

COLD ADAPTION BEHAVIORS OF *VIBRIO VULNIFICUS* AND *VIBRIO  
PARAHAEMOLYTICUS* IN OYSTERS

A Thesis

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## **DEDICATION**

To my parents and my brother for their support and encouragement

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## ABSTRACT

*Vibrio vulnificus* is an estuarine bacterium that can cause primary septicemia as well as other serious wound infections. *Vibrio parahaemolyticus* is a gram-negative, halophilic bacterium that may cause gastroenteritis, diarrhea, headache, vomiting, nausea, abdominal cramps and is the leading cause of gastroenteritis in United States. Consumption of raw oysters is believed to closely relate with infection with *Vibrio vulnificus* and *Vibrio parahaemolyticus*. All strains of *V. parahaemolyticus* produce *tlh* genes and the major virulence factors of *V. parahaemolyticus* are the *tdh* and *trh* genes.

This study was conducted to determine if *Vibrio vulnificus* and *Vibrio parahaemolyticus* clinical and environmental strains display different behaviors at refrigeration temperature. The effects of three different temperature treatments were investigated in this study. In addition, the cold adaption behaviors of different gene-containing *Vibrio parahaemolyticus* in broth under three different temperatures 5, 8, and 10°C were also studied. Results obtained from this study showed clinical and environmental strains of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in oysters had significant differences in growth and survival at specific days under different refrigeration temperature treatments. When different gene-containing *Vibrio parahaemolyticus* strains were stored at 5°C over 10 days, all strains were able to survive but not grow. At 8°C all of the *V. parahaemolyticus* strains were able to survive and

grow. At 10°C, two of the *V. parahaemolyticus* strains survived and grew while the *V. parahaemolyticus* 132 X 5 strain survived but did not grow.

**CHAPTER 1**  
**INTRODUCTION**

*Vibrio vulnificus* is Gram-negative, motile, curved, and rod-shaped, and is an invasive human pathogen with worldwide distribution. It is an estuarine bacterium that can cause primary septicemia as well as serious wound infections. In general, clinical isolates have a high lethal effect compared with environmental isolates (Tsuchiya and others 2007). It is the leading cause of death in US due to consumption of uncooked seafood. *Vibrio parahaemolyticus* is a Gram-negative, curved, rod-shaped facultative anaerobic, with growth temperature from 5°C to 43°C, pH range from 7.6 to 9.0, halophilic and is commonly found in coastal marine waters and seafood throughout the world. It may cause gastroenteritis, diarrhea, headache, vomiting, nausea, abdominal cramps and is a common cause of food-borne illnesses in Asian countries, especially in Japan. In the United States, *Vibrio parahaemolyticus* is the leading cause of gastroenteritis. Its virulence is believed to be related to hemolysins such as thermo stable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) (Pan and others 2007).

Acute foodborne disease infections are more of a concern to governments and the food industry today than a few decades ago and seafood has been the most common food vehicle implicated in outbreaks. During July to August 1997, the largest reported outbreak in North America of *Vibrio parahaemolyticus* infections occurred. Illness in 209 persons was associated with eating raw oysters harvested from California, Oregon, and Washington in the United States and from British Columbia (BC) in Canada; one person died (CDC, 1998).

Oyster is an important medium of infection with *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Depaola et al. (1990) indicated that *V. parahaemolyticus* density was more than 100 times greater in oysters than in water. Another study carried out by (Gooch and others 2002) showed that *Vibrio parahaemolyticus* can grow rapidly in unrefrigerated oysters. Many cases have identified that improper refrigeration temperature of seafood allow proliferation of *Vibrio*. A national survey showed that densities of both *V. vulnificus* and *V. parahaemolyticus* in market oysters from their chosen harvest regions followed a seasonal distribution, with highest densities in the summer (Cook and others 2002). In the draft assessment conducted by Food and Drug Administration, they addressed the factors associated with handling and processing of oysters. Important Factors included the temperatures of ambient air at time of harvest, time from harvest that oysters remained unrefrigerated, time required to cool oysters once placed under refrigeration, and the length of time oysters were stored in refrigeration until consumption (Force, 2000).

The objective of this study was to determine if any differences exist in the growth and survival of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in refrigeration temperature by utilizing different cooling methods and to inquire if different gene-containing strains of *Vibrio parahaemolyticus* showed different cold temperature adaptation behaviors at three different storage temperatures.

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**CHAPTER 2**  
**LITERATURE REVIEW**

## ***VIBRIO VULNIFICUS***

*Vibrio vulnificus*, previously called *Beneckeia vulnificus* is a motile lactose-positive *Vibrio* (Bowdre and others 1981), Gram-negative, curved rod-shaped bacterium with a single polar flagellum. It is distinguished from other members of the *Vibrio* genus in its ability to ferment lactose. It's presence in the estuarine environment is not due to pollution or other forms of contamination. It often causes serious vomiting, diarrhea, abdominal pain, and fetal infections like septicemia.

*Vibrio vulnificus* is responsible for over 95% of seafood related deaths in the United states (Day and Oliver, 2004). Immunocompromised persons are especially at risk when they eat uncooked seafood like oysters. Persons with elevated serum iron levels are vulnerable to infection (Oliver and others 1983). FDA (1995) has issued a warning that people with HIV or liver disease should avoid eating raw oyster to prevent the infection of *Vibrio vulnificus* and proper cooking is always recommended. In addition, proper handling temperature is essential to control the microbial load. However, even with proper storage, levels of *Vibrio vulnificus* may remain sufficiently higher in shell-stock oysters to produce infection in compromised hosts (Murphy and Oliver, 1992)

*Vibrio vulnificus* is divided into two biotypes based on phenotypic and host range differences (Tison and others 1982). Biotype 1 strains produce indole and ornithine decarboxylase, exhibit several immunologically distinct lipopolysaccharide (LPS) types, and

are typically associated with shellfish colonization and human illness. *Vibrio vulnificus* biotype 2 strains are negative for indole and ornithine decarboxylase production, and express a common LPS type. There are two colony morphotypes of *Vibrio vulnificus*, which are translucent and opaque. *Vibrio vulnificus* with the ability to utilize transferrin-bound iron and the presence of a surface polysaccharide were found to correlate with colony opacity and virulence (Simpson and others 1987).

### ***VIBRIO PARAHAEMOLYTICUS***

*Vibrio parahaemolyticus* is a human pathogen distributed in marine environments. It is facultative anaerobic, with a growth temperature from 5°C to 43°C, optimum pH range from 7.6 to 9.0, and halophilic (Beuchat, 1973). It causes gastroenteritis, diarrhea, headache, vomiting, nausea, and abdominal cramps. It is a common cause of foodborne illnesses in Asian countries, especially in Japan, the most frequent cause of foodborne disease. Also, it is the leading cause of human gastroenteritis associated with seafood consumption in the United States (Su and Liu, 2007). During July to August 1997, the largest reported outbreak in North America of *Vibrio parahaemolyticus* infections occurred. Illness in 209 persons was associated with eating raw oysters harvested from California, Oregon, and Washington in the United States and from British Columbia (BC) in Canada; one person died (CDC, 1998). The infection is due to consumption of raw, improperly cooked, or cooked, re-contaminated fish and shellfish. Furthermore, improper refrigeration of seafood allows its proliferation. Usually,

symptoms occur within 24 hours of ingestion and are self-limited, lasting 3 days. Severe disease is rare, occurring in persons with weak immune systems.

The factors causing infections by *Vibrio parahaemolyticus* include that cells have an enterotoxin in the bacterial cell walls, and the ability to produce a thermostable direct hemolysin (*tdh*) - termed the Kanagawa phenomenon (Miyamoto and others 1969). The thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) are the main virulence factors of *V. parahaemolyticus* (Cabrera-Garcia and others 2004). Only Kanagawa-positive (possess TDH gene) strains cause infection. Fortunately, only 1% of this type exists in the natural ocean environment.

## **TEMPERATURE RESPONSE**

Temperature is always a great concern in food safety. Proper storage temperature of food is essential to ensure food safety and temperature effects on *Vibrio* species have been widely studied. Under heat shock *Vibrio parahaemolyticus* had leakage of nucleic acids from the cells which increased the demand of NaCl for recovery (Chang and others 2004). Another study carried out by Chiang (2005) pointed out that heat shock could cause a change in the proportions of the unsaturated and saturated fatty acid. In order to reduce microbial load in food product, cold temperature treatments have been applied for a long time. However, there are still some problems during cold storage. Bryan et al. (1999) indicated that *Vibrio vulnificus* survival and tolerance at cold temperatures could be due to the expression of

cold-adaptive proteins. They also found that *Vibrio vulnificus* exposure to 15°C followed by much colder temperature (6°C) would cause a cold adaptive response. Heat treatment is the other way to reduce the microbial load. Hesselman et al.(1999) found that *Vibrio vulnificus* in oysters (*Crassostrea virginica*) subjected to a commercial heat-shock process, which is 1 to 4 minutes at internal oyster meat temperatures exceeding 50°C, were significantly reduced.

Hot water pasteurization followed by cold shock has proven to be successful in reducing *Vibrio vulnificus* and *Vibrio parahaemolyticus* and a total processing time of at least 22 minutes at 52°C was recommended to reduce *Vibrio parahaemolyticus* 03:K6 CFU g<sup>-1</sup> oyster meat to non-detectable levels (< 3 g<sup>-1</sup> oyster meat) (Andrews and others 2003)

## **IDENTIFICATION METHODS**

The development of rapid and sensitive molecular techniques for the detection of *Vibrio* species would be useful for the surveillance of sporadic infections and management of major outbreaks (Tracz and others 2007). Several new methods have been developed to identify the *Vibrio* rapidly. DNA probe-based detection methods were considered as an alternative to time-consuming protocols such as MPN. Banerjee et al.(2002) showed that Colony (Southern) hybridization analyses carried out using hydrophobic grid membrane filters (HGMFs) was successful to identify and enumerate *Vibrio vulnificus* and *Vibrio parahaemolyticus* in which the whole procedure can be complete within 1 day. (Overman and others 1985; Overman and Overley, 1986) have shown among the API 20E, API Rapid E, and API Rapid NFT system,

API 20E was one of the valid methods for use in the identification of the more commonly occurring members of the family *Vibrionaceae* and was the most accurate and efficient of the three systems tested. Tarr et al. (2007) also indicated that the PCR assay provides a simple, rapid, and reliable tool for identification of the major *Vibrio* pathogens in clinical samples, and *rpoB* sequencing provides an additional identification tool for other species in the genus *Vibrio*.

## **COLD STORAGE**

Bradshaw et al. (1974) indicated that 12.8°C seems to be the borderline temperature for *Vibrio parahaemolyticus* to grow on cooked seafood. A Study carried out by Cook (1994) indicated that at 13°C and below, *Vibrio vulnificus* failed to multiply in the oyster after harvest. This implies that temperature control is important to inhibit *Vibrio vulnificus*. Other researchers (Gooch et al., 2002) found that if water temperatures at harvest were > 20°C, the geometric mean harvest density of *Vibrio parahaemolyticus* was 130 CFU/g compared with 15 CFU/g when water temperature was < 20°C . Johnson et al. (1973a) showed that *Vibrio parahaemolyticus* survive storage for at least 3 weeks at 4°C. Kaysner et al. (1989) indicated that *Vibrio vulnificus* can survive in the oysters stored at 10°C and below. Furthermore, they found that numbers of endogenous *Vibrio vulnificus* cells could still be found after 7 days at both 0.5 and 10°C in oysters. They pointed out that oysters allowed to take up *Vibrio vulnificus* from seawater could retain the bacterium for 14 days at 2°C and would possibly

cause potential human disease. However, refrigeration temperature is not a guarantee for food safety. There is evidence that oysters kept on ice do not seem to be a major factor in the epidemiology of *Vibrio vulnificus* infection. The reason may be due to the standard method of homogenizing oysters for examining bacteriological quality because toxic compounds are released from the oysters during this process (Oliver, 1981). Other researchers (Quevedo and others 2005) also showed that the use of ice immersion as a post-harvest method is not effective in reducing *Vibrio vulnificus* because of the relatively small declines (Ren and Su, 2006); however using electrolyzed oxidizing (EO) water treatment on postharvest oysters could efficiently reduce *Vibrio vulnificus* and *Vibrio parahaemolyticus*.

## **FOODBORNE DISEASE ON SHIPS**

Food borne disease outbreaks on ships are also a big concern because of their potentially high costs to the industry. Rooney et al. (2004a) indicated that water supply on ships differs tremendously from that of water supply on land. Risks of contamination can come from source water at the port or during loading, storage, or distribution on the ship. Rooney et al. (2004b) also pointed out that *Salmonella* spp. were most frequently related with outbreaks on ships. In addition, Foodborne outbreaks on ships have been associated with enterotoxigenic *E. coli* spp., *Shigella* spp., noroviruses (formally called Norwalk-like viruses), *Vibrio* spp., *Staphylococcus aureus*, *Clostridium perfringens*, *Cyclospora* spp., and *Trichinella* spp.. Some of the risk factors to cause the outbreaks are inadequate temperature

control, infected food handlers, contaminated raw ingredients, cross-contamination, inadequate heat treatment, and onshore excursions.

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### **CHAPTER 3**

## **GROWTH AND SURVIVAL OF DIFFERENT CLINICAL AND ENVIRONMENTAL STRAINS OF *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* WITH DIFFERENT TEMPERATURE TREATMENTS**

## INTRODUCTION

Food safety has been a great issue in the global community. In order to eliminate the pathogenic microorganisms and therefore prolong the shelf life of food products, there are many methods for control of pathogens that have been developed to meet this goal. One example would be by applying modified atmosphere packaging (MAP) conditions that can significantly extend the shelf life of food products. Another method like using aqueous ozone as a water additive may be useful as a decontaminant for small fruits (Bialka and Demirci, 2007). Furthermore, the uses of Iodophor under acidic conditions has proven to be effective in destroying *Vibrio parahaemolyticus* (Rodney and Da Vid). Another study carried out by Andrews (2000) showed that low temperature pasteurization was effective in reducing *Vibrio vulnificus* and *Vibrio parahaemolyticus* to non-detectable levels in less than ten minutes of processing. High pressure treatment is another method that has been shown to be an effective method to extend the shelf life of seafood (Cruz-Romero and others 2004).

Controlling temperature is another way to ensure the quality of food products. Contamination of oysters with *Vibrio vulnificus* and *Vibrio parahaemolyticus* is a great concern in the food safety area. However, by far, environmental control of growth of *Vibrio* has not yet been thoroughly investigated. From a previous study, it was believed that *Vibrio parahaemolyticus* was sensitive to both heat and cold, generally displaying more resistance to freezing than chilling (Beuchat, 1975). Johnson et al. (1973b) indicated that *Vibrio*

*parahaemolyticus* in Maryland waters was able to survive storage for at least 3 weeks at 4°C, and to multiply after being held for 2 to 3 days at 35°C. *Vibrio vulnificus*, another common foodborne pathogen; however, has had more attention due to its high mortality rate on patients. It often causes two distinct disease syndromes, a primary septicemia and wound infections can occur from *V. vulnificus* infections. According to Simpson et al. (1987), all avirulent strains produced only translucent colonies while all virulent strains, with the exception of biogroup 2 (eel pathogens), exhibited both opaque and translucent colonies. Their study also indicated that *V. vulnificus* ability to utilize transferrin-bound iron and the presence of a surface polysaccharide was correlated to colony opacity and virulence.

In this study, we investigated whether different temperature treatments affect the survival or growth rate of different *Vibrio vulnificus* and *Vibrio parahaemolyticus* strains in oysters.

## **MATERIALS AND METHODS**

### **Media**

All of the media used during this study were based on U.S. Food and Drug Administration Bacteriological Analytical Manual Online (2001). Tryptic Soy Broth supplemented with 2% NaCl plus kanamycin (TSBN<sub>2</sub> + kanamycin) (Kanamycin Monosulfate, Sigma Chemicals, K-1377) and Tryptic Soy Agar supplemented with 2% NaCl plus kanamycin (TSAN<sub>2</sub> + kanamycin) were used for culturing and enumerating *Vibrio*

*vulnificus* and *Vibrio parahaemolyticus*.

## **Bacterial Cultures**

Two green fluorescent protein (GFP) transformed *Vibrio vulnificus* and two GFP transformed *Vibrio parahaemolyticus* strains were used during this study. The GFP transformed *Vibrio parahaemolyticus* isolates used were a clinical strain 49529 that was isolated from feces in California and obtained from American Type Collection Center (ATCC), Manassas, Va. Environmental isolates 541(O) 57C was isolated from oysters in Louisiana and obtained from Louisiana State University (LSU) - Baton Rouge, LA.

Stock strains were maintained on 2% NaCl tryptic soy agar (TSAN<sub>2</sub>) slants at room temperature. In order to maintain *Vibrio* GFP strains viability, *Vibrio* GFP strains were transferred every 3 months to fresh TSAN<sub>2</sub> plus kanamycin slants and incubated at 37°C for 18 - 24 hours. After incubation, sterile mineral oil was added to the slants and stored in the dark.

## **Culture Preparation**

One loop of GFP transformed cultures were placed into 10 ml tryptic soy broth supplemented with 2% NaCl plus kanamycin (TSBN<sub>2</sub> + kanamycin) TSBN<sub>2</sub> tubes and was incubated at 37°C for 12 hours. After incubation, cultures were centrifuged 3,300 g for 10 minutes, the supernatant was discarded and 10 ml of PBS was added.

## **Oysters Preparation and Inoculation**

Holes were drilled into 114 oysters with a drill (Black and Decker 9.6 V) obtained from Wal-mart using 0.38cm diameter drilling bit. After drilling, 114 oysters were divided into clinical and environmental groups. Each group contained 57 oysters. The 57 oysters were inoculated with 100 µl of the clinical *Vibrio vulnificus* and *Vibrio parahaemolyticus* cultures and the rest were inoculated with 100 µl of the environmental *Vibrio vulnificus* and *Vibrio parahaemolyticus* cultures at room temperature (25°C).

## **Different Temperature Treatments**

After inoculation, three oysters from both the clinical and environmental groups were shucked and approximately 25 grams was added to stomacher bags, and homogenized with the same amount of Alkaline Peptone Water (APW). Dilutions of the samples were plated onto Tryptic Soy Agar supplemented with 2% NaCl plus kanamycin (TSAN<sub>2</sub> + kanamycin). After 24 hours incubation, the GFP colonies were counted under UV light. The rest of the oysters were pooled into three sampling groups according to three different protocols: (Q) direct submersion into ice water until a temperature of 5°C was reached; (M) placed into a 5°C refrigeration unit; and (S) placed into a refrigeration unit with the temperature gradually dropped to 5°C over 9.5 hours. Thermocouples were placed into the oyster meat of two or three representative specimens to monitor the internal temperature of the oysters as they reached the target temperature of 5°C. Samples were taken at time 0 (when oysters were first

inoculated), at the point when the internal target temperature was reached, and at day 2, 4, 6, 8, and 14.

For plating and enumeration, samples were serially diluted and spread plated onto Tryptic Soy Agar supplemented with 2% NaCl plus kanamycin (TSAN<sub>2</sub> + kanamycin). All experiments were done in triplicates.

### **Statistical Analysis**

Differences in growth/survival of clinical and environmental strains of *Vibrio vulnificus* and *Vibrio parahaemolyticus* under three different temperature treatments at each time interval were analyzed for significance using Student's t test following one - way analysis of variance (ANOVA) JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA). The statistical difference was set at  $p < 0.05$ . All experiments were done in triplicate.

## **RESULTS**

### ***Vibrio vulnificus***

Significant differences among the different strains and treatments were observed on a daily basis. For *Vibrio vulnificus*, there were no significant differences in the three different temperature treatments among similar strains, but clinical strains had lower counts than environmental strains at day 1 for iced and gradual temp decline treatments (Table 1). By day 2, there was significantly higher *V. vulnificus* counts in oysters that had the temperature gradually dropped to 5°C than the other two clinical strains under directly icing and

refrigerated temperature treatments; whereas, there were no significant differences for the environmental *V. vulnificus* strains among the three different temperature treatments. The clinical *V. vulnificus* counts in oysters that were treated by refrigerated temperature treatment had the highest counts of 6.21 log CFU/ml by day 4 as compared with the other treatments, followed by the directly icing and gradual temperature treatments. As for the environmental strains of *V. vulnificus* in oysters on day 4, the results showed that refrigerated temperature treatments had the highest plate counts, followed by the gradual temperature treatment and directly icing treatment. On Day 6, there were no significant differences in the *V. vulnificus* clinical strains among the three different temperature treatments; however, the *Vibrio vulnificus* environmental strains under refrigerated treatment had the lowest plate counts of 3.6 log CFU/ml compared with the other two treatments. On Day 8, there were no significant differences in the *V. vulnificus* clinical strains among the three different temperature treatments. As for *V. vulnificus* environmental strains, the results indicated that the gradual temperature treatment had a significant lower bacterial counts of 4.09 log CFU/ml than that of refrigerated temperature treatment with 5.02 log CFU/ml. By Day 14, the results indicated that there were no significant differences among the three different temperature treatments for clinical and environmental strains.

**Table 1. *Vibrio vulnificus* inoculated into oysters then subject to three different temperature treatments**

Treatment <sup>a</sup>	log CFU/ml					
	Day 1	Day 2	Day 4	Day 6	Day 8	Day 14
QC	3.97 ± 0.71 c d	3.49 ± 0.16 b	6.13 ± 0.04 a	5.42 ± 0.83 a b	5.09 ± 1.29 a b	4.86 ± 1.14 a
QE	5.64 ± 0.74 a	5.54 ± 0.93 a	4.84 ± 0.08 d	4.65 ± 0.08 b	3.40 ± 0.35 b c	4.74 ± 0.12 a
MC	4.15 ± 0.21 b c d	3.30 ± 0.00 b	6.21 ± 0.24 a	5.44 ± 0.64 a b	5.34 ± 0.90 a b	4.15 ± 2.46 a
ME	5.27 ± 0.10 a b	6.31 ± 0.27 a	5.26 ± 0.04 c	3.60 ± 0.00 c	5.02 ± 1.33 a b	4.04 ± 0.71 a
SC	3.39 ± 0.12 d	5.40 ± 0.88 a	5.84 ± 0.09 b	5.88 ± 0.55 a	5.66 ± 0.42 a	4.10 ± 1.26 a
SE	4.72 ± 0.61 a b c	5.41 ± 0.04 a	5.12 ± 0.04 c d	4.88 ± 0.03 b	4.10 ± 0.05 c	3.42 ± 1.94 a

<sup>a</sup> Abbreviations of treatments are as follows: QC, clinical strains submerged into ice; QE, environmental strains submerged into ice; MC, clinical strains stored at refrigerated unit; ME, environmental strains stored at refrigerate unit; SC, clinical strains temperature gradually dropped; SE, environmental strains temperature gradually dropped

All analyses were based on three separate experiments with each mean ± standard deviation being the average of three determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ( $P \leq 0.05$ ). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

### ***Vibrio parahaemolyticus***

Our result showed that on day 1 there were significantly higher bacterial counts for the clinical strains of *V. parahaemolyticus* under gradual temperature treatment, with 6.01 log CFU/ml, followed by the refrigerated and directly icing treatments and the lowest counts were the *V. parahaemolyticus* environmental strains that were submerged directly into ice compared with the other temperature treatments. In addition, the results indicated that directly icing treatment had the lowest counts compared with the other two temperature treatments.

On day 2, the counts for the *V. parahaemolyticus* among the environmental strains were higher with the gradual temperature treatment than any other treatment, followed by the refrigerated and directly icing temperature treatments. However, there were no significant differences for *V. parahaemolyticus* clinical strains among the three different temperature treatments. On Day 4, there were no significant differences with the *V. parahaemolyticus* clinical strains for the three different temperature treatments while the *V. parahaemolyticus* environmental strains inoculated into oysters under gradual treatment had significantly higher plate counts compared with refrigerated and directly icing treatments. On Day 6, we found that the *V. parahaemolyticus* clinical strains under directly icing had the lowest counts of 4.22 log CFU/ml compared with the other two treatments and there were no significant differences in the *V. parahaemolyticus* environmental strain counts in oysters under three

different temperature treatments. On Day 8, the results indicated that the *V. parahaemolyticus* clinical strains under refrigerated temperature had the highest bacterial counts, followed by the gradual and directly icing treatments. The *V. parahaemolyticus* environmental strains inoculated into oysters had the lowest counts when placed directly into ice compared with the other two treatments on day 8. On the last day, 14, the results showed that for both clinical and environmental strains, the gradual temperature treatment had the highest counts, with 4.98 and 5.00 log CFU/ml.

**Table 2. *Vibrio parahaemolyticus* inoculated into oysters then subject to three different temperature treatments**

Treatment <sup>a</sup>	Log CFU/ml					
	Day 1	Day 2	Day 4	Day 6	Day 8	Day 14
QC	5.06 ± 0.02 d	6.09 ± 0.07 b	5.22 ± 0.04 b	4.22 ± 0.02 c	4.27 ± 0.01 c	3.85 ± 0.07 b
QE	5.23 ± 0.04 c	5.09 ± 0.02 d	5.42 ± 0.30 b	5.95 ± 0.04 a	3.87 ± 0.04 c	3.61 ± 0.43 b
MC	5.45 ± 0.07 b	6.04 ± 0.06 b	5.29 ± 0.02 b	5.22 ± 0.03 b	5.42 ± 0.04 a	3.60 ± 0.13 b
ME	6.02 ± 0.07 a	5.93 ± 0.04 c	5.22 ± 0.06 b	5.99 ± 0.06 a	5.33 ± 0.02 a b	3.96 ± 0.08 b
SC	6.01 ± 0.01 a	6.04 ± 0.05 b	5.48 ± 0.06 b	5.17 ± 0.04 b	4.84 ± 0.51 b	4.98 ± 0.11 a
SE	6.07 ± 0.06 a	6.95 ± 0.07 a	6.14 ± 0.09 a	6.02 ± 0.09 a	5.22 ± 0.01 a b	5.00 ± 0.32 a

<sup>a</sup> Abbreviations of treatments are as follows: QC, clinical strains submerged into ice; QE, environmental strains submerged into ice; MC, clinical strains stored at refrigerated unit; ME, environmental strains stored at refrigerate unit; SC, clinical strains temperature gradually dropped; SE, environmental strains temperature gradually dropped

All analyses were based on three separate experiments with each mean ± standard deviation being the average of three determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ( $P \leq 0.05$ ). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

## DISCUSSION

Our results showed temperature fluctuation affects the survival of *V. vulnificus* and *V. parahaemolyticus* in oysters. Eiler et al. (2007) indicated that temperature alone did not significantly affect abundances of total *Vibrio* spp. or individual *Vibrio* populations. However, temperature fluctuation does affect the distribution of microorganisms (Miterpakova and others 2006). According to Zhang et al.(2006) , the fluctuation of temperatures resulted in biogas production variation and significantly influenced the anaerobic process. Bacteria are well known to adapt to environmental changes such as temperature fluctuations (Obuchowski and others 1997). McGovern and Oliver (1995) indicated that temperature shifts ( 23 to 13°C) are within the permissive growth range of *V. vulnificus*. Another study carried out by Bryan et al. (1999) showed that cold-adaptive proteins may enhance survival and tolerance at cold temperatures and iron played an important role during the cold response. They indicated exposure to an intermediate cold temperature (15°C) causes a cold adaptive response, helping *V. vulnificus* remain in cultivable state when exposed to a much colder temperature (6°C). In our study, we found that there is different cold response behavior among different species of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. The reason may be due to the time it took for *V. vulnificus* in oysters to reach the target temperature of 5°C from room temperature. Mizunoe et al. (2000) indicated that *V. parahaemolyticus* cells at 4°C reached the non-cultivable stage in about 12

days. According to Jiang and Chai (1996) , *V. parahaemolyticus* could reach the non-cultivable stage in 50 to approximately 80 days during starvation at 3.5°C. In addition, they also pointed out that surviving cells under low temperature were capable of growth and multiplication with limited nutrients at an extraordinary rate when the temperature was increased, which means that refrigerated temperature can't be relied upon for destroying microorganisms even under proper refrigerated temperature.

The presence of capsular polysaccharide (CPS) is an important virulence determinant in *V. vulnificus*. The presence and amount of CPS on any given virulent isolate has been proved to be correlated with quantitative measures of virulence of the organism in the mouse model (Strom and Paranjpye, 2000). In addition, CPS expression is visible in the colony morphology of *V. vulnificus*, with colonies of cells expressing high levels of CPS appearing opaque while those of cells lacking CPS, or expressing it in low levels, are translucent (Rosche and others 2006). This is probably another explanation of the different cold response behaviors between the clinical and environmental strains of *V. vulnificus* observed in our studies.

Our research has shown that there are different cold response behaviors at specific days in clinical and environmental strains of *Vibrio vulnificus* and *Vibrio parahaemolyticus* under different temperature fluctuation treatments and strains vary significantly in their ability to survive and grow at refrigeration temperatures. These data may be useful for updating FDA

risk assessment models for these pathogens.

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**CHAPTER 4**  
**ADAPTION DIFFERENCES OF DIFFERENT GENE-CONTAINING STRAINS OF**  
***VIBRIO PARAHAEMOLYTICUS* DURING COLD STORAGE**

## INTRODUCTION

*Vibrio parahaemolyticus* is a gram-negative, halophilic bacterium that occurs naturally in estuarine environments (DePaola and others 2003). It often causes diarrhea along with abdominal cramping, nausea, vomiting, fever, chills and is a leading cause of gastroenteritis in United States. Several cases have been reported in the United States mainly due to shellfish consumption, especially in oysters (Kaysner and others 1990). Its density was affected by various factors such as temperature, salinity and geographical distributions (Kaneko and Colwell 1975; DePaola, Hopkins et al. 1990). Its higher density was observed in a warmer season, especially in summer time.

The pathogenicity of *Vibrio parahaemolyticus* is believed to be related to hemolysins such as thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) (Pan et al., 2007). Kanagawa phenomenon refers to the strains that can produce *tdh* gene and can be identified by  $\beta$ -type hemolysis on Wagatsuma blood agar (Bej and others 1999). Shirai et al.(1990) indicated that both *trh* and *tdh* genes are important virulence factors of *Vibrio parahaemolyticus*.

Cold-shock response has been widely studied in many microorganisms. From prokaryotes to plants, their response to cold shock occurs in a similar manner. The general response of cells to cold stress is the elite and rapid over-expression of a small group of proteins, so called CSPs (cold-shock proteins) (Al-Fageeh and Smales, 2006). It is well

known that temperature downshift generally suppresses protein synthesis in both eukaryotic and prokaryotic cells (Fujita, 1999). Furthermore, cold stress-induced changes of the RNA degradosome determine a drastic stabilization of the cold-shock transcripts and cold shock-induced modifications of the translational apparatus determine their preferential translation in the cold condition (Gualerzi and others 2003).

This study was conducted to determine if *Vibrio parahaemolyticus* strains that contain different genes exhibit a different cold temperature adaptation response.

## **MATERIALS AND METHODS**

### **Preparation of Inoculums**

Three *Vibrio parahaemolyticus* strains containing either the *trh*, *tlh*, or *tdh* genes were used during this study. Of these strains, *trh*-containing strain AQ 4037 and *tlh*-containing strain 132 X 5 were obtained from North Carolina University; *tdh*-containing strain 70802 was obtained from the Food Safety / Food Microbiology laboratory at Louisiana State University Agricultural Center.

Stock strains were maintained on TSAN<sub>2</sub> slants at room temperature. Before use, each strain was transferred two consecutive times in TSBN<sub>2</sub> and incubated at 37°C for 12 hours. After incubation, the strains were centrifuged at 9,300 g for 10 minutes. After centrifugation, the supernatant was discarded, and the bacterial pellet was re-suspended into 10 ml of PBS two consecutive times. Serial 10-fold dilutions of suspended cultures were performed to

achieve a final count of  $10^8$  CFU/ml.

### **Cold Adaption Study**

Ninety-nine ml of tryptic soy broth supplemented with 2% NaCl was added to specimen cups and then the cups were pre-chilled to 5°C. The cups were inoculated with 1 ml of washed *V. parahaemolyticus* cultures. This gave an initial count of  $10^6$  log CFU/ml. The cups were stored at 5, 8, or 10°C for 10 days. Bacterial counts were determined every other day by plating on tryptic soy agar supplemented with 2% NaCl.

### **Statistical Analysis**

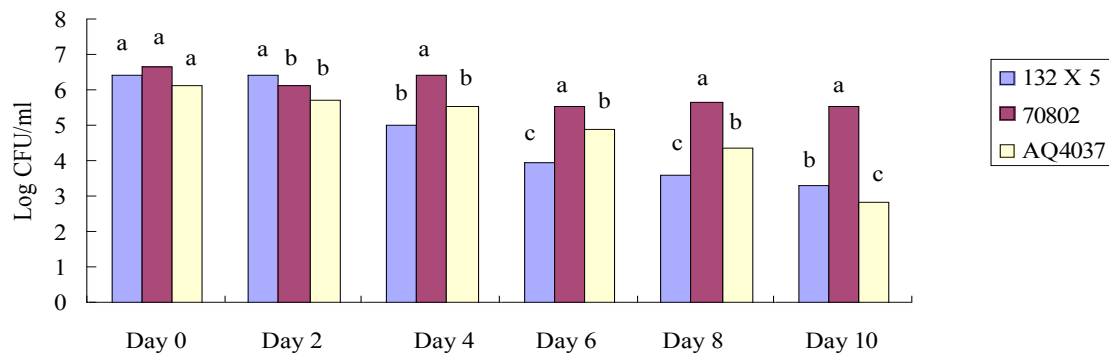
Differences in growth rate among three different gene-containing *V. parahaemolyticus* strains were analyzed for significance by using Student's t test following one-way analysis of variance (ANOVA) JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA). The statistical difference was set at  $p < 0.05$ . All experiments were done in triplicate.

## **RESULTS**

### **Three Gene-Containing *Vibrio parahaemolyticus***

*Vibrio parahaemolyticus* strains stored in tryptic soy broth at 5°C for 10 days survived for the duration of the experiment, with *tlh* and *trh* strains having a steady decline in visible counts over the 10 days (Figure 1). Differences in survival at various stages of storage were evident among the various strains at this temperature. All three different gene-containing strains stored in tryptic soy broth at 5°C for 10 days showed significant differences in survival

at different stages of storage throughout the duration of the experiment. At day 2, *V. parahaemolyticus tlh* had significantly higher viable counts compared with the other two strains, with 6.45 log CFU/ml. At day 4, *V. parahaemolyticus tdh* counts were higher than those of the other two strains. At day 6 and day 8, the results showed that *V. parahaemolyticus tdh* had the highest counts, followed by the *V. parahaemolyticus trh* and *V. parahaemolyticus tlh* strains. By day 10, *V. parahaemolyticus tdh* had the highest counts, followed by the *V. parahaemolyticus tlh* and *V. parahaemolyticus trh* strains. From day 0 to day 4, a significant reduction of *V. parahaemolyticus trh* strains counts was observed.

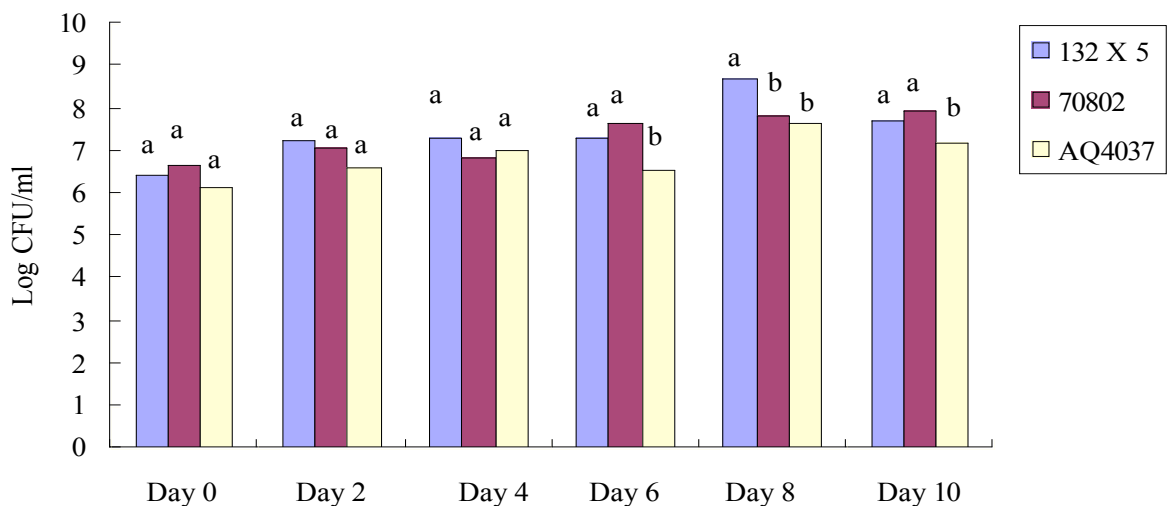


**Figure 1. Visible counts of *V. parahaemolyticus* strains after cold storage at 5°C in tryptic soy broth supplemented with 2% NaCl for 10 days**

Abbreviations of strains are as follows: 132 X 5 = *Vibrio parahaemolyticus tlh* strain, AQ4037 = *Vibrio parahaemolyticus trh* strain, 70802 = *Vibrio parahaemolyticus tdh* strain

All analysis were based on three separate experiments. Means within day followed by the same letter are not significantly different ( $P = 0.05$ ) from each other. Statistical comparisons of all pairs were analyzed using Student's *t*-test following one-way analysis of the variance (ANOVA) (SAS Institute, Inc., Cary, NC).

All strains survived well during the experiment at 8°C (Figure 2). At day 6, *V. parahaemolyticus trh* strain had the lowest counts compared with the other two strains, with 6.57 log CFU/ml. At day 8, *V. parahaemolyticus tlh* strain had the highest counts compared with the other two strains, with 8.68 log CFU/ml. At day 10, *V. parahaemolyticus trh* strains had the lowest counts compared with the other two strains, with 7.18 log CFU/ml. The *V. parahaemolyticus trh* strain had a significant increase in visible counts from day 0 to day 4.

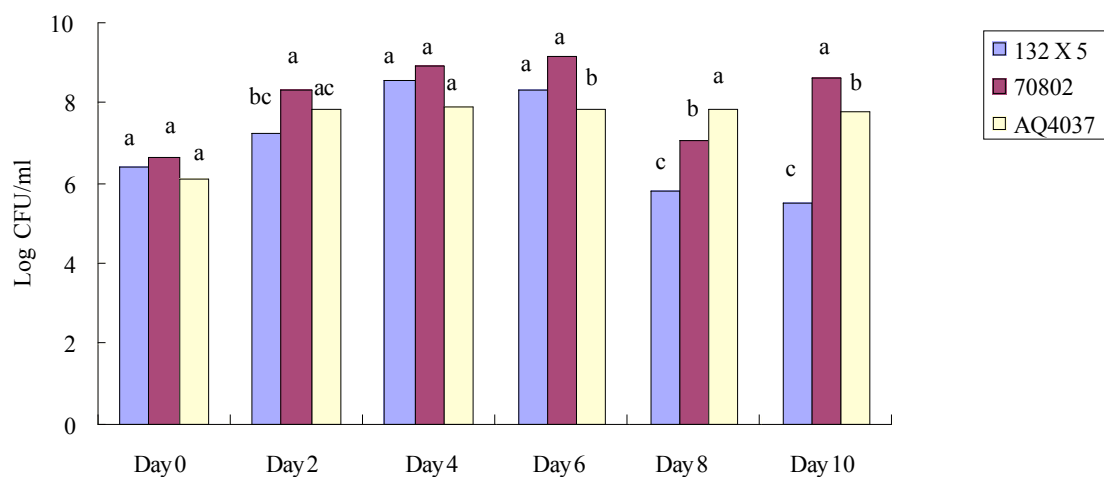


**Figure 2. Visible counts of *V. parahaemolyticus* strains after cold storage at 8°C in tryptic soy broth supplemented with 2% NaCl for 10 days**

Abbreviations of strains are as follows: 132 X 5 = *Vibrio parahaemolyticus tlh* strain, AQ4037 = *Vibrio parahaemolyticus trh* strain, 70802 = *Vibrio parahaemolyticus tdh* strain

All analysis was based on three separate experiments. Means within day followed by the same letter are not significantly different ( $P = 0.05$ ) from each other. Statistical comparisons of all pairs were analyzed using Student's *t*-test following one-way analysis of the variance (ANOVA) (SAS Institute, Inc., Cary, NC).

There were differences in growth among the various strains throughout the duration of the experiment at 10°C (Figure 3). At day 2, *V. parahaemolyticus tlh* strain had lower counts than those of *V. parahaemolyticus tdh* strain, with 7.26 log CFU/ml and 8.33 log CFU/ml, respectively. At day 6, *V. parahaemolyticus trh* strain had lower counts compared with the other two strains, with 7.85 log CFU/ml. At day 8, *V. parahaemolyticus trh* strains had the highest counts of 7.84 log CFU/ml followed by the *V. parahaemolyticus tdh* and *V. parahaemolyticus tlh* strains. At day 10, *V. parahaemolyticus tdh* strain had the highest counts of 8.62 log CFU/ml, followed by the *V. parahaemolyticus trh* and *V. parahaemolyticus tlh* strains.



**Figure 3. Visible counts of *V. parahaemolyticus* strains after cold storage at 10°C in tryptic soy broth supplemented with 2% NaCl for 10 days**

Abbreviations of strains are as follows: 132 X 5 = *Vibrio parahaemolyticus tlh* strain, AQ4037 = *Vibrio parahaemolyticus trh* strain, 70802 = *Vibrio parahaemolyticus tdh* strain

All analysis was based on three separate experiments. Means within day followed by the same letter are not significantly different ( $P = 0.05$ ) from each other. Statistical comparisons of all pairs were analyzed using Student's *t*-test following one-way analysis of the variance (ANOVA) (SAS Institute, Inc., Cary, NC).

## DISCUSSION

Different *V. parahaemolyticus* strains containing different genes grown at 5, 8 or 10°C showed significantly different growth and survival at different stages of storage at all the temperatures tested. These differences were evident throughout the 10 days of the experiment. Our results showed that at 5°C all three strains were able to survive but not grow. On the other hand, all three strains were able to survive and grow at 8°C and 10°C, except the *Vibrio parahaemolyticus* strain 132 X 5 at day 8 and day 10 with 10°C storage. It seems that survival or growth differed among the *Vibrio parahaemolyticus* strains under different refrigeration temperature storage.

The results showed that the three different *V. parahaemolyticus* cultures stored at 5°C were not able to grow. The reasons may be the dying of cells or with the temperature downshift, the cells entering a viable but nonculturable (VBNC) state and the ability to produce hemolysin did not affect entrance into the VBNC state (Bates and Oliver, 2004). VBNC state is a survival strategy adopted by bacteria when exposed to environmental stresses capable of inducing cell growth inhibition and cell death (Del Mar Lleo and others 2007) The VBNC cells are highly resistant to adverse environmental conditions such as low temperature, low salinity, and low pH (Wong and Wang, 2004) and therefore it's a great threat to human health. The ability may occur because of the presence of rpoS in *Vibrio parahaemolyticus*; alternate sigma factor of RNA polymerase is believed to potentially play

an important role in the survival of *Vibrio parahaemolyticus* under conditions of cold stress (Vasudevan and Venkitanarayanan, 2006). In our study, the bacterial counts under 8 and 10°C compared with those at 5°C were relatively higher, which is probably due to the fact that VBNC cells could be resuscitated by temperature up-shift with and without the presence of nutrition (Du and others 2007). Our results agreed with Miles et al. (1997) who reported that the minimum observed temperature for growth of *V. parahaemolyticus* was 8.3°C. With the ability for *Vibrio parahaemolyticus* to grow above 8°C, the enhanced stress resistance of the VBNC cells should draw our attention to the increased risk presented by *V. parahaemolyticus* in the food industry.

Variation in survival rates that have been seen among the different strains could be due to the different structure of different strains. Our research has shown that various *V. parahaemolyticus* strains containing different genes vary significantly in their ability to survive and grow at 5°C, 8°C and 10°C. These data may be useful in updating risk assessment models for these pathogens.

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**CHAPTER 5**  
**CONCLUSION**

Results obtained from this study have shown that there were significantly different cold adaption behaviors in clinical and environmental strains of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in oysters. For *Vibrio vulnificus*, on day 4, 6, 8, and 14, the latter stages of the experiment, clinical strains tended to have higher counts than those of environmental strains. This observation indicates that *Vibrio vulnificus* clinical strains may be more adaptable to refrigerated temperature and may pose increased risk from a food safety standpoint than environmental strains. As for temperature treatment effects on *Vibrio vulnificus*, even though differences were observed on day 2, 4, and 6, they were not consistent, reflecting that the cooling methods may not have major effects on *Vibrio vulnificus*.

The effect of different cold adaption behaviors between *Vibrio parahaemolyticus* clinical and environmental strains were not consistent enough to prove there were differences between clinical and environmental strains of *Vibrio parahaemolyticus* in their ability to adapt to refrigerated temperature. However, *Vibrio parahaemolyticus* that had been gradually cooled had generally higher counts than the other two cooling methods indicating that *Vibrio parahaemolyticus* subjected to the gradual temperature reduction method was more likely to cause food safety problems.

Results obtained from the second part of this study showed that *V. parahaemolyticus* strains with different genes grown in tryptic soy broth exhibit significant differences in

growth and survival on a daily basis when stored at 5, 8 and 10°C. The *V. parahaemolyticus* strains survived but did not grow when shifted from 37°C to storage at 5°C. At 8 and 10°C however; *Vibrio parahaemolyticus* strains were able to grow except for the *Vibrio parahaemolyticus* strain 132 X 5. Adaptation responses varied between strains. At 5°C, the counts of strain 70802 were significantly higher than the other *V. parahaemolyticus* strains, indicating that the 70802 strain had greater adaptability at 5°C. At 8°C and 10°C, there were no consistent differences between strains. However, the results showed that the 132X5 strain on the last two days of storage at 10°C had significantly lower counts than the other two strains. In addition, even though the different storage temperatures weren't statistically compared, 8°C storage temperature appeared to have the highest counts for some of the strains at specific days among the three different temperatures.

These results may be useful for providing evidence for cold adaption behaviors of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in oysters at refrigerated temperature and for *Vibrio parahaemolyticus* stains that possess different genes.

## **VITA**

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