *V. vulnificus*. Different cell lysis methods were tested before it was determined that direct amplification of non-lysed cultures allowed for sensitive detection of the target DNA. Because the limiting step for improving the sensitivity of this assay is the ability to detect target DNA in shellfish tissue, a variety of different DNA extraction and separation procedures were tested.

B. Bacterial assay

The overall approach for developing an assay for the detection of *V. vulnificus* in shellfish involved use of emerging PCR technology to develop a rapid, sensitive assay that did not include any culture steps. The DNA from *V. vulnificus* was hybridized to primers for the cytotoxin/hemolysin gene and the PCR was run to amplify the target gene sequence. Work with pure cultures of *V. vulnificus* was necessary to establish optimal PCR conditions, screen environmental isolates, to test different primers, and as a control to assure that steps in the eventual protocol for detection in shellfish tissue were not limited by factors associated with the bacterial cells by themselves. Details of this approach are described below.

1. Cell lysis and DNA extraction from pure cultures:

Cultures of the ATCC type strain of *Vibrio vulnificus* were grown on LB medium and three different textbook techniques for lysing cells and purifying DNA were tested. Modifications based on our findings were incorporated into a modified lysis/DNA extraction procedure that incorporated different aspects of the three procedures. Centrifuged cells were suspended in SET buffer and lysozyme and RNase A were added. The pellets were then extracted with phenol/chloroform/isoamyl alcohol prior to ethanol precipitation.

A new cell lysis procedure for liberating DNA that is more rapid and easy to perform than the first procedure was tested. The ATCC type strain of *V. vulnificus* was then grown in five different media to determine if culture conditions had any effect on the efficiency of the new cell lysis procedure. *V. vulnificus* was grown in five different commonly used media: brain heart infusion broth, alkaline peptone water, buffered peptone water, 1% peptone/1% salt, and autoclaved estuarine water. 100 µl samples were collected, centrifuged, and the pellet was lysed using the rapid procedure. This involved addition of 1 N KOH to pelleted cells, incubation at 70°C for 20 minutes, neutralization of the base with 1 N HCl, and addition of 1 M Tris HCl at pH = 7.6. Lysis was confirmed microscopically. The liberated DNA was then amplified using the PCR and

the same DNA band that was amplified using the first lysis method was amplified in these samples, indicating that the new, more rapid cell lysis procedure worked with *V. vulnificus* cultures grown in five different media.

Both cell lysis procedures were repeated and the DNA from ATCC cultures were quantified and checked for purity. Both methods proved to yield similar quantities of DNA of acceptable purity. We finally tested the efficacy of adding *V. vulnificus* cultures directly to the PCR tube and found this most rapid and easy treatment of the culture to give acceptable PCR results.

2. PCR conditions:

The DNA from the ATCC type strain of *V. vulnificus* was hybridized to the primers for the cytotoxin/hemolysin gene and the polymerase chain reaction (PCR) was run under one set of time and temperature conditions in a thermal cycling machine for amplification of the target gene sequence.

The primers were the same as used by Dr. W. Hill of the FDA in Wash., DC. The primers used for amplifying a 519 base pair sequence of target DNA had the following sequences:

sense: 5' CCG GCG GTA CAG GTT GGC GC 3' (20 bp)

antisense: 5' CGC CAC CCA CTT TCG GGC C 3' (19 bp)

These primers were named AM 1 (20 bp sense primer) and AM 2 (19 bp antisense primer). The initial PCR conditions were as follows:

1) denaturation- 94°C for 1.75 minutes;
2) reannealing- 67°C for 2 minutes;
3) elongation- 72°C for 2 minutes.

Concentrations of PCR reagents were as recommended by Perkin Elmer Cetus for their reagents. After running the amplified DNA from 1 µl of a sample that contained approximately 10^9 cfu/ml on an agarose gel, we found one clear band of amplified DNA. Attempts made to increase the sensitivity of the assay by varying some of the PCR reagent concentrations showed that recommended concentrations were the best of the range of concentrations of primers, template DNA, polymerase, and $MgCl_2$. Increasing the cycles of amplification from 25 to 35 did not improve the detection of target DNA bands/further amplify the DNA.

We tested the sensitivity of these conditions by diluting DNA isolated from *V. vulnificus* (12.5 µg DNA/ml) and found that the limit of detection was 1:1000 of what we started with. The amplified band in a 0.8% agarose gel migrated after ~45 minutes was the same distance as the 500 bp DNA fragment amplified in the Hind3 cut lambda DNA control lane. It also migrated the same distance as a control primer probe of a 500 bp DNA fragment supplied by a Dupont PCR kit.

However, we felt that the sensitivity could be improved. The lowest concentration of bacteria that could be detected visually on the gels corresponded to 11 to 500 cells in the PCR tube.

We then pursued our observations that low MW DNA, smaller than the target sequence, was being amplified. We ran blanks without template DNA and still observed low MW bands. Blanks were run that included exclusion of template and primer DNA and polymerase in different combinations. The results showed that the primers were needed to see the extra bands. For better resolution of the low MW bands we ran the electrophoresis using 2.0% agarose. A sample containing primers without polymerase gave one low MW band, but running primers with the polymerase gave two extra bands. These results suggest that the primers had characteristics (homology at 3' ends, etc.) that enhanced formation of primer-primer dimers and possibly trimers.

We compared the sequence of the two primers we were using to the published sequence and found that the sense primer (AM 1) that contained 20 bp had an extra guanosine near the 5' end. We then had new primers made that would include a sense primer without the extra G and with the last 5 bases on the 3' end removed (AM 12), and another sense primer (AM 13) with the sequence shifted 5 base pairs past the 5' end, thus cutting off the last 5 base pairs on the 3' end and adding the previous 5 base pairs on the 5' end. The new sequences are as follows:

AM 12: 5'-CCG CGG TAC AGG TT-3'

AM 13: 5'-ACG TAC CGC GGT ACA GGT T-3'

We also ordered more of the original sense (AM 1) and antisense (AM 2) primers. The concentrations of the primers received were (in μg per ml): 270, 250, 270, and 200 for AM 1, AM 2, AM 12, and AM 13, respectively.

We compared the different sense primers with a relatively high concentration of bacteria (1:10 dilution of overnight culture) and 1.5 mM $MgCl_2$. The results suggested that the original primer, AM1, gave the best amplification, and that the other primers were not an improvement. However, it was clear that the new primers, and especially AM 12, gave significantly less amplification of low MW DNA, suggesting that the homology between the 3' ends of AM 1 & 2 may have led to dimer and trimer formation and amplification. Using higher $MgCl_2$ concentrations (1.5 to 4 mM) showed AM 12 and 13 to give better amplification with increasing $MgCl_2$ concentration than AM 1. Amplification was poor or undetectable with AM 12 and AM 13 at 1.5 mM $MgCl_2$. We tried lowering the reannealing temperature in the PCR cycle from 67-69°C to 55°C. The higher temperature was still superior, especially at lower concentrations of bacteria. The different primers were then compared using 6 mM $MgCl_2$ and purified *V. vulnificus* DNA. AM 12 was most sensitive, showing a detectable band at a 10^{-5} dilution, and gave the cleanest gel.

This was even more apparent when PCR was run for 15 extra (50 total) cycles.

3. Assay sensitivity:

We determined the concentration of cells where we no longer could detect the bacterial DNA. *V. vulnificus* was grown overnight in 1% peptone/1% salt and a 100 µl sample was lysed using the rapid procedure. A series of ten decimal dilutions of the lysed DNA was made and the DNA in the dilutions subjected to the PCR using AM 1 and AM 2 primers. The characteristic band of amplified DNA was visible on an agarose gel at dilutions down to and including the 10^{-7} dilution of the culture. This dilution contained DNA from the equivalent of approximately 1.1 x 10^2 cfu, and only 1/10th of the sample was actually amplified. Thus, successful amplification of DNA from approximately 11 cells was apparent. Further tests using the initial PCR conditions and primers showed the sensitivity of the assay to be 11 to 500 cells in the PCR tube.

The sensitivity of the three sense primers with pure bacterial cultures was tested. AM 12 gave the best sensitivity and the cleanest gel. The original culture contained 7.1 x 10^8 total cells per ml, and a band was visible in the 10^{-6} dilution. This means that a culture containing 710 cells per ml was detectable with our method. Addition of 2.5 µl of the culture meant that only 1.8 cells were added to the PCR tube.

4. Separation of bacterial cells/target DNA from shellfish tissue:

The effect of oyster tissue and/or juice on amplification was tested. Tissue samples were centrifuged in a microfuge for 15 minutes to remove solids and the supernatant was collected as oyster juice. Juice and total tissue samples were diluted from 10^{-1} to 10^{-7}, and the same amount of bacteria was added to each tube. Amplified DNA bands were only visible in samples where either juice or tissue were diluted 10^{-3} or more. Boiling to inhibit enzymatic activity or increasing the $MgCl_2$ concentration to 2.25 mM had no effect on the inhibitory effect of the oyster tissue.

We then tested the effects of different $MgCl_2$, KCl, and Tris buffer concentrations on amplification in the presence of oyster tissue. AM 12 was more sensitive than AM 1 and AM 13, and increasing the Tris concentration/decreasing the KCl concentration gave poorer detection.

The easiest PCR assay would be one where a small volume of oyster tissue could be added directly to reaction mixture for PCR. However, oyster tissue apparently inhibits the activity of the polymerase. Thus, either the bacteria need to be separated from the tissue or the total DNA needs to be isolated from the rest of the tissue.

Oyster tissue was seeded with *V. vulnificus* cells, different separation methods were tested, the bacterial fraction was processed and PCR was run, and bacterial cells in different fractions

were usually plated onto agar media and counted to determine the efficiency of the different separation methods. The problems with these experiments were that the bacteria stick to the oyster tissue and cannot be separated from the tissue, or tissue remained associated with bacteria separated from the major portion of the tissue and caused inhibition of the PCR assay. The sticking of bacteria to particles was confirmed using fluorescence microscopy.

We first tried to isolate bacteria from tissue by washing the seeded tissue with saline and various other solutions. Low plate counts were observed in the recovered bacterial fraction, sometimes even lower numbers than in the unseeded tissue. This illustrates one problem in relying on selective and non-selective agar media and plate counts for determining recoveries. We tried using seeded, autoclaved tissue, and recovered 1/10 of the bacteria (cfu) used for seeding. Another experiment where we seeded with different concentrations of bacteria also gave low recoveries.

Another protocol recommended for use with PCR of blood cells which also contain inhibitory substances was tested. A lysing buffer was added that is supposed to lyse cells but not nuclei, which would have hopefully minimized collection of oyster DNA. After several washings and treatment with Protease K, the suspension was tested in the PCR assay. Several different concentrations of seeded bacteria gave no amplification. The tissue was then diluted 2:3 in peptone water, but this also gave no amplification. Suspecting that the oyster cells did not lyse, the assay was modified to include freezing the suspension at -70 °C and thawing at 37°C every time the lysis buffer was added. This also gave no amplification, and it was repeated again without success.

We next tried an assay used for separating bacterial cells from soil. This protocol involved mixing the sample with polyvinylpolypyrrolidone (PVPP) and washing with sodium hexametaphosphate. This was tested twice. Neither of the seeded samples gave positive results, but neither did extracts seeded with bacteria after the isolation protocol was run. This suggests that the sample suspension still contained inhibitory substances from the oyster tissue. Continuation of this protocol would involve identifying and separating the inhibitory substance from tissue or further purification of the bacterial DNA. One method that could be used if specific antibodies were available for *V. vulnificus* involves the use of antibody-coated magnetic beads. Another possibility is to run samples on Percoll gradients.

Direct extraction of DNA was tested using an assay developed for extracting and purifying DNA from sediments. Both ethanol and PEG precipitation options were tested. The result was a turbid solution, indicative of impure DNA. No amplification of DNA from these samples was apparent from gels. We checked the solution for toxicity to the PCR by adding fresh bacteria, and observed an amplified band on the gels.

Another assay developed for isolating bacteria from mouse brains was tested. Seeded tissue was suspended in lysing buffer (dilution = 1:10) and boiled, and samples of the resulting supernatant were run directly for PCR. This was not successful for us. Reseeding the supernatant gave amplified bands after diluting the supernatant 1:100. Increasing the $MgCl_2$ concentration increased sensitivity ten-fold. Repeating with the higher $MgCl_2$ concentration gave negative results when the supernatant was not reseeded.

In a new protocol, the tissue was then suspended in water before boiling, boiled in a microwave oven, washed several times with deionized water, and the pooled supernatant was precipitated with ethanol. The extracted DNA gave no positive results. Tissue samples were then autoclaved instead of boiling in the microwave oven, but the results were still negative. Another variation involved suspending the tissue in Tris-EDTA (TE) buffer, boiling in the microwave, treatment with Proteinase K, then precipitation. Samples for PCR were taken before and after Proteinase K treatment but gave no positive results. Dilution of the ethanol precipitated DNA by 1:10 and using primer AM 12 gave an amplified band. This was the first positive result.

Another protocol involving use of guanidine isothiocyanate (GITC) was then tested. Two volumes (0.1 and 1.0 ml) of tissue were extracted and the DNA precipitated with ethanol before running PCR. The 1.0 ml sample gave a smear of amplified DNA, while a clear band of amplified target DNA was observed for the 0.1 ml sample. This is the protocol that is currently being used in continuation studies on the assay in oyster tissue.

The maximum amount of shellfish tissue that can be processed using the GITC protocol was 100-300 mg of homogenate. Higher amounts gave too much interference for the PCR assay to be efficiently and routinely used for detection of *V. vulnificus*.

5. Screening of environmental isolates:

Initial PCR tests included using the assay to detect different environmental *V. vulnificus* strains isolated from the Great Bay Estuary during 1989-90. Two of the five isolates were hemolysin positive, like the ATCC type strain, and three of the isolates were hemolysin negative. The purpose of running the assay on these strains was three-fold: 1) we were interested in determining if the assay worked with environmental isolates; 2) we were interested in determining if strains that tested negative for hemolysin activity using conventional culture techniques would be missing or would show the presence of the hemolysin gene; 3) if hemolysin negative strains were missing the gene, then the detection method for *V. vulnificus* using the hemolysin gene would not be useful for detecting these strains. We found that the target DNA was clearly amplified for the two hemolysin positive environmental strains using 10^0 to 10^{-2} dilutions of cultures grown in 1%

peptone/1% salt broth to approximately 10^9 cfu/ml. For the three hemolysin negative strains, no apparent amplification of the target sequence was observed for one, the target band and a different band were apparent in another, and clear amplification of the target DNA was apparent for the third strain.

We found that even though *V. vulnificus* could not be cultured directly from Great Bay Estuary oysters after November, or when water temperatures declined to below 10°C, it could be cultured if oysters from previously contaminated areas were first subjected to warm (20°C) water for 48 hours. Follow-up experiments gave evidence that appearance of culturable *V. vulnificus* after a 48 hour resuscitation in warm water was probably not a result of a few cells growing to a larger, detectable population. Rather, it appears that viable, non-culturable cells in cold oysters became viable during the warming time.

One remaining concern with the assay as developed was that it was specific for *V. vulnificus* that had an intact hemolysin gene. This toxin has been both implicated as the virulence factor and refuted as such. Other factors may also be involved in disease incidence, and this needs to be determined with larger, epidemiological and clinical studies. Thus, it is unclear if *V. vulnificus* cells that are hemolysin negative are still virulent. We have isolated a number of hemolysin negative *V. vulnificus* strains from the Great Bay Estuary, as well as many other hemolysin positive strains. Of the 47 isolates tested by the PCR assay, 7 were hemolysin negative. All of the hemolysin positive and 5 of the 7 hemolysin negative isolates were detectable with the PCR assay. Thus, only 2 of 7 hemolysin negative strains were not detectable with the PCR assay. This could be significant if the nondetectable strains are still virulent. Further characterization of these hemolysin negative strains is now underway.

V. Results

A. Viral Assay

1. Hybridizations:

Initial results of the hybridization reaction had shown that one of the ssDNA fragments for the purification recognition sequence was degraded. Because of this, the strand had to be resynthesized before any further work could be performed . Once the new fragment was obtained the hybridizations were done as previously described. Results from the hybridizations show that the electrophoretic mobility of the hybridized strands is less than the mobility of either of the individual components that compromise the double stranded molecule. The decrease in mobility of

the band is a direct indication that hybridization had occurred. In addition, hybridization stringency conditions as described above, assured that strand hybridization was complete and not partial. The hybridized fragments were used in subsequent ligation reactions as discussed in the next section.

2. Ligation:

What had been initially characterized as being a ligated fragment during the last report, was determined to be an artifact. All subsequent attempts at duplicating the conditions that originally produced the supposedly ligated band could not be reproduced. Further attempts at ligation by: 1) reducing the Na^+ ion concentration during hybridization 2) adding additional magnesium chloride to the ligation reaction, thinking that the EDTA in the buffer that the DNA was suspended in, was chelating the magnesium chloride in the ligase reaction buffer. The magnesium chloride is necessary for the ligation reaction to occur. 3) It was possible that the cohesive ends after hybridization were blocked, due to small contaminating oligonucleotides. If this was the case, then the ligation would be inhibited by a lack of cohesive termini. Because this specially constructed cohesive area is also recognized by a restriction endonuclease (Sal 1), an attempt was made to do a restriction digest after the hybridization reaction. The restriction digest would cut the hybridized DNA at the cohesive site, removing anything that may have non-specifically hybridized to it, producing the ligatable cohesive ends.

All of these attempts to ligate the synthetic DNA were found to be unsuccessful. Controls had been utilized indicating that the activity of the ligase was adequate to ligate Hind III digested DNA. Since an exhaustive number of reaction conditions were utilized in the attempts to ligate the probe fragments, other areas were then looked at to determine what could be going wrong. One of the possibilities was that: 1) either part or all of the cohesive termini on one or both of the fragments was missing. 2) there was one or more misincorporated bases in the cohesive ends preventing annealing and subsequent ligation. 3) since synthetic pieces of DNA are most routinely used as promoters or probes directly and not in constructing larger molecules of DNA, the way in which they are synthesized may prevent ligation from occurring. Initially a more in-depth look into the way the DNA was synthesized was undertaken. Through a literature search, it was discovered that during synthesis of the DNA the starting nucleotide does not have a 5' phosphate on the ribose molecule, but only a 3' hydroxyl group which is necessary for any subsequent elongation. The 5' phosphate is necessary for the formation of the 3' to 5' phosphodiester bond. The phosphodiester bond involves the linking of the 3 hydroxyl with the 5' phosphate of adjacent nucleotides. The phosphodiester bond is the way in which adjacent bases are covalently linked to one another, without the formation of this bond adjacent bases or segments of nucleic acid cannot

be joined.

It was because of the missing 5' phosphate that any prior attempts at ligation were unsuccessful. Without the 5' phosphate the complementary cohesive termini on each of the two fragment could hybridize but the two molecules could not be covalently linked by the T-4 ligase. Because the hydrogen bonding strength between the tetrad of the cohesive termini was weak, the molecules would readily disassociate into the original two individual fragments. This discovery explains why the control Lambda DNA fragments which were generated from a restriction digest of the Lambda DNA, yielded ligatable fragments with a 5' phosphate and 3' hydroxyl and the synthesized DNA could not be ligated.

In order to ligate the synthetic fragments the 5' ends must first be modified by adding a 5' phosphate group. The addition of the phosphate to the 5' nucleotide is accomplished by an enzymatic reaction, the enzyme used is T-4 polynucleotide kinase. T-4 kinase exhibits a 5' phosphorylating and a 3' phosphatase activity. The enzyme catalyzes the transfer of phosphate from ATP to a terminal 5' nucleotide.

As illustrated, the probe consists of four individual fragments, each of which lacks a 5' phosphate. Two approaches were evaluated prior to phosphorylating the fragments: 1) All four fragments could be phosphorylated or 2) only the two terminal 5' nucleotides that make up the cohesive termini of the ligation region can be phosphorylated. The approach taken was to only phosphorylate the 5' nucleotides in the Sal I cohesive region. By not phosphorylating the 5' of the other two fragments, the degree of non specific ligations could be minimized.

Assembly of the probe was now attempted by: 1) phosphorylation the two fragments which have 5' termini in the ligatable region according to conditions stated by the manufacturer. 2) following phosphorylation the DNA was ethanol precipitated to concentrate and to remove the kinase buffer. 3) complementary fragments to each of the phosphorylated fragments were then added and the DNA was allowed to hybridize at 37° C for 2 hours. 4) ligase buffer and T-4 ligase were then added and the reaction was incubated at 16° C overnight. Polyacrylamide gel electrophoresis on the ligated DNA along with non-ligated but hybridized fragments was carried out. Results had shown that there were two bands present with an electrophoretic mobility less than either of the two individual hybridized fragments. What was also seen was a decrease in the intensity of the two individual phosphorylated double stranded
fragments which indicated that the bands are a result of the two individual fragments ligating and not merely an artifact. This procedure was then repeated and the same results had been produced in all subsequent reactions.

Now that all the necessary factors for ligation appear to have been resolved, the next step is

the identification of the ligated bands. The way in which the probe was constructed, only one band would be expected from a ligation reaction of the two individual fragments. Results of the gel had shown two distinct bands, there are a number of possible factors that may have resulted in this: 1) there may have been some kinase activity occurring during the overnight ligation reaction. The additional phosphorylation of the ligated blunt end 5' termini and the 5' cohesive Pst 1 termini may result in the two bands. The reasoning behind this theory is that the electrophoretic mobility of DNA with a 5' phosphate is greater than that of DNA without a 5' phosphate. What could have happened is some of the ligated DNA was phosphorylated and some was not, generating two bands on the gel. 2) since the T-4 ligase also has a blunt end ligation function it is possible that there is some blunt end ligation occurring thereby producing ligated fragments of different molecular weights. 3) if one of the original phosphorylated fragments was in excess so that some remained single stranded after hybridization the single stranded species could ligate to the cohesive end of other double stranded fragment. In this case two species could be generated, a fully double stranded molecule and a partially single stranded and double stranded molecule.

Some work has been started in determining the reason why two ligated bands were being generated. Phosphorylated and non-phosphorylated single and double stranded fragments of the same type were run side by side on a polyacrylamide gel. Results had shown that the fragments had a slight to no detectable change in mobility. Due to the degree of distance between the two ligated bands it does not appear the two bands are a result of one containing phosphorylated termini and the other non-phosphorylated.

Currently we are looking into whether one of the bands is a result of a partially double stranded molecule. Each of the individual fragments were first run on a polyacrylamide gel in concentrations based on previously obtained spectrophotometric readings. The gel results had shown that one of the fragments was in significant excess when compared to the other three. The fragment in excess was one which was phosphorylated prior to ligation. There is a good likelihood that this fragment, being in excess, exists as both a single stranded and double stranded species in the ligation reaction. Both the single and double stranded DNA would be in competition with the other double stranded fragment that it is ligated to, consequently generating species of different electrophoretic mobilities. To alleviate this problem, the concentration of the excess fragment must be reduced, prior to duplication of the reaction conditions, to determine if only one band would then be produced in the ligation reaction.

If all of the above do not answer the question as to why the two bands are being generated, a more detailed approach will have to be undertaken. One approach would be to individually isolate the two fragments and sequence them. Sequencing would be the most definitive way of

determining the actual base composition of the two bands.

B. Bacterial Assay

A number of important findings resulted from this project. We showed that the PCR assay could be successful in amplifying target *V. vulnificus* DNA from pure cultures and in shellfish tissue without prior cultivation of the cells. Pure cultures could be added directly from a variety of nutrient media and estuarine water to PCR tubes without prior lysis, while shellfish tissue, even in supernatants of centrifuged homogenates, inhibited amplification of the target DNA. This interference by the shellfish tissue remains the main limitation for improving the sensitivity of the PCR assay.

The best sensitivity of the assay for pure cultures was approximately 2 cells in a PCR tube. This is just about as low as we can go, and we must now work to better concentrate shellfish tissue, probably using the GITC procedure, so that extractions of larger samples can be added to PCR tubes. None of the procedures for first separating bacterial cells from tissue appeared to be successful. Theoretically, this would be a useful step, and warrants further study as more is known about the attachment of bacteria to shellfish tissue.

A number of problems with primers were apparent. First, the sense primer used first was an incorrect sequence. Subsequent tests with alternative primers showed another sequence for the sense primer gave better results. We also suspect that amplification of low molecular weight DNA using the first sense primer resulted from formation of primer-primer dimers and trimers, because of homology at the 3' ends.

The assay was tested against *V. vulnificus* strains isolated from the Great Bay Estuary. We found that all isolates that were hemolysin positive were also successfully detected using the developed PCR assay. However, only 5 of 7 hemolysin negative strains were detectable. The inability to detect these strains is not surprising, and probably depends on the genetic basis of the hemolysin negative phenotype. These results raise a serious question about the virulence of hemolysin negative strains, and whether the developed assay can detect all virulent *V. vulnificus* cells.

VI. Evaluation

Protection of public health and adequate safety of the food supply is a concern of the Federal Government. New detection methods developed will provide the shellfish consumer with

added protection from pathogenic bacteria and viruses and should therefore be funded by the Federal Government.

The Federal and State Governments are responsible for monitoring commercial molluscan shellfish beds through the ISSC by means of the National Shellfish Sanitation Program. The potential product resulting from this work may assist government personnel to better identify beds which pose threats of virus contamination to shellfish, but remain open due to the inability of the indicator (coliform) organism to accurately reflect the presence of viruses, and conversely to open beds which may have marginal indicator levels but no detectible level of viral contamination.

The development of a new PCR method based on this work will allow for a better understanding of the colonization, seasonal disappearance, and potential removal of this pathogen from shellfish. This type of information is critical to the shellfishing industry at this time because of the adverse public perception associated with the consumption of raw shellfish. Using the fully developed assay, shellfish markets can screen shellfish to assess the potential public health threat of each lot. The absence of *V. vulnificus* and enteric viruses in shellfish, either because of growth in uncontaminated waters or because of removal by some future processing strategy will allow the shellfishing industry to assure the public of the sanitary safety of their food product.

The shellfishing industry has benefitted from the results on both assays because the basic research required before routine assays are developed has progressed to a great extent through this project. Much progress has been made, and many critical new questions have been raised. Eventual development of a rapid detection method for bacteria and viruses will benefit the entire shellfishing industry. Furthermore, once the technique is proven to work with seafood, the methodology could be modified to make it useful for all appropriate food-producing industries. Since the project will potentially benefit all seafood industries, support of this research should not be the responsibility of one individual, company, or sector of the seafood industry.

VII. Conclusion:

The work described in this report represents the initial stages of developing an assay for the rapid detection of low levels of virus in shellfish. Development of the capture probe was successful and rapid since it was simply the 1100 and 1600 base pair segments of the viral cDNA that were denatured and kept single stranded. Future production of the capture probe would be easier and faster by use of the polymerase chain reaction, and to that the correct primers were produced.

Development of the second probe was much more difficult and there were several

unexpected problems which were encountered through the course of developing the assay, they have been described in this report, along with the methods for solving these problems. To complete the development of this assay there are still several areas of research which must be completed. Once the probe has been ligated, it would prudent to sequence it. This will help determine if the required sequence are present for the production of RNA transcripts. In addition, the probe needs to be passed through a suitable host, such as E. coli, to ensure correct spacial orientation of the constructed strands of DNA. Once this has been accomplished, and there has been successful production of RNA from the T-7 promoter, the probe must be evaluated when used in conjunction with the capture probe. Due to stearic hinderance, there may not be sufficient room for the T-7 enzyme to effectively bind to the DNA. If this happens, the probe must be modified to allow ample room for the enzyme. This can be accomplished by providing a spacer sequence after the viral recognition sequence.

Once the probe has been shown to work when used with the capture probe, the assay needs to be optimized, i.e., correct amount of T-7 polymerase, maximum time for production of RNA transcripts, minimum molarity of dNTP precursors, etc. The final step of assay development will be to test the probe with seeded shellfish eluent and homogenate, to determine how the probe will operate under these conditions. Completion of this part of the assay will allow field testing of the probe with shellfish from closed, restricted and open waters.

This project has provided a basic assay that allows for rapid and easy detection of *V. vulnificus* from a variety of media without any cultivation. As is the case with all applications of molecular detection techniques to natural environments, work is still needed to develop the best method for making low levels of target DNA available for amplification. Further modifications should soon provide the final basis for development of routine assays of not only *V. vulnificus* and enteric viruses, but a battery of all important, specific microbial pathogens. Further food safety and ecological work is also needed to give a better understanding of the extent of shellfish sanitation problems and to suggest possible strategies to overcome these problems.