Investigation into Rising *Vibrio* Populations in Eastern Coast Oysters

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Abstract

Vibrio parahaemolyticus (*Vp*) is a gram-negative, rod-shaped, and halophilic bacteria that is the leading cause of seafood-borne bacterial gastroenteritis. The gastroenteritis is caused when *Vp* is consumed with raw oysters and various other forms of seafood. The gastroenteritis is mainly characterized by watery diarrhea along with nausea, vomiting, abdominal cramps, chills, and fever.[1] *Vibrio vulnificus* (*Vv*) causes the same gastroenteritis, but can infect the bloodstream of immunocompromised people causing fever, chills, lowered blood pressure, and skin lesions.[2] Fifty percent of people infected with *Vv* succumb to the infection.[2]

Introduction

Currently, the oysters are harvested and put through a process called depuration to cleanse them of fecal bacteria (coliforms). In this process, oysters or other shellfish are put in a tank with filtered and sterilized seawater under conditions that better facilitate the filter feeding of the oysters. This causes the expulsion of the contents from the oysters. It also enhances the separation of these contaminants from the shellfish and prevents recontamination. The depuration cleans the oysters of fecal bacteria like *E. coli*, but is less effective at removing other pathogens and viruses in its current design. It is also inconsistent or inefficient at removing biotoxins, and water native bacteria like *Vibrio*.[3] A study by the Aquaculture Research Station at Louisiana State University Agricultural Center, while inconclusive, showed evidence that cooling oysters on dry ice has an effect on *Vibrio* populations, however, icing is not a common practice.[4] In addition, icing the oysters has a high chance of killing them depending their age and size.

Connecticut has seen an increase in *Vibrio* infections over the last two oyster harvesting seasons. As water temperatures increase *Vibrio* bacteria populations expand but the time and rate of expansion is not known. Late May is the best time to perform this kind of experiment because it will give the best *Vibrio* population data. In addition to this, it's also the time when oyster reproduction is at its highest.5 This means that there could be a rise in foodborne *Vibrio* infections during the summer (as seen in the past two years). It's estimated that there are about 4500 cases of *Vp* and many of those not reported.[1] It's also reported that there 95 cases of *Vv* per year only half of which are culture confirmed.[2] In addition to this, evidence shows that these numbers are rising.[6-7]

From 1996 to 2010, *Vibrio* infections reported to COVIS and FoodNet were recorded. According to COVIS, the number of infections annually per 100,000 people increased from 0.09 to 0.28. The number of annual infections per 100,000 people according to FoodNet increased 0.15 to 0.42.[6]

Testing for presence of microorganisms in food is usually carried out by growing the bacteria on artificial media. This can be time consuming and unreliable. Recently, there have been attempts to confirm the presence of *Vibrio* species using Polymerase Chain-Reaction procedures. There have been published articles that suggest using the *toxR* gene to select for *Vibrio vulnificus* and the *vpm* gene to select for *Vibrio parahaemolyticus*.[10,11] The use of PCR instead of culturing allows for a faster determination of the presence of *Vibrio* and eliminates a large margin of error that results from culturing.

Materials and Methods

Oyster Collection:

16-18 oysters were collected weekly from June 6th to July 31st at oyster beds at the Sound School in New Haven, Connecticut. These oysters were scrubbed with a scrub brush to prevent interior contamination with shell-based organisms. Samples were collected and processed within an hour of collection.

Oyster Processing:

A Waring commercial blender (Model: 51BL32) was placed on a balance and its mass was taken. The oysters were then shucked and their tissue was placed in the blender to create a pooled sample. The blender was massed once more with the tissue inside and the difference between the two measurements was used to determine the mass of the oysters. 100mL of 18ppt artificial seawater was added into the blender and the tissue was blended at full speed for 1 minute. The total volume of the pooled sample was taken by pouring it from the blender into a graduated cylinder.

Vibrio Testing:

50uL aliquots of tissue were taken from the blended sample and placed onto plates containing Chromagar *Vibrio* media. The plates were then placed in a 37°C incubator for 24-48 hours. The plates were then removed from the incubator and the number of colony forming units (CFUs) were counted. This count was used to find the number of CFUs per 50uL. Plates containing more than 300 CFUs were to confluent to count. The presence of *Vibrio* was confirmed using PCR.

DNA Extraction:

Boiling Method – Isolated colonies of *Vibrio parahaemolyticus* and *Vibrio vulnificus* were taken from the plates and placed in separate 1.5mL centrifuge tubes

containing 40uL of sterile water. The tubes were then placed in a centrifuge and spun at 13,200 rpm for 5 minutes in order to pellet the bacteria. The supernatant was discarded and 200uL of sterile water was added to each tube. The tubes were then placed in a 100°C water bath for 10 minutes. The tubes were removed and centrifuged at 13,200 rpm for 2 minutes. The supernatant was used for PCR.

Chelex Method – Isolated colonies of V*ibrio parahaemolyticus* and *Vibrio vulnificus* were taken from the plates and added to separate 1.5mL centrifuge tubes containing 200uL of 10% chelex solution. These tubes were boiled for 10 minutes. The tubes were then centrifuged at 13,200 rpm for 5 minutes. The tubes were removed and the supernatant from each was transferred to separate 1.5mL centrifuge tubes.

PCR Confirmation:

Tox-130 and Tox-200 primers were used to confirm presence of Vv and VPM-1 and VPM-2 primers were used to confirm presence of Vp.[9, 10] PCR reactions were loaded into Ready-To-Go PCR tubes with 3uL of DNA, 1uL of each primer (forward and reverse), and 21uL of distilled water. The PCR tubes were loaded with each DNA sample being loaded as according the above table. The tubes were then briefly centrifuged. They were then placed in the thermocycler with the following program:
Initial incubation: 94°C for 5 minutes, 94°C for 1 minute, 53.4°C for 1 minute, 72°C for 1 minute, 35 cycles and final anneal of 72°C for 5 minutes. The PCR products were held at 4°C. 3uL of loading dye was mixed with 7uL of each PCR product. 1.5% agarose gel was prepared and each mixture was loaded into a well. Electrophoresis was run at 100V for 30-45 minutes.

Results and Discussion

Oyster Collection

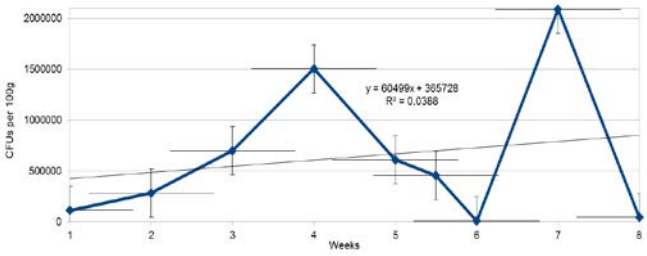

Figure 1. Number of *Vibrio vulnificus* CFUs counted per 50uL of hemolymph over 8 weeks.

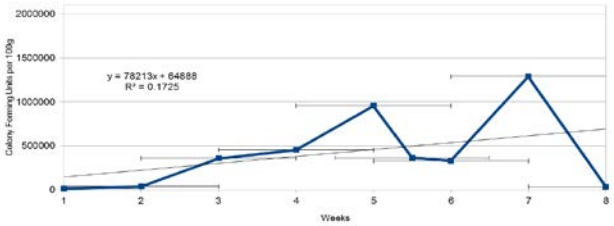

Figure 2. Number of *Vibrio parahaemolyticus* CFUs counted per 50uL of hemolymph over 8 weeks

Our results show an overall increase in *Vibrio* CFUs over the course of the summer. Our first four collections show a constant increase in number of *Vv* CFUs until our fifth collection. The fifth collection was performed after Hurricane Arthur passed through the Connecticut area around the 4th of July. The hurricane contributed to the drop in *Vv* numbers by decreasing the salinity of the water and increasing the amount of sediment and runoff from the mainland. A second collection of eight oysters (Collection 5.5) was performed during the same week. The number of CFUs from the second collection dropped slightly from the previous (Collection 5) confirming the large drop in *Vv* CFUs from week 4 to week 5. The numbers continued to drop for the sixth collection, but increased again to a much higher number for collection 7. The weekend after collection 7, heavy rain came through the area and lowered the *Vibrio* numbers for collection 8.

The trend displayed by the *Vp* CFUs mimicked the trend set by the *Vv*, except for the fifth collection which was performed after Hurricane Arthur. The *Vp* numbers continued to climb while the *Vv* numbers dropped dramatically. The confirmatory collection showed *Vp* numbers that were more on par with the trend set by the *Vv* numbers. The rise in numbers may have arisen from environmental factors such as lowered salinity from the passing storm allowing for the drop in *Vv* and proliferation of the *Vp*. The high number could have also arisen from a human error.

PCR Confirmation:

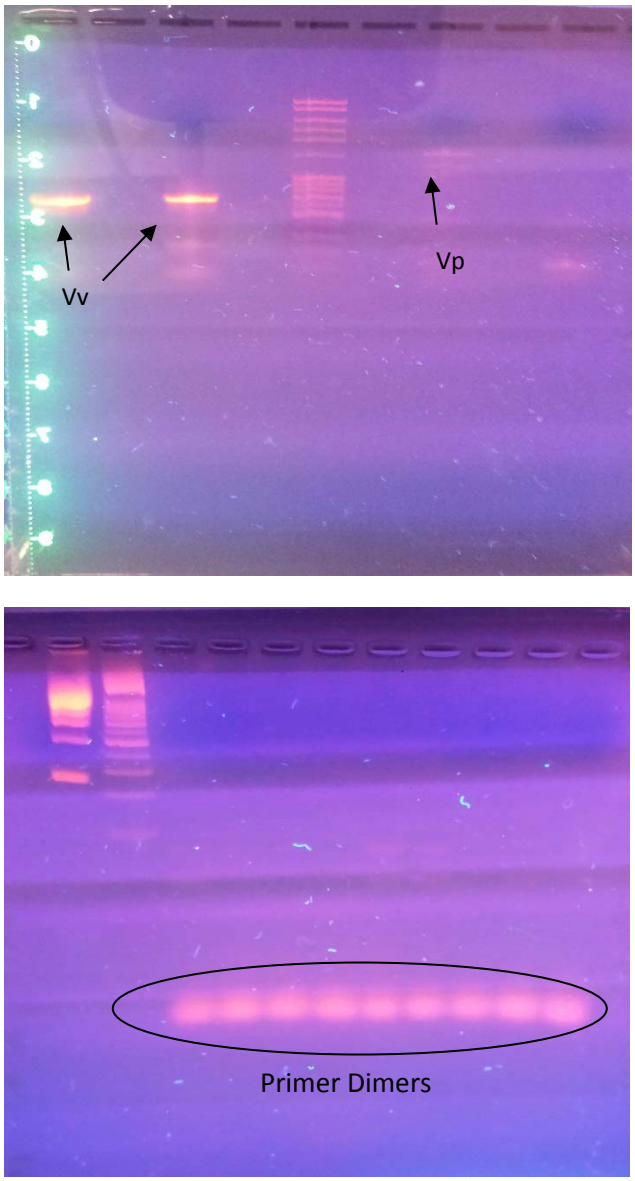

Figure 3. 1.5% agarose gel showing positive results for presence of *Vv* and *Vp*.

The initial Polymerase Chain Reaction confirmed for presence of *Vv* and *Vp*. Subsequent runs of the reaction also confirmed presence of *Vibrio*, but were marred by the presence of primer dimers. Optimization of the primers is required in order to increase the signal from the *Vibrio* bacteria.

Conclusion

The study found an increasing trend of both *Vibrio parahaemolyticus* CFUs and *Vibrio vulnificus* CFUs. This shows that the risk of infection with *Vibrio* bacteria via raw oysters rises during the summer months. In addition, heavy rainfall seems to have an effect on *Vibrio* populations. After Hurricane Arthur passed, the CFUs for *Vibrio vulnificus* dropped sharply. This occurred again after heavy rainfall during the eighth week of the study. Given these results, routine surveillance of oyster beds should be instituted. In addition, a more thorough means of cleaning the oysters post-harvest should be applied as the current process, depuration, is inefficient with removing *Vibrio* bacteria.

The PCR confirmed the presence of *Vibrio vulnificus* and *Vibrio parahaemolyticus*, however, some reactions were marred by the presence of primer dimers. These occur when the primers used in the PCR stick to each other rather than to the template DNA. The primers need to be better optimized for efficient use in confirming *Vibrio* species.

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Biography

Eric Grigg is a senior Biology major with concentrations in General Biology and Pre-Med. He is from St. Croix in the Virgin Islands. He wants to graduate and move on to a dual M.D.-Ph.D. program with specializations in oncology and microbiology. He wants to study the Ebola virus and study how bacteria can affect cancer growth.