Tracking the Presence of *Vibrio* Pathogens in Louisiana Seafood

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Abstract

In Louisiana, the sale and consumption of seafood is a critical economic industry for the state, generating 2.4 billion dollars for Louisiana. As one of the top shippers of domestic seafood, monitoring the seafood is important for consumer safety. One of the main dangers to consumer health is bacteria in the *Vibrio* family. Two disease causing members of the family are *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Combined, these two are responsible for 500 hospitalizations and 100 deaths per year. Over the years, the amount of *Vibrio* present in seafood across the world has been studied in depth. The ecology of environments where it is found and the cultures' susceptibility to antibiotics are just two of the many examples of studies done on different strains of *Vibrio* found on local seafood. Working with the John Folse Culinary Institute, the primary objective of this study is to isolate colonies of *V. parahaemolyticus* and *V. vulnificus* from the seafood the Louisiana Seafood class uses, and to test for antibiotic resistance and pathogenicity. To test for antibiotic resistance, Kirby-Bauer Assays will be used and to test for antibiotic resistance, PCR and gel electrophoresis will be used to test for the pathogenicity. A secondary objective is to quantify the amount of *vibrio* present on the surface of seafood and to compare the known values of *vibrio* present in the environment and in the seafood itself. The results will be used to educate the general public of South Louisiana on the risks of *Vibrio* infections from seafood.

Introduction

In Louisiana, one out of every 70 jobs is related to the seafood industry and the seafood industry also has an annual economic impact of $2.4 billion. Louisiana's seafood is shipped all over the country and nearly a third of all the domestic seafood consumed in the United States is from Louisiana's waters. Louisiana is the number one provider of shrimp,

oysters, crabs, and crawfish in the nation (1). Because of the importance of this industry, it is important to monitor the state of the seafood. If the seafood is contaminated, whether with pollutants or bacteria, it becomes dangerous to consume and hurts the economy. One of the most common bacterial contaminants of seafood is *Vibrio* spp.

Vibrios are gram-negative, rod-shaped aerobic bacteria that live in warm, marine or estuarine environments. Studies have shown that *Vibrio* are found most often in water temperatures ranging from 13°C to 27°C and in salinity as low as 11 ppt to as high as 27 ppt (2). The disease causing species of V*ibrio* are responsible for approximately 500 hospitalizations and 100 deaths per year in the United States. Infection is typically caused after exposure to seawater or consuming raw seafood. This infection is called Vibriosis and its typical symptoms are diarrhea, primary septicemia, wound infections, and other extra intestinal infections (3). In the waters of south Louisiana, two common species of *Vibrio* that cause disease are *Vibrio parahaemolyticus* and *Vibrio vulnificus*.

Vibrio parahaemolyticus typically causes gastrointestinal illness in humans and the symptoms of its infection include diarrhea, abdominal cramps, nausea, vomiting, headache, fever, and chills (4). The virulence factors in *V. parahaemolyticus* are thermostable direct hemolysin (TDH) and thermostable direct hemolysin-related hemolysin (TRH). These two factors are both used to identify virulent strands of *V. parahaemolyticus* (4). *Vibrio vulnificus* can cause potentially fatal complications in immunocompromised individuals by infecting open wounds. Because of the severity of these symptoms, it is the leading cause of seafood associated fatalities in the United States (5)

To treat *Vibrio* infections, a cocktail of antibiotics are normally used in the clinical settings. However, past studies have shown that environmental strains of *Vibrio*

parahaemolyticus and *V. vulnificus* can show some resistance to the antibiotics used to treat the infections. *V. parahaemolyticus* isolates from the Chesapeake Bay area were shown to have varying levels of resistance to ampicillin, penicillin, chloramphenicol, streptomycin, and cephalothin. The *V. vulnificus* isolates from the same area had lower levels of resistance present, but still showed some intermediate resistance to streptomycin and chloramphenicol (6). Another study tested the antimicrobial susceptibility of *V. parahaemolyticus* isolates from seafood caught in Lagos Lagoon in Nigeria. These isolates were resistant to ampicillin, but were susceptible to the other antibiotics tested (7). According to the CDC, the best antibiotics to treat Vibriosis are doxycycline, third-generation cephalosporins, fluoroquinolones, and trimethoprim-sulfamethoxazole.

Because of the prevalence of *Vibrio* bacteria on seafood and the water they are grown in, a large majority of seafood is treated before it can be cooked or purchased. This treatment is very commonly found in oysters because of the prevalence of raw consumption. Physical, chemical, and biological methods have been studied extensively as treatment methods for seafood. Some physical methods include relaying, depuration, temperature control, irradiation, and high pressure. The chemical methods shown to reduce *vibrio* bacterial loads include electrolyzed oxidizing water, chlorination, organic acids, chitosan, and essential oils. The biological methods use probiotics and bacteriophages to control the amount of *vibrio* present in seafood (8). Some of these methods, plus advantages and limitations, are presented in Table 1, which is adapted from Wang's Review (8) on Intervention Strategies for Reducing *Vibrio parahaemolyticus*.

Table 1: Advantages and Limitations of Seafood Treatment Methods

Method	Advantages	Limitations
Relaying	No seafood injury	Not effective in short time periods
Depuration	Long history of use	Not efficient as a standalone procedure
Heat Treatments	Avoid seafood death	Protein denaturation and costly
Cold Treatments	Inhibits Bacterial growth	Temperature dependent
High Pressure Process	Effective inactivation for short time treatment	Costly
Chlorine	Effective antimicrobial activity	Not permitted for seafood processing
Biocontrols	No chemical residue	Limited research into the public's reaction to this treatment method

Most of the seafood for this experiment is provided by the John Folse Culinary Institute and Chef Jean Paul Daigle. The seafood swabbed will be used for the Louisiana Seafood Class, and some of the seafood, such as the oysters, is treated using some of the above methods before being sold to the Culinary Institute. Also, most of the seafood is stored, frozen, until the class is ready to use it. While this period of cold temperature varies in time, studies have shown that temperatures below 10°C lead to a moderate decline in the *V. parahaemolyticus* population over time (8).

This research was conducted using the grant funding from the Louisiana SeaGrant's Undergraduate Research Opportunity Program. While plenty of research has been done on the amount and types of *Vibrio* spp. in seafood before and after consumption, very little research has been done on the *Vibrio* spp. found on the surface of Louisiana Seafood. Louisiana's culture and cuisine focuses heavily on seafood and seafood dishes and safety should always be a priority when handling seafood. While professional chefs often understand this importance, most people preparing and eating seafood are not aware of this. Too often, people will handle raw and live seafood with little regard to the possibility that cross contamination could potentially be

occurring during this process. This research focuses on the preliminary experiments in understanding the amount and types of *Vibrio* spp. present on the raw and live seafood of Louisiana.

Materials

The seafood swabbed from the John Folse Culinary Institute's Louisiana Seafood Class included Grouper, Red Snapper, Blue Crabs, Cobia, Mahi, Shrimp (both peeled and not peeled), Catfish, Alligator, Turtle, Flounder, Oyster, and Crawfish. The seafood collected from Rouses Supermarket included fresh unpeeled Shrimp, live Blue Crabs, and Oysters.

The media used for this experiment included Alkaline Peptone Water (APW), Vibrio CHROMagar™, Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar, Mueller-Hinton (MH) agar, Biolog Universal Growth (BUG) agar w/ 5% sheep blood, Inoculation Fluid B (IF-B), and Phosphate-Buffered Saline (PBS). Deionized Water was also needed for the majority of these experiments.

For the sample collecting, sterile cotton swabs, an incubator (37°C), sterile test tubes, and sterile inoculation loops were used. For the TCBS identification portion of the experiment, sterile inoculation loops and an incubator (37°C) were used in addition to TCBS agar. For the Kirby-Bauer Assays, the following antibiotic discs were used: Tetracycline (TE 30), Ampicillin (AM 10), Sulfamethoxazole Trimethoprim (SXT), Nalidixic Acid (NA 30), Gentamycin (GM 10), Cefoxitin (FOX 30), and Cefazolin (KZ 30). Also, a sterile inoculation loop, a sterile antibiotic disc stamper, and an incubator (37°C) were used.

For the Biolog Microbial indentification, a Biolog Microbial ID system with the GEN III Database, a sterile inoculation loop, a sterile pipettor, and an incubator (37°C) were also needed in addition to the BUG agar and the IF-B solution. For the PCR identification, an incubator

(37°C), micro-centrifuge tubes, and a centrifuge were needed. Also, tlh gene primer set, trh gene primer set, tdh gene primer set, iNtRON's Maxime PCR Premix Kit (*i*-Taq), a PCR machine, loading solution, 1.5% agarose gel, ethidium bromide, 1x TBE, an electrophoresis machine, and an UV camera were used. For the bacterial load experiment, sterile test tubes, sterile pipettes, sterile petri dishes, sterile blenders, an oyster shucking knife, and a bacteria colony counter were needed.

Methods

Procedure 1 – Sample Collecting

Seafood was swabbed on a weekly basis as it was used by the John Folse Culinary Institute's Louisiana Seafood class. Sterile Q-tips were soaked in DI water before swabbing the outside of the seafood. After swabbing the seafood, the cotton swab was incubated in Alkaline Peptone Water (APW) for 24 hours. After incubating, the APW was quadrant streaked onto Vibrio CHROMagar™. The Vibrio CHROMagar™ was then incubated at 37°C for 24 hours. Colonies of *Vibrio parahaemolyticus* and *Vibrio vulnificus* were then isolated from the Vibrio CHROMagar™. *V. parahaemolyticus* colonies are a purple color on the agar and *V. vulnificus* colonies are a green/blue color. The isolated colonies were then incubated in APW for 24 hours at 37°C.

Procedure 2 – TCBS identification

The *V. parahaemolyticus* and *V. vulnificus* colonies isolated from the seafood samples were streaked onto Thiosulfate-citrate-bile salts-sucrose (TCBS) agar. The TCBS plates were then incubated for 24 hours at 37°C. After 24 hours, the colonies were examined.

Procedure 3 – Kirby-Bauer Assays

The *V. parahaemolyticus* and *V. vulnificus* colonies isolated from the seafood samples were then streaked onto Mueller-Hinton (MH) agar for Kirby-Bauer antibiotic resistance assays. The antibiotics used for the Kirby-Bauer Assay were Tetracycline, Ampicillin, Sulfamethoxazole Trimethoprim, Nalidixic Acid, Gentamicin, Cefoxitin, and Cefazolin. Each *Vibrio* colony was streaked onto a MH agar plate and then the 7 antibiotic discs were applied to the plate using a sterile Antibiotic disc stamper. The plates were then incubated for 24 hours at 37°C. After incubating, the zone of inhibition around each disc was measured in mm.

Procedure 4 – Biolog Microbial Identification

The *V. parahaemolyticus* colonies isolated from the grouper, shrimp, and cobia seafood samples were then run through the Biolog Microbial ID system using the GEN III Database. The *V. parahaemolyticus* colonies were streaked on Biolog Universal Growth (BUG) agar w/ 5% sheep blood and then incubated for 24 hours at 37°C. After incubating, the colonies were suspended in the IF-B solution for use with the GEN III microplates. After suspending in the IF-B, the liquid was pipetted into the GEN III Microplate using a sterile pipette tips and pipettor. The Microplates were then incubated for 24 hours at 37°C. After incubating for 24 hours, the Microplates were read using the MicroLog 3/5.5.01 35 software. The microplates were then put back in the incubator for another 24 hours (48 hours total) and read again using the Microlog software.

Procedure 5 – Multiplex PCR Identification

The *V. parahaemolyticus* colonies isolated from the oyster samples were run through a multiplex PCR analysis. The colonies were grown overnight in fresh APW at 34°C. One ml of culture was centrifuged for 3 minutes at 15,000 x g in a micro-centrifuge tube. The pellet was then washed twice using a physiological saline. The pellet was then suspended in one ml of DI

H_2O and boiled for 10 mins. The template DNA was then stored at 20°C until use. Three primer sets were used for the PCR identification: tlh gene primer set, trh gene primer set, and tdh gene primer set. iNtRON's Maxime PCR Premix Kit (*i*-Taq) was used a master mix solution for the PCR identification of the tlh gene. To each tube of *i*-Taq, 5 µl of the Template DNA was added, 2.5 µl of the primer set for the tlh gene, and 12.5 µl of DI H_2O. iNtRON's Maxime PCR Premix Kit (*i*-Taq) was again used a master mix solution for the PCR identification of the trh and tdh genes. To each tube of *i*-Taq, 5 µl of the Template DNA was added, 2.5 µl of the primer set for the trh gene, 2.5 µl of the primer set for the tdh gene, and 10 µl of DI H_2O. Each *i*-Taq tube was then run in a PCR machine under the conditions described in the Bacteriological Analytical Manual, Chapter 9. 10 µl of each PCR product was mixed with 2 µl of loading gel and loaded into sample wells of 1.5% agarose gels with 1 µg/ml ethidium bromide submerged in 1x TBE. The Tlh gene PCR products were run on a separate gel from the trh and tdh gene PCR products to prevent the tlh and trh genes from interfering with each other due to similar base pair lengths. The voltage was a constant 100 volts for 45 minutes. After 45 minutes, the gels were viewed under a UV camera to identify the bands for each gene.

Procedure 6 – Serial Dilution and Bacterial Load

Fresh samples of Oyster, Shrimp, and Blue Crab were obtained. Three samples of each species were soaked in 100 ml of Phosphate-Buffered Saline (PBS) for 20 minutes. After 20 minutes, the seafood samples were removed and the 100 ml of PBS solution was shaken. After shaking, 1 ml of the 100 ml was added to 9 ml of PBS to create a 10^{-3} dilution. This step was repeated until dilutions up to 10^{-7} were made for each sample. 1 ml of each sample was put in a sterile Petri dish, and then 10 ml of Vibrio CHROMagar™ was poured into each Petri dish, and then spun for uniformity. After cooling, the plates were incubated for 24 hours at 35°C. After 24

hours, the number of individual colonies of each color (blue, white, and purple) was counted on each plate. A second serial dilution was performed with 12 shucked oysters as well. 12 oysters were aseptically shucked into a blender and an equal amount of PBS was poured in as well to create a 1:2 dilution. The oyster-PBS mixture was blended for 3 minutes. From this mixture, dilutions were created for 10^{-2} to 10^{-7}. One ml from each of these dilutions was also poured into a Petri dish to be mixed with 10 ml of Vibrio CHROMagar™. After cooling, these plates were also incubated for 24 hours at 35°C. After 24 hours, the number of individual colonies of each color (blue, white, and purple) was counted on each plate.

Results

Sample Collecting

Using the Vibrio CHROMagar™, numerous *Vibrio* pathogens were found to be on the surface of most of the seafood used during the Louisiana Seafood cooking class. However, a lack of *Vibrio* spp. was found in the frozen seafood, such as the alligator filets used by the class. Some of the freshwater seafood, such as turtles and catfish, did not have *Vibrio* spp.

TCBS Identification

All colonies isolated from the Vibrio CHROMagar™ that were thought to be *Vibrio* spp. also grew on the TCBS agar as well.

Kirby-Bauer Assays

The results from the Kirby-Bauer assays can be found in Table 1. For the tetracycline, the average zone of inhibition for the *V. parahaemolyticus* was 24.2 mm $\pm$ a standard error of 1.79 mm and the average zone of inhibition for the *V. vulnificus* was 28.5 mm $\pm$ 1.09 mm. For the ampicillin, the average zone of inhibition for the *V. parahaemolyticus* was 10 mm $\pm$ 2.91 mm and the average zone of inhibition for the *V. vulnificus* was 6.4 mm $\pm$ 4.35 mm. For the

sulfamethoxazole trimethoprim, the average zone of inhibition for the *V. parahaemolyticus* was 27.7 mm ± 0.96 mm and the average zone of inhibition for the *V. vulnificus* was 29.6 mm ± 0.58 mm. For the nalidixic acid, the average zone of inhibition for the *V. parahaemolyticus* was 17.9 mm ± 4.66 mm and the average zone of inhibition for the *V. vulnificus* was 29.9 mm ± 2.19 mm. For the gentamycin, the average zone of inhibition for the *V. parahaemolyticus* was 19.0 mm ± 1.77 mm and the average zone of inhibition for the *V. vulnificus* was 22.8 mm ± 0.58 mm. For the cefoxitin, the average zone of inhibition for the *V. parahaemolyticus* was 13.8 mm ± 3.67 mm and the average zone of inhibition for the *V. vulnificus* was 18.4 mm ± 1.96 mm. For the cefazolin, the average zone of inhibition for the *V. parahaemolyticus* was 14.6 mm ± 2.50 mm and the average zone of inhibition for the *V. vulnificus* was 16.8 mm ± 2.58 mm.

Table 1: Average Zone of Inhibition and Standard Error of each antibiotic tested during the Kirby-Bauer Assay.

Antibiotics		Avg. Zone of Inhibition	Standard Error
V. parahaemolyticus	TE 30	24.23	1.79
	AM 10	10.00	2.91
	SXT	27.68	0.93
	NA 30	17.91	4.66
	GM 10	19.00	1.77
	FOX 30	13.82	3.67
	KZ 30	14.59	2.50
Vibrio vulnificus	TE 30	28.50	1.10
	AM 10	6.40	4.35
	SXT	29.60	0.58
	NA 30	29.90	2.19
	GM 10	22.80	0.58
	FOX 30	18.40	1.96
	KZ 30	16.80	2.58

Biolog Microbial identification

The possible *V. parahaemolyticus* colony isolated from the grouper was identified by the Biolog Microbial Identification as *Aeromonas salmonicidia* after 28 hours with a probability of 0.19 but after 48 hours with a probability of 0.62. The possible *V. parahaemolyticus* colony isolated from the cobia seafood sample was identified as an *Enterococcus faecalis* after 28 hours with a 0.97 probability and after 48 hours with a 0.60 probability. The first possible *V. parahaemolyticus* isolated from the shrimp sample came back with a no ID after 28 and 48 hours. The other three possible *V. parahaemolyticus* colonies all came back as *Vibrio metschnikovii* with a probability of over 0.60 after 28 hours and 48 hours. The FDA and ATCC *V. parahaemolyticus* known controls were also run through the Biolog Microbial Identification Software and both came back as possible *V. parahaemolyticus* colonies. The FDA sample had a probability of 0.50 after 24 hours and the ATCC sample had a probability of 0.36 after 24 hours.

Multiplex PCR Identification

All the possible *V. parahaemolyticus* colonies isolated from oyster samples did not have bands present for the tdh, trh, or tlh genes (Figures 1 and 2).

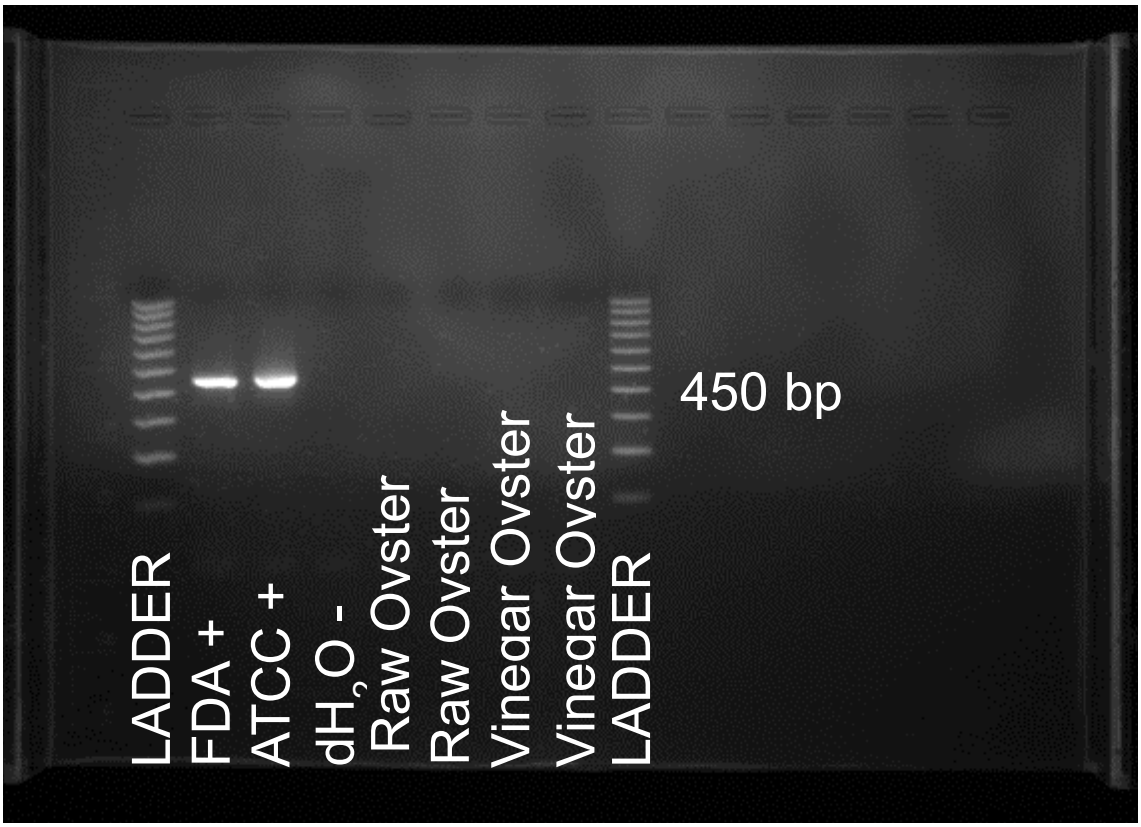

Figure 1: Gel Electrophoresis for TLH gene in *V. parahaemolyticus.* Ladder is 1000 bp, with 100 bp bands.

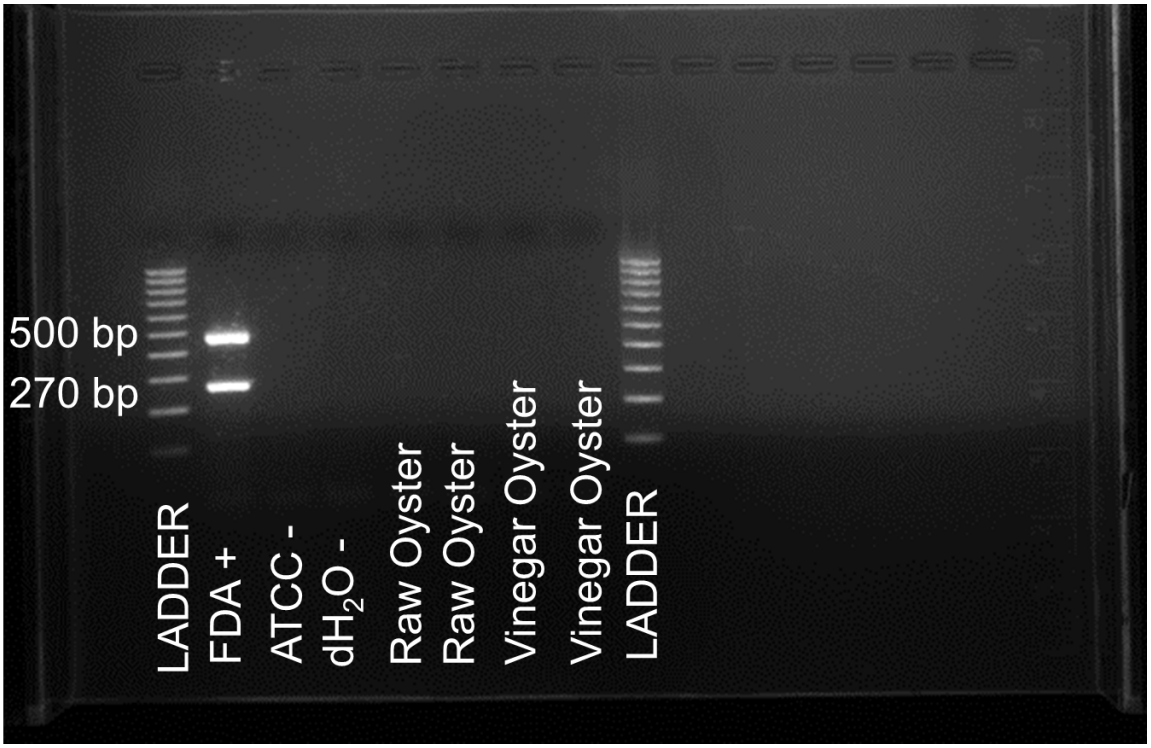

Figure 2: Gel Electrophoresis for TDH and TRH tox genes in *V. parahaemolyticus.* Ladder is 1000 bp, with 100 bp bands.

Serial Dilution and Bacterial Load

The blended oyster control showed no *Vibrio* spp. growth on any of the MH plates after 24 hours. On the shrimp dilution plates, no *Vibrio* growth was seen on any of the plates past a dilution of $1x10^{-2}$. On the crab plates, *Vibrio* growth was on all plates up to $1x10^{-6}$ and then *V. parahaemolyticus* was found on two plates up to the 1×10^{-7} dilution. On the shrimp plates, *Vibrio* growth was found only on the $1x10^{-2}$ plates.

Discussion

Vibrio spp. were found on the surface of the majority of the seafood, especially the marine seafood and the seafood was not kept on on ice for long. *Vibrio* spp. do not like cold temperatures and also live in salt water environments. Some of the seafood had already been prepackaged and frozen before it could be swabbed, so a lack of *Vibrio* pathogens was found in these samples. An example of this was the alligator filets used by the class. Another reason *Vibrio* was possibly not isolated from the alligator filets is that alligators live in fresh water environments, which is not the suitable saltwater environment for *Vibrio* spp. This could also explain the lack of *Vibrio* spp. on the turtles and catfish as well.

Potential *V. parahaemolyticus* and *V. vulnificus* colonies isolated from the Vibrio CHROMagar™ were also streaked onto TCBS agar. Growth on this agar is also a good indicator that the targeted colony is *Vibrio* spp.

For the Kirby-Bauer Assay, very little resistance was seen among the isolated *Vibrio* spp. to the antibiotics tested. For the tetracycline, the average zone of inhibition for *V. parahaemolyticus* and *V. vulnificus* was much higher than the 11mm zone of inhibition expected for Tetracycline resistant *Vibrio*. Large susceptibility was seen for the antibiotics Sulfamethoxazole Trimethoprim and Gentamicin as well. For Cefoxitin and Nalidixic Acid, *V.*

vulnificus isolates were mainly susceptible, but the *V. parahaemolyticus* colonies showed intermediate resistance to these antibiotics. Both species of *Vibrio*, however, did show a large amount of varying resistance to both the Ampicillin and the Cefazolin. It's also important to look at the standard error as well of these averages. The high standard error of the Cefoxitin, Ampicillin, Cefazolin, and Nalidixic Acid zone of inhibition averages show a mixture of both highly resistant and highly susceptible *Vibrio* spp. in the samples.

While the Biolog Microbial ID system is a very good tool to use in species identification, it may not always be right in its diagnostic. Proof of this is the two known *V. parahaemolyticus* samples from the FDA and ATCC both came back with relatively low probabilities (0.50 and 0.36 respectively) that they were actually *V. parahaemolyticus*. However, I do think the diagnostic that the cobia and grouper samples were not *Vibrio parahaemolyticus* based on how different the results were from *V. parahaemolyticus* expected results. And while the shrimp samples did not come back as *V. parahaemolyticus* either, the ID of another *Vibrio* sp. can mean that other species of Vibrio grow on the Vibrio CHROMagar™ and the TCBS agar and could potentially grow similar to the targeted *Vibrio* spp.

For the gel electrophoresis, no bands were present in any of the isolates from the Oyster samples taken. The tlh, trh, and tdh genes were present in the positive controls, so the procedure works, but no bands were available from the isolates. The lack of bands could potentially be from a lack of DNA available in the isolates' DNA templates. A lack of bands could also signify that the isolates thought to be *V. parahaemolyticus* on Vibrio CHROMagar are in fact another species of *Vibrio* that grows similar to *V. parahaemolyticus*.

The lack of *Vibrio* spp. growth on the dilution plates from the control sample of blended oysters shows that modern oyster sterilization techniques do eliminate the *Vibrio* from the edible

center of the oyster. However, *Vibrio* spp. growth was seen in the dilution plates taken from the oysters soaking in 100 ml of PBS. This presence of *Vibrio* on the surface could potentially be contamination from the shipping or handling processes between the sterilization procedure and the soaking in 100 ml of PBS. The oysters were being sold in the same area as other types of seafood, so it is possible the *Vibrio* seen could be from this area. However, the bacterial loads present on both the surface of the Oysters and the shrimp were much lower than those present on the Blue crabs. Part of this could be because the shrimp and oysters were being kept on ice, while the blue crabs were live and not iced at the time of procurement. Freezing seafood is known to limit *Vibrio* growth because it only survives in warmer temperatures (2).

All of these preliminary trials do show that more studies are needed on the seafood of south Louisiana to assure safety and public health. A large focus of future research in this area does need to focus on the *Vibrio* spp. present on the surface of seafood, because of the high probability of cross contamination when preparing seafood for consumption.

Acknowledgements

Many thanks to Dr. Ramaraj Boopathy, Dr. Rajkumar Nathaniel, Dr. Michelle Thiaville, and Ms. Angela Corbin for their help in the lab and to Chef Jean Pierre Daigle of the John Folse Culinary Institute for his help in the culinary department. This work was funded by a grant from the Louisiana Sea Grant under Undergraduate Research Opportunity Program.

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