

2010

Antimicrobial Susceptibility, Genotypic Characterization, and Molecular Detection of *Vibrio parahaemolyticus* and *Vibrio vulnificus* from Louisiana Oysters

Feifei Han

Louisiana State University and Agricultural and Mechanical College, fhan1@lsu.edu

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_dissertations



Part of the [Life Sciences Commons](#)

Recommended Citation

Han, Feifei, "Antimicrobial Susceptibility, Genotypic Characterization, and Molecular Detection of *Vibrio parahaemolyticus* and *Vibrio vulnificus* from Louisiana Oysters" (2010). *LSU Doctoral Dissertations*. 2549.

https://digitalcommons.lsu.edu/gradschool_dissertations/2549

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Doctoral Dissertations by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.

**ANTIMICROBIAL SUSCEPTIBILITY, GENOTYPIC
CHARACTERIZATION, AND MOLECULAR DETECTION OF
VIBRIO PARAHAEMOLYTICUS AND *VIBRIO VULNIFICUS*
FROM LOUISIANA OYSTERS**

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Food Science

by
Feifei Han
B.S., China Agricultural University, 2004
August 2010

ACKNOWLEDGEMENTS

The major academic accomplishments of this project could not have been achieved without many people. I am deeply grateful to my advisor Dr. Beilei Ge for her mentoring, encouragement, and support throughout my Ph.D. study. There is no doubt in my mind that her talent, diligence, and persistence have profoundly shaped me and will continue to impact me in the future. I also would like to thank all of my committee members for their suggestions and support. I thank Dr. Witoon Prinyawiwatkul for his guidance through my Ph.D. study and for supporting me for all scholarship application. I thank Dr. Joan King for instructing my courses and advising my research. I thank Dr. Aixin Hou for allowing me to use the gel document system. I also would like to thank Dr. Samithamby Jeyaseelan as dean's rep. It has been an honor and privilege for me to have them on my committee, which I could not have asked for a better one. I wish to express my most sincere gratitude to our Graduate Secretary Ms. Terri Gilmer for all her support and encouragement during my graduate studies. Thanks the U.S. Department of Agriculture Aquaculture Special Grant Program and the Louisiana Sea Grant Omnibus Program for funding my dissertation research.

Thanks also go to my laboratory fellows and friends, Mr. Fei Wang, Ms. Siyi Chen, Ms. Lin Jiang, Ms. Qianru Yang, and Mr. Shuaihua Pu for their help on experiments. Thanks to my friend Dr. Fei Yao and Ms. Yunyun Lu for all their help during my study in the U.S.

I am deeply indebted to my parents, Mr. Zhende Han, Ms. Zengxiu Li, and my sister Yafei Han, for their patience, encouragement, understanding, and deep love. They support every single decision I made and they are proud of every tiny achievement I earned. They deserve my deepest appreciation and respect.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	ii
LIST OF TABLES	v
LIST OF FIGURES	vii
ABSTRACT.....	ix
CHAPTER 1: INTRODUCTION.....	1
CHAPTER 2: LITERATURE REVIEW.....	4
Epidemiology.....	5
Microbiology	7
Clinical Symptoms.....	8
Prevalence in Seafood.....	9
Antimicrobial Susceptibility Profiles	10
Virulence Properties.....	12
Isolation and Identification Methods	14
References	19
CHAPTER 3: PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITY OF <i>VIBRIO</i> <i>PARAHAEMOLYTICUS</i> AND <i>VIBRIO VULNIFICUS</i> FROM LOUISIANA GULF AND RETAIL RAW OYSTERS.....	28
Introduction	29
Materials and Methods	31
Results.....	35
Discussion	41
Conclusion	46
References	47
CHAPTER 4: GENOTYPIC CHARACTERIZATION OF <i>VIBRIO VULNIFICUS</i> ISOLATES FROM LOUISIANA GULF AND RETAIL RAW OYSTERS	51
Part I: Characterization of Clinical and Environmental Types of <i>Vibrio vulnificus</i> Isolates from Louisiana Oysters	52
Introduction	53
Materials and Methods	56
Results.....	59
Discussion	65
Conclusion	69
References	69
Part II: Multiplex PCR Assays for Simultaneous Detection and Characterization of <i>Vibrio</i> <i>vulnificus</i> Strains.....	73
Introduction	74
Materials and Methods	76
Results.....	77

Discussion	82
Conclusion	86
References	86
CHAPTER 5: MOLECULAR DETECTION OF <i>VIBRIO VULNIFICUS</i> ISOLATES FROM LOUISIANA GULF AND RETAIL RAW OYSTERS	89
Part I: Evaluation of a Loop-Mediated Isothermal Amplification Assay for Detecting <i>Vibrio vulnificus</i> in Retail Raw Oysters.....	90
Introduction	91
Materials and Methods	93
Results.....	99
Discussion	102
Conclusion	105
References	106
Part II: Quantitative Detection of <i>Vibrio vulnificus</i> in Raw Oysters by Real-Time Loop-Mediated Isothermal Amplification	110
Introduction	111
Materials and Methods	112
Results.....	117
Discussion	120
Conclusion	125
References	125
Part III: Quantitative Detection of Virulent-Type <i>Vibrio vulnificus</i> in Raw Oysters by Real-Time Loop-Mediated Isothermal Amplification	130
Introduction	131
Materials and Methods	133
Results.....	139
Discussion	144
Conclusion	147
References	147
CHAPTER 6: CONCLUSIONS	151
APPENDIX: COPYRIGHT PERMISSION LETTERS	154
VITA.....	169

LIST OF TABLES

Table 3 - 1 Prevalence of <i>Vibrio parahaemolyticus</i> and <i>Vibrio vulnificus</i> in 94 Louisiana Gulf and Retail Oysters.....	38
Table 3 - 2 Prevalence of <i>Vibrio parahaemolyticus</i> and <i>Vibrio vulnificus</i> by month of isolation, in oyster samples obtained from the Louisiana Gulf Coast and retail outlets in 2005-2006	38
Table 3 - 3 Antimicrobial susceptibility testing ranges, breakpoints, and MIC distribution for 319 <i>Vibrio parahaemolyticus</i> and <i>Vibrio vulnificus</i> isolates for eight antimicrobials tested	40
Table 3 - 4 The presence of ampicillin-resistant and intermediate <i>Vibrio parahaemolyticus</i> isolates from Louisiana Gulf and retail oysters among 168 <i>Vibrio parahaemolyticus</i> isolates included in the susceptibility testing.....	41
Table 4 - 1 <i>Vibrio vulnificus</i> reference strains used in this study, their source of isolation, and genotypic characteristics based on the virulence-correlated gene (<i>vcg</i>), 16S rRNA, capsular polysaccharide operon (CPS), and the <i>vluB</i> -associated fragment	56
Table 4 - 2 PCR primers used to characterize <i>Vibrio vulnificus</i> genotypes.....	58
Table 4 - 3 Distributions of genotypic profiles among 349 <i>Vibrio vulnificus</i> oyster isolates based on genotypic characteristics of three loci (<i>vcg</i> , 16S rRNA, and CPS)	60
Table 4 - 4 Associations between environmental and clinical <i>Vibrio vulnificus</i> genotypes based on analysis of each of the three loci (<i>vcg</i> , 16S rRNA, and CPS) and the presence/absence of the <i>vluB</i> -associated fragment among 349 <i>V. vulnificus</i> oyster isolates	62
Table 4 - 5 Predictions of clinically important <i>Vibrio vulnificus</i> isolates from Louisiana oysters based on various combinations of the four biomarkers used in this study	63
Table 4 - 6 Ten <i>Vibrio vulnificus</i> reference strains used for multiplex PCR.....	78
Table 4 - 7 <i>Vibrio vulnificus</i> oyster isolates used in the specificity test of multiplex PCR.....	79
Table 4 - 8 Multiplex PCR primers to simultaneously detect and characterize <i>Vibrio vulnificus</i> strains	80
Table 5 - 1 Bacterial strains used in this study, their source, and specificity of the <i>Vibrio vulnificus</i> cytolysin/hemolysin (<i>vvhA</i>) gene amplification by LAMP and PCR.....	95
Table 5 - 2 Comparison of sensitivities of LAMP and PCR in detecting <i>Vibrio vulnificus</i> 1007 in pure cultures and seeded oyster samples	101

Table 5 - 3 Bacterial strains used in real-time LAMP study to detect total *Vibrio vulnificus* .. 113

Table 5 - 4 Bacterial strains used in LAMP to detect virulent-type *Vibrio vulnificus*..... 134

LIST OF FIGURES

Figure 2 - 1 Relative rates of laboratory-confirmed infections with <i>Campylobacter</i> , STEC O157, <i>Listeria</i> , <i>Salmonella</i> , and <i>Vibrio</i> compared with 1996--1998 rates, by year --- Foodborne Diseases Active Surveillance Network (FoodNet), United States, 1996—2009 (Centers for Disease Control and Prevention, 2010)	5
Figure 2 - 2 <i>Vibrio parahaemolyticus</i> and <i>Vibrio vulnificus</i> cases (excluding toxigenic <i>Vibrio cholerae</i>) reported to COVIS, 1997-2007	6
Figure 2 - 3 Numbers of cases of <i>Vibrio</i> illnesses (excluding toxigenic <i>V. cholerae</i>), by month of illness onset or specimen isolation, 2007 (N = 549) (Cholera and Other <i>Vibrio</i> Illness Surveillance, 2009)	7
Figure 2 - 4 Loop-mediated isothermal amplification (LAMP) procedure	18
Figure 4 - 1 Ratios of clinical-type and environmental-type of <i>Vibrio vulnificus</i> oyster isolates by the month of isolation when analyzed by <i>vcg</i> (A), 16S rRNA (B), CPS (C), <i>viuB</i> -associated fragment (D), and a combination of all four markers (E).....	64
Figure 4 - 2 An agarose gel showing multiplex PCR amplification products using typical virulent- and non-virulent-type <i>Vibrio vulnificus</i> strains.....	81
Figure 5 - 1 Partial nucleotide sequence of the cytolysin/hemolysin (<i>vvhA</i>) gene of <i>Vibrio vulnificus</i> EDL174 (accession number M34670), six target regions, and four primers used in the LAMP assay.....	99
Figure 5 - 2 Sensitivity of <i>vvhA</i> LAMP (A) and PCR (B) for detecting <i>Vibrio vulnificus</i> 1007 in pure cultures.....	100
Figure 5 - 3 SYBR Green I fluorescent dye-mediated visualization of LAMP amplification products by the naked eye under normal light (A) and UV (B).....	102
Figure 5 - 4 Sensitivity of the fluorescence-based real-time LAMP when detecting <i>Vibrio vulnificus</i> ATCC 27562 in pure culture.....	118
Figure 5 - 5 Sensitivity of the turbidity-based real-time LAMP when testing the same set of <i>Vibrio vulnificus</i> ATCC 27562 DNA templates.....	119
Figure 5 - 6 Quantitative detection of <i>Vibrio vulnificus</i> ATCC 27562 in spiked oysters by using fluorescence-based and turbidity-based real-time LAMP.....	121
Figure 5 - 7 Partial nucleotide sequence of the <i>vcgC</i> type of <i>Vibrio vulnificus</i> (accession number AY626575).....	139
Figure 5 - 8 16S rRNA-LAMP using <i>Vibrio vulnificus</i> ATCC 33815 and WR1.....	140

Figure 5 - 9 Comparison between optimized LAMP and prototype LAMP using *Vibrio vulnificus* ATCC 33815. 141

Figure 5 - 10 Sensitivity of *vcgC* real-time LAMP and PCR when testing serial-diluted *Vibrio vulnificus* ATCC 33815 DNA templates. 142

Figure 5 - 11 Quantitative detection of *vcgC* real-time LAMP and PCR using *Vibrio vulnificus* ATCC 33815 in spiked oysters. 143

ABSTRACT

Members of the genus *Vibrio* are Gram-negative, halophilic bacteria that inhabit warm coastal and estuarine waters worldwide. Among pathogenic vibrios, *Vibrio parahaemolyticus* is the leading cause of seafood-related illnesses and *Vibrio vulnificus* causes the highest number of seafood-related deaths in the United States. Moreover, according to the U.S. Centers for Disease Control and Prevention, the incidence of infections of the two vibrios due to the consumption of oysters has shown a sustained increase since 2001, indicating further measures are needed to prevent human *Vibrio* illness.

In this dissertation research, a total of 622 *Vibrio* isolates, consisting of 252 *V. parahaemolyticus* and 370 *V. vulnificus*, were recovered from 82 Louisiana Gulf and retail raw oysters between 2005 and 2006. A selected subset of the isolates (168 *V. parahaemolyticus* and 151 *V. vulnificus*) was determined for antimicrobial susceptibility profiles. In addition, *V. vulnificus* isolates ($n = 349$) were characterized by the presence/absence of a *viuB*-associated fragment and genotypes of three biomarkers: the virulence-correlated gene (*vcg*), 16S rRNA, and the capsular polysaccharide operon (CPS). Then multiplex PCR assays using three biomarkers: *vcg*, 16S rRNA and CPS, as well as species-specific *vvhA* were developed to simultaneously detect and characterize *V. vulnificus*. Finally, loop-mediated isothermal amplification (LAMP) assays were developed and evaluated to detect total or virulent-type *V. vulnificus* in raw oysters. Compared to PCR, LAMP assay developed were highly specific, sensitive and quantitative.

The dissertation research provided comprehensive information on the genotypes, population dynamics, and antimicrobial resistance profiles of the two important vibrios. The rapid, specific, sensitive, and cost-effective molecular detection assays developed provided invaluable tools for the regulatory agencies and seafood industry to facilitate better control of

Vibrio in seafood, therefore reducing the incidence of foodborne illnesses and deaths resulted from the consumption of raw oysters.

CHAPTER 1: INTRODUCTION

Members of the genus *Vibrio* are Gram-negative, halophilic, and curved-rod shape bacteria that inhabit warm coastal and estuarine waters worldwide, which are especially abundant in the gut of filter-feeding shellfish such as oysters, clams, and mussels. Among pathogenic vibrios, *Vibrio parahaemolyticus* causes the most seafood-associated bacterial gastroenteritis in the United States and Asian countries, while *Vibrio vulnificus* is responsible for more than 95% of seafood-related deaths in the U.S. Therefore, multi-faceted strategies are needed to reduce the number of human infections caused by the two important vibrios.

In this dissertation research, detection methods, genotypes, and antimicrobial susceptibility profiles of these two vibrios were investigated. Firstly, we isolated *V. parahaemolyticus* and *V. vulnificus* from Louisiana Gulf and retail oysters between 2005 and 2006. Secondly, we characterized their antimicrobial susceptibility profiles and genotypic patterns. Finally, we developed sensitive and specific molecular detection assays for the detection and quantification of total and pathogenic *V. vulnificus*, in order to facilitate the regulatory agencies and the seafood industry to better control the potential *Vibrio* risks in raw oyster and reduce the incidence of oyster-related foodborne illnesses and deaths.

This dissertation is organized as following:

Chapter 1-Introduction.

Chapter 2-Literature review on general information of *V. parahaemolyticus* and *V. vulnificus*, antimicrobial resistance profiles, and molecular detection.

Chapter 3-Prevalence and antimicrobial susceptibilities of *V. parahaemolyticus* and *V. vulnificus* isolates from Louisiana Gulf and retail raw oysters from 2005 to 2006.

Chapter 4-Genotypic characterization of *V. vulnificus* isolates from Louisiana Gulf and retail raw oysters. Part I shows the genotypic characterization of *V. vulnificus* from Louisiana

oyster isolates, focusing on the differentiation of clinical-type from environmental-type strains. Part II describes a multiplex PCR assay for the simultaneous detection and characterization of *V. vulnificus* strains.

Chapter 5-Development of loop-mediated isothermal amplification (LAMP) assays to detect total and virulent-type *V. vulnificus* strains. The LAMP assays developed include one conventional LAMP for total *V. vulnificus*, a real-time LAMP on two real-time platforms for the quantification of total *V. vulnificus*, and a real-time LAMP for the detection and quantification of virulent-type *V. vulnificus* strains in raw oysters.

Chapter 6-Conclusions.

CHAPTER 2: LITERATURE REVIEW

Epidemiology

Human infections with *Vibrio* occur all over the world (Bauer *et al.*, 2006; Dalsgaard *et al.*, 1996; Hoi *et al.*, 1998; Wong *et al.*, 1999). In the United States, it is estimated that *Vibrio* cause 8,000 cases, 200 hospitalizations, and 50 deaths each year (Mead *et al.*, 1999a). According to the Centers for Disease Control and Prevention (CDC)'s report, *Vibrio* infections due to the consumption of raw or undercooked oysters have shown an increase since year 2001, indicating the need to control human *Vibrio* infections (Figure 2-1) (Centers for Disease Control and Prevention, 2010). Among pathogenic vibrios, *V. parahaemolyticus* causes the highest number of seafood-associated bacterial gastroenteritis in the United States and Asian countries (Mead *et al.*, 1999b) while *V. vulnificus* is responsible for more than 95% of seafood-related deaths in the U.S. (Oliver, 2006).

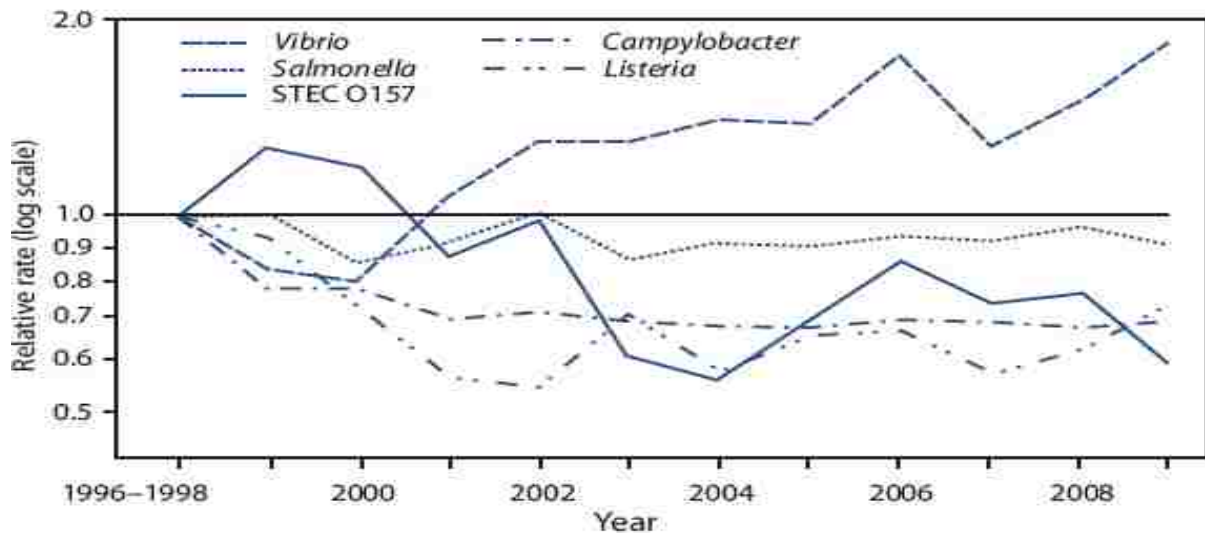


Figure 2 - 1 Relative rates of laboratory-confirmed infections with *Campylobacter*, STEC O157, *Listeria*, *Salmonella*, and *Vibrio* compared with 1996--1998 rates, by year --- Foodborne Diseases Active Surveillance Network (FoodNet), United States, 1996—2009 (Centers for Disease Control and Prevention, 2010)

Initiated by CDC, U.S. Food and Drug Administration (FDA), and the Gulf Coast states (Alabama, Florida, Louisiana, Mississippi, and Texas) in 1988, the Cholera and Other *Vibrio*

Illness Surveillance System (COVIS) has been collecting the information on *Vibrio* human infections in order to obtain reliable information on illnesses associated with *Vibrio* species. Figure 2-2 list the infection caused by *V. parahaemolyticus* and *V. vulnificus* reported to COVIS from 1997 to 2007.

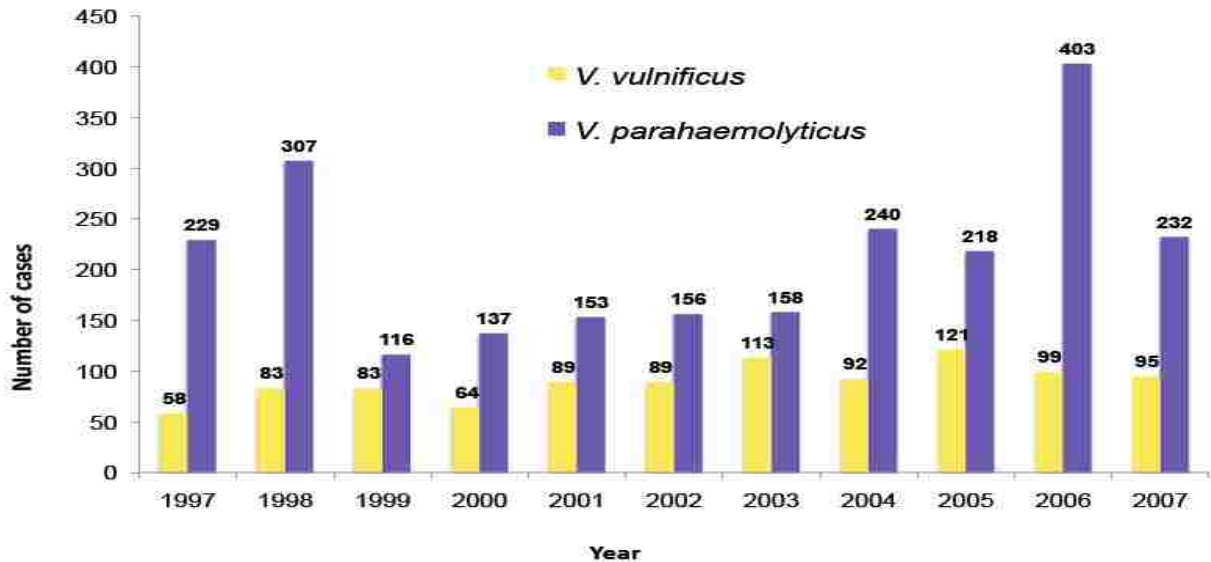


Figure 2 - 2 *Vibrio parahaemolyticus* and *Vibrio vulnificus* cases (excluding toxigenic *Vibrio cholerae*) reported to COVIS, 1997-2007

In 2007, a total of 549 *Vibrio* cases (excluding toxigenic *Vibrio cholerae*) were reported to COVIS, with 39% of patients hospitalized and 7% dies (Cholera and Other *Vibrio* Illness Surveillance, 2009). Among all of these cases, *V. parahaemolyticus* was the most frequently reported *Vibrio* species, isolated from 42% (232 of 549) of the patients; while *V. vulnificus* was isolated from 17% (95 of 549) of the patients, with a 36% mortality rate. Most *V. parahaemolyticus* and *V. vulnificus* infections had a clear seasonal peak during summer, with most cases (70%) occurring during May to September, and the greatest frequency happening during August (Figure 2-3).

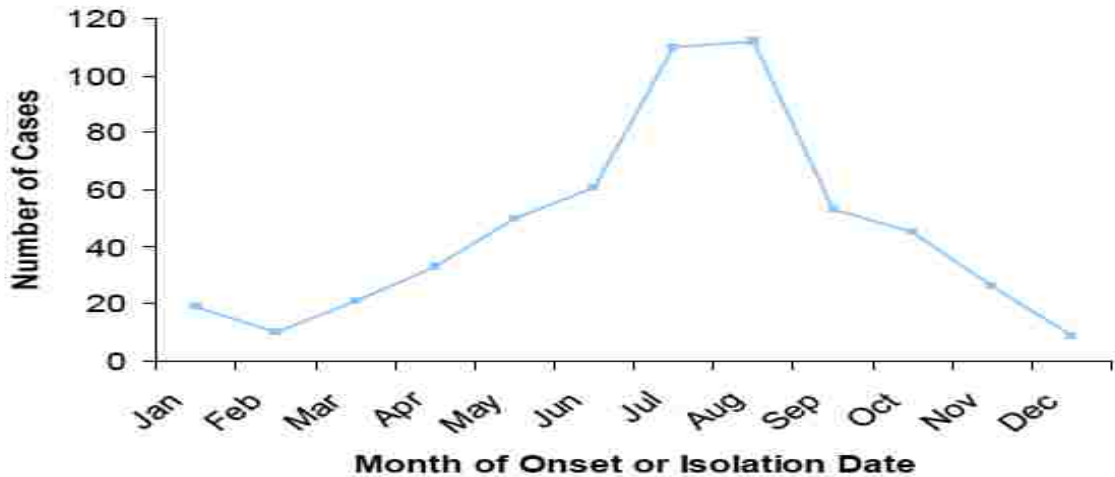


Figure 2 - 3 Numbers of cases of *Vibrio* illnesses (excluding toxigenic *V. cholerae*), by month of illness onset or specimen isolation, 2007 (N = 549) (Cholera and Other *Vibrio* Illness Surveillance, 2009)

Louisiana, as one of the five Gulf Coast states, reported a total of 1,007 *Vibrio* infections to the Infectious Disease Epidemiology Department of the Louisiana Office of Public Health between 1980 and 2005, with 249 (25%) *V. parahaemolyticus* and 257 (25%) *V. vulnificus* cases (Thomas *et al.*, 2007). The fatality rate for *V. parahaemolyticus* and *V. vulnificus* was 6% and 31%, respectively.

Given the number of illnesses and deaths caused by these two vibrios, research is needed to provide data and strategies to better control the potential *Vibrio* risks in seafood, particularly oysters.

Microbiology

V. parahaemolyticus and *V. vulnificus* are Gram-negative, motile bacteria that inhabit warm coastal and estuarine waters worldwide, especially in the gut of filter-feeding shellfish such as oysters, clams and mussels (Barbieri *et al.*, 1999; Hervio-Heath *et al.*, 2002; Hoi *et al.*, 1998). Both *V. parahaemolyticus* and *V. vulnificus* are halophilic, requiring a minimum of 0.5% NaCl for growth (Kaysner & DePaola, 2001). They are mesophilic and proliferate in warm water

(Cook *et al.*, 2002b). A seasonal preference has been demonstrated on all coasts in the U.S., with the most abundant presence during warm weather ($> 20^{\circ}\text{C}$) and moderate salinity (5 to 25 ppt) (Bryan *et al.*, 1999; DePaola *et al.*, 2003a; Kaspar & Tamplin, 1993). In winter when the temperature is below 10°C , they would enter a status termed viable but nonculturable (VBNC), during which they will lose the ability to be cultured on routine media but retain their viability (Bates & Oliver, 2004; Oliver *et al.*, 1995)

Both of these two vibrios are sucrose-negative, thus forming green colonies on thiosulfate citrate bile salts sucrose agar (TCBS) (Kobayashi *et al.*, 1963). However, *V. vulnificus* is cellobiose positive while most other vibrios including *V. parahaemolyticus* are cellobiose negative. Therefore, modified cellobiose polymyxin B colistin (mCPC) agar, which takes advantage of the resistance of *V. vulnificus* to colistin and polymyxin B and the fermentation of cellobiose, has been reported to be effective for the isolation of *V. vulnificus* from environmental sources (Massad & Oliver, 1987; Tamplin *et al.*, 1991).

Clinical Symptoms

The clinical symptoms of *V. parahaemolyticus* infection include watery diarrhea, abdominal cramps, nausea, vomiting, headache, fever, and chills, which may last for 2 to 10 days and are normally self-limiting (Iida *et al.*, 2006).

V. vulnificus, on the other hand, causes gastroenteritis, wound infections, and primary septicemia. Gastroenteritis is the mildest human syndrome among the three, usually occurring within 16 hours of ingesting the organism and mostly needing no hospitalization. In contrast, primary septicemia, the most significant form of *V. vulnificus* infection, had $> 50\%$ motility rate, especially for at-risk groups, which include people with immunocompromising conditions, diabetes, and elevated serum iron concentrations due to chronic liver disease or alcohol abuse

(Wright *et al.*, 1981). Furthermore, most cases (> 88.5% of the U.S. cases between 2000 and 2003) of septicemia related to *V. vulnificus* infection are males over the age of 50, suggesting the protective effect of women's estrogen (Oliver, 2006). Symptoms of wound infection are pain, erythema, and edema, with 20% to 25% fatality rate.

Prevalence in Seafood

The prevalence of *V. parahaemolyticus* and *V. vulnificus* in seafood varies greatly depending on the variety of seafood, geographic area and temperature. It has been reported that *V. vulnificus* abundance was 32-2,511 cells/ml in Mobile Bay, Alabama (DePaola *et al.*, 1994), 0-200 cells/ml in Chesapeake Bay, Delaware (Wright *et al.*, 1996), and 0-7,000 cells/ml in Apalachicola Bay, Florida (Tamplin *et al.*, 1982). In oyster sample, it was reported that 0-12,000 colony forming units per gram (CFU/g) of *V. parahaemolyticus* was found in Alabama oysters from March 1999 to September 2000 (DePaola *et al.*, 2003a). A national survey from June 1998 to July 1999 on *V. parahaemolyticus* and *V. vulnificus* in 370 oysters was conducted in coastal and markets throughout the U.S. and the highest densities of both organisms were found in oysters harvested from the Gulf Coast, which normally exceeded 10,000 MPN/g (Cook *et al.*, 2002b). One following study carried from 1999 to 2000 in Atlantic and Gulf Coast molluscan shellfish found 5% of the samples contained more than 1,000 CFU/g *V. parahaemolyticus*, also Gulf Coast had higher densities of *V. parahaemolyticus* in shellfish than that from the Atlantic (Cook *et al.*, 2002a). A recent study by FDA showed approximately 44% and 38% of samples harvested from Louisiana in 2007 sampled from different lots exceeded 10,000 MPN/g for *V. vulnificus* and *V. parahaemolyticus*, respectively, and Gulf oysters had significantly higher *V. vulnificus* levels than either the North Atlantic and Pacific regions during each season (DePaola *et al.*, 2010).

The occurrence of *V. parahaemolyticus* and *V. vulnificus* in Norwegian blue mussels was 10.3% and 0.1%, respectively after examining 885 blue mussel samples (Bauer *et al.*, 2006). In Japan, 2-13% *V. parahaemolyticus* and 1-4% *V. vulnificus* was dominant in shrimp samples from multiple shrimp farm environment (Gopal *et al.*, 2005). In China, a study showed 73.3% (165 of 225) of seafood samples were positive for *V. parahaemolyticus*, with a range of 0-719 CFU/g (Luan *et al.*, 2008).

Since multiple studies have showed Gulf Coast oysters normal contained significantly high level of *V. parahaemolyticus* and *V. vulnificus*, and most current studies sampled oysters from Coast regions, lacking the information from restaurant site, it is important to monitor both of the two species from oysters sampled in Gulf Coast and retail sites. However, the last comprehensive study could only trace to FDA's studies in 2000s, so the prevalence of *V. parahaemolyticus* and *V. vulnificus* in Gulf Coast oysters need to be studied to provide more information for the risk assessment of the two species.

Antimicrobial Susceptibility Profiles

Since the introduction of penicillin in the 1920s, hundreds of antimicrobial agents have been discovered, synthesized, and applied for either clinical use or food animals for therapy, prophylaxis, and growth promotion (Aarestrup & Wegener, 1999). However, as a result, the occurrence of antimicrobial resistance of bacteria in food animals has been reported to be associated with the consumption of antimicrobial agents in animal husbandry, which could cause the failure of clinical therapy (Aarestrup, 1999).

Traditionally, *Vibrio* is considered to be susceptible to all antibiotics, except that ampicillin-resistance was observed in *V. parahaemolyticus* and *V. vulnificus* (Joseph *et al.*, 1978; Zanetti *et al.*, 2001). However, some studies indicated that antimicrobial resistance has been

surfaced into *V. parahaemolyticus* and *V. vulnificus* in Austria (Akinbowale *et al.*, 2006), the Philippines and Thailand (Maluping *et al.*, 2005) by characterizing the antimicrobial susceptibility profile of these two vibrios. One study in the past found very high ampicillin resistance rate (> 90% for *V. parahaemolyticus*), indicating the exhibition of β -lactamase activity (Joseph *et al.*, 1978). Another study in Italy tested antibiotics against eight *V. parahaemolyticus*, six *V. vulnificus* and some other *Vibrio* strains isolated in the coastal waters of Italy. They found that more than 80% of *Vibrio* isolates were resistant to ampicillin (Zanetti *et al.*, 2001). A similar susceptibility profile was found in *V. parahaemolyticus* from patients in Indonesia with 98% resistance rate to ampicillin while all isolates (100%) were sensitive to chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, gentamicin, and ciprofloxacin (Lesmana *et al.*, 2001). In 2008, a study screened 151 coastal isolated *V. vulnificus* and 10 primary septicemia isolates against 26 antimicrobial agents. Surprisingly, 45% (68 of 151) of the environmental sourced *V. vulnificus* strains were resistant to three or more classes of antibiotics (Baker-Austin *et al.*, 2009). However, whether *V. parahaemolyticus* and *V. vulnificus* has become more resistant is unknown.

In clinical therapy, tetracycline has been recommended as the antimicrobial of choice to treat severe *V. parahaemolyticus* and *V. vulnificus* infections (Morris & Tenney, 1985), and alternative treatments are a combination of a third-generation cephalosporin (e.g., ceftazidime) and doxycycline, or a fluoroquinolone alone (Tang *et al.*, 2002). Trimethoprim-sulfamethoxazole plus an aminoglycoside are used to treat children in whom doxycycline and fluoroquinolones are contraindicated (Centers for Disease Control and Prevention, 2005).

Considering few recent studies have investigated the antimicrobial susceptibility testing on these two species, especially information is lacking in the U.S. since the 1990s, research is

needed to test first-line drugs if they remain highly effective against both *V. parahaemolyticus* and *V. vulnificus*.

Virulence Properties

About 400-500 cases of *Vibrio* infections were reported to CDC annually; however, considering millions pounds of oyster meats consumed in the U.S. each year, it is hypothesized that not all environmental *V. parahaemolyticus* and *V. vulnificus* are virulent (Chatzidaki-Livanis *et al.*, 2006a).

For *V. parahaemolyticus*, the thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) have been considered the major virulent factors by multiple studies, and most clinical strains carry one or both of the two hemolysins (DePaola *et al.*, 2000; Takahashi *et al.*, 2000), while less than 1% of food or environmental strains have the *tdh* gene coding for TDH (DePaola *et al.*, 2003a). One study showed that most clinical isolates from the Pacific Northwest of the U.S. are likely to have both *tdh* and *trh* genes (DePaola *et al.*, 2003b). The prevalence of pathogenic *V. parahaemolyticus* varies from 6% in Atlantic and Gulf coast mollusk shellfish (Cook *et al.*, 2002a) to 21.8% in Alabama oyster (DePaola *et al.*, 2003a) depending on the environmental parameters such as area, water temperature, and salinity.

For *V. vulnificus*, several potential virulent factors have been examined which included the lipopolysacchride (LPS), flagella, and capsular polysaccharide (CPS) (Lee *et al.*, 2004; Linkous & Oliver, 1999; Strom & Paranjpye, 2000). LPS, endotoxin, was the factor reported to cause shock and death by *V. vulnificus* infection (Oliver, 2006). A study tested intravenous injections of *V. vulnificus* LPS into rats and the results showed decreased heart rate and blood pressure within 10 min and further decline lead to death in 30 to 60 min (McPherson *et al.*, 1991). A follow up study found the lethal effect was reversed after using a LPS-induced enzyme

(nitric oxide synthase) (Elmore *et al.*, 1992). The presence and amount of CPS on any given virulent isolate has been positively correlated with measures of virulence of *V. vulnificus* in mouse model (Wright *et al.*, 1990; Yoshida *et al.*, 1985), which were demonstrated that the presence of CPS protected the bacteria by conferring resistance to the bactericidal effects of serum and phagocytosis by macrophages (Johnson *et al.*, 1984; Yoshida *et al.*, 1985). However, whether one or more of those factors play an important role in the virulence of *V. vulnificus* remains to be determined.

Given that unique virulence markers have not been identified, alternative strategies are sought. Recently, several biomarkers have been explored to differentiate virulent- (i.e., clinical-) from non-virulent- (i.e., environmental-) type *V. vulnificus* strains with varied degree of success. A 200- to 178- bp segment was observed always present in the clinical strains while the segment was occasionally present in the environmental isolates (Warner & Oliver, 1999). A follow up study using the virulence-correlated gene (*vcg*) to screen 55 strains found that 90% of *vcgC*-type (correlates with clinical origin) strains were clinical isolates, while 93% of environmental isolates were *vcgE*-type (correlates with environmental origin) (Rosche *et al.*, 2005). The same group analyzed the RAPD genotype of *V. vulnificus* isolates from oysters and seawater and a total of 880 isolates from oysters confirmed the previous study by showing 84.4% of the isolates revealed the *vcgE* type, while 292 isolates from the seawater showed almost equal distribution among the two genotypes of *vcgE* and *vcgC* (Warner & Oliver, 2008). Meanwhile, the polymorphisms discrimination of the 16S rRNA has been reported as a virulence indicator between the clinical and the environmental *V. vulnificus* isolates (Nilsson *et al.*, 2003). One study in 2003 examined 33 nonclinical isolates and 34 clinical isolates by terminal restriction fragment length polymorphism (T-RFLP) of the 16S rRNA gene, and they found the majority (31 of 33) of

the nonclinical strains belonged to 16S rRNA A type, while 26 of 34 clinical ones were 16S rRNA B type (Nilsson *et al.*, 2003). The same group did a following study by combining the T-RFLP with real-time to be a new assay to analyze a total of 86 *V. vulnificus* isolates including 67 from the previous study strain collection (Vickery *et al.*, 2007). Surprisingly, they found 15 strains, which were previously found as 16S rRNA A type showed as both A and B. A recent study characterized the polysaccharide operon by identifying two CPS allele 1 and 2 (Chatzidaki-Livanis *et al.*, 2006b), and a following study indicated significant relationship between the clinical and capsular 1, while environmental isolates were predominantly capsular 2 (Chatzidaki-Livanis *et al.*, 2006a). Beside the three biomarkers, a vulnibactin-encoding *viuB* gene was reported to closely correlate with clinical-type *V. vulnificus* isolates in shellfish (Panicker *et al.*, 2004c), one of the siderophore genes involved in iron acquisition (Litwin *et al.*, 1996). However, none of those could serve as a unique virulence marker that to be used to screen for virulent *V. vulnificus* isolates from oysters.

Given the lack information of the virulent factors, research is needed to apply single or a combination of biomarkers for the identification of virulent-type *V. parahaemolyticus* and *V. vulnificus* in seafood, especially oysters.

Isolation and Identification Methods

Traditional isolation and detection methods for *V. parahaemolyticus* and *V. vulnificus*, approved by FDA as standard methods, are mainly based on microbiological culturing methods; however, they are labor-intensive and time-consuming (Kaysner & DePaola, 2001). DNA probe hybridization, also approved by FDA, is more rapid and specific, but it still takes 2-3 days (Morris *et al.*, 1987; Nordstrom *et al.*, 2006). The detection limit by DNA hybridization was between 10^2 to 10^4 CFU/g of oyster (Raghunath *et al.*, 2007; Wright *et al.*, 1993).

Immunological-based assays, such as enzyme-linked immunosorbent assay (ELISA) used the monoclonal antibody and achieved a detection limit of 2.0×10^3 cells (Tamplin *et al.*, 1991). A recent study on the use of anti-flagella protein monoclonal antibody to detect *V. parahaemolyticus* exhibited about 35% to 45% binding of 10^2 to 10^3 cells in phosphate-buffered saline (Datta *et al.*, 2008). One problem of conventional ELISA is that the antibody for antigen capture is nonspecifically and physically adsorbed onto ELISA plates, thus the antigen captured by the antibody may easily detach from the ELISA plates during subsequent processing, so chemically immobilizing antibody on nylon was developed to ensure the stable immobilization of sufficient amounts of antibodies on nylon (Honda *et al.*, 1995).

ELISA to detect *V. vulnificus* hemolysin has been reported, however, cross-reaction with other *Vibrio* (*Vibrio campbelli*, *Vibrio harveyi*, *Vibrio pelagius*, and *Vibrio splendidus*) (Nishibuchi & Seidler, 1985), or non-*Vibrio* species (*Acinetobacter calcoaceticus*, *Achromobacter ichthyoderms*, *Aeromonas hydrophila*, and *Pseudomonas aeruginosa*) (Parker & Lewis, 1995) were found, indicating specificity problem.

Compared to conventional method, immunological-based assays greatly eliminate the lengthy and labor-intensive cultural assays for *Vibrio* identification, but the cross-reactions were distinguished owing to the nature of the test method, which limits the application of such assays.

Molecular-based DNA detection assays, mainly PCR and real-time PCR (Campbell & Wright, 2003; Gordon *et al.*, 2008; Nordstrom *et al.*, 2007; Panicker *et al.*, 2004b; Panicker *et al.*, 2004c; Panicker & Bej, 2005), have been widely employed for the rapid and specific detection of *V. parahaemolyticus* and *V. vulnificus* worldwide. A multiplex PCR detecting the total and virulent strains of *V. parahaemolyticus* was developed by targeting *tlh*, *tdh*, and *trh*, and the detection limit was found 10-100 CFU per 10 g of alkaline peptone water enriched seeded

oyster tissue homogenate after 6 h enrichment (Bej *et al.*, 1999). Compared to conventional PCR, real-time PCR is more sensitive and rapid, and also quantitative. It is reported the real-time PCR could detect 1 CFU *V. parahaemolyticus* in oyster tissue homogenate after overnight enrichment (Ward & Bej, 2006). Another group developed a real-time multiplex PCR assay for the simultaneous detection of *tlh*, *tdh*, and *trh* of *V. parahaemolyticus*, and the multiplex assay detected <10 CFU/reaction of pathogenic *V. parahaemolyticus* in the presence of >10⁴ CFU/reaction of total *V. parahaemolyticus* bacteria (Nordstrom *et al.*, 2007). When applied to oyster samples, compared to MPN, the multiplex real-time PCR assay detected more tubes positive for total and pathogenic *V. parahaemolyticus* bacteria (Nordstrom *et al.*, 2007).

Similar results were found by using PCR and real-time PCR for the detection of *V. vulnificus*. Back to 1991, two studies have been reported by PCR for detecting general (Hill *et al.*, 1991) and nonculturable *V. vulnificus* cells (Brauns *et al.*, 1991), all targeting *vvhA*. Later, Multiplex PCR analysis for simultaneous identification of *V. vulnificus* and other vibrios, have been reported (Dalmasso *et al.*, 2009; Panicker *et al.*, 2004a; Teh *et al.*, 2009). When applied in real-time, SYBR Green-based real-time PCR (Panicker *et al.*, 2004b) and Taqman real-time PCR (Campbell & Wright, 2003) both targeting *vvhA* have been reported to quantify *V. vulnificus* from oysters, both of the assays are highly specific. SYBR Green-based real-time PCR was able to detect the equivalent of 10² cells in pure culture and 10²/ml in seawater without enrichment, however, after 5h enrichment, the detection limit was found to one cell (Panicker *et al.*, 2004b). Taqman real-time PCR could detect the equivalent of six cells in pure culture, 10² CFU/g from oyster homogenates using purified DNA templates (Campbell & Wright, 2003).

Compared to PCR, real-time offers quantitative analysis while reducing the need for post-processing, thus providing more rapid analysis. However, for both PCR and real-time PCR, a

dedicated thermal cycler is needed, which is rather expensive especially for real-time PCR, and hinders the wide application of such assays.

A newer molecular method, loop-mediated isothermal amplification (LAMP), developed by Tsugunori Notomi in Japan in 2000, applied four to six primers to specifically recognize six to eight distinct sequences on the target DNA under isothermal conditions in less than one hour (Notomi *et al.*, 2000). Also this novel nucleic acid amplification method could be observed by naked eye or real-time turbidity meter due to the formation of magnesium pyrophosphate as a by-product (Mori *et al.*, 2001). Since its invention, LAMP has been applied to detect multiple bacterial and viral agents, including those of major food safety concerns, such as *Salmonella* (Hara-Kudo *et al.*, 2005; Ohtsuka *et al.*, 2005), *Campylobacter* (Yamazaki *et al.*, 2008c), *Flavobacterium* (Yeh *et al.*, 2006), *E. coli* (Hara-Kudo *et al.*, 2007; Yano *et al.*, 2007), *Staphylococcus aureus* (Goto *et al.*, 2007; Misawa *et al.*, 2007), *Vibrio* (Chen & Ge, 2010; Yamazaki *et al.*, 2008a; Yamazaki *et al.*, 2008b), and virus (Fukuda *et al.*, 2007; Mekata *et al.*, 2006; Yamada *et al.*, 2006). Meanwhile, real-time LAMP has been developed by using a fluorescent DNA-intercalating dye in a real-time PCR format (Aoi *et al.*, 2006) or measuring the turbidity change during LAMP amplification in a turbidimeter (Chen & Ge, 2010; Mori *et al.*, 2001). Figure 2-3 shows the LAMP procedure (Eiken Genome Site, 2000).

Advantages of LAMP detection include: 1) isothermal (60-65°C), no special thermal cycling instrument is required; 2) rapid, the assay can be completed in 15-60 min (Nagamine *et al.*, 2002); 3) specific, the assay targets 6-8 regions of the target gene sequence; 4) sensitive, extremely large amount of DNA can be amplified from a few target cells; 5) detection by the naked eye due to the formation of large quantities of a by-product, magnesium pyrophosphate, which turns positive reaction tubes turbid (Mori *et al.*, 2001); 6) direct amplification, there is no

need to denature DNA templates before amplification (Nagamine *et al.*, 2001); 7) robust, LAMP is less subjective to inhibition by biological substances (Kaneko *et al.*, 2007); and 8) quantitative, real-time LAMP is used to quantify DNA in the reaction (Chen & Ge, 2010).

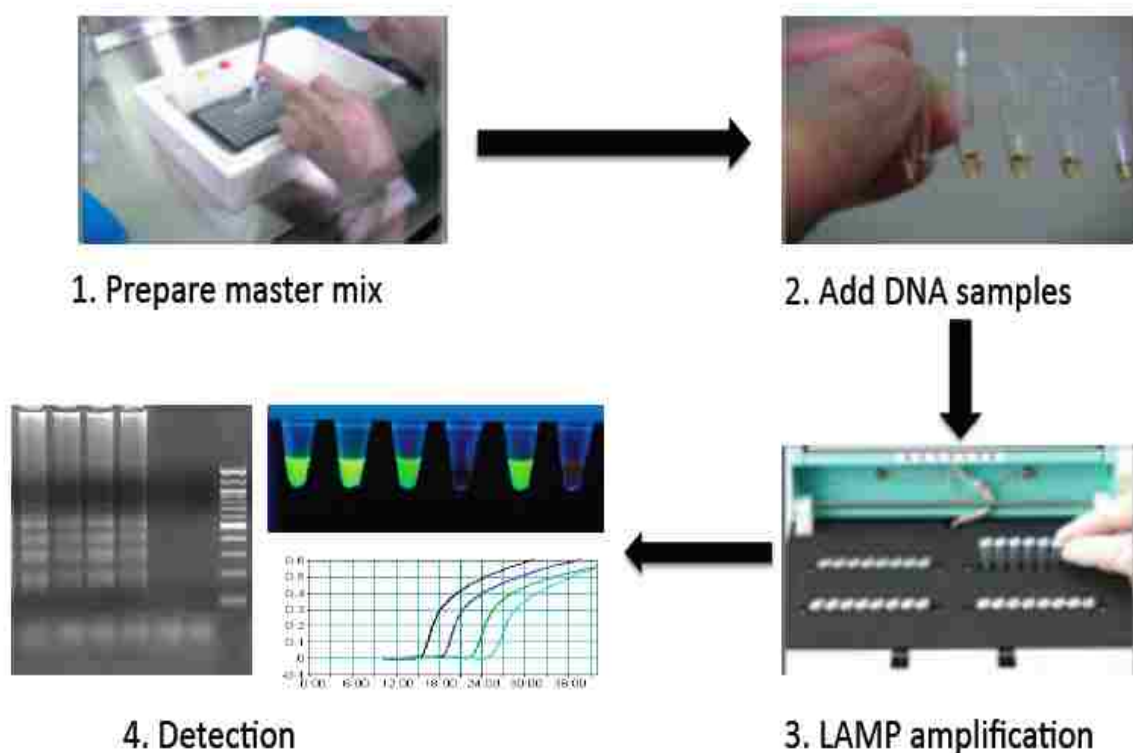


Figure 2 - 4 Loop-mediated isothermal amplification (LAMP) procedure

Given the lack of information on both the epidemiology and sensitive and cost-effective method for the rapid detection of general and virulent-type of *Vibrio*, this dissertation research was carried out to fulfill three objectives: 1) To isolate *V. parahaemolyticus* and *V. vulnificus* from Louisiana Gulf and Retail oysters; 2) To characterize the *V. parahaemolyticus* and *V. vulnificus* isolates, including antimicrobial susceptibility profile and genotypic patterns; and 3) To develop and evaluate LAMP assays for real-time detection of general and virulent-type *V. vulnificus*. The successful complete of this dissertation generated baseline data on the prevalence, genotypes, and antimicrobial susceptibility profiles of the two vibrios from Louisiana oysters,

and rapid, specific, sensitive, and yet cost-effective molecular detection assays for both general and virulent *V. vulnificus* were developed, which could contribute to developing effective control strategies to reduce the incidences of human *Vibrio* infections due to the consumption of oysters in the long run.

References

- Aarestrup, F. M. (1999).** Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. *Int J Antimicrob Agents* **12**, 279-285.
- Aarestrup, F. M. & Wegener, H. C. (1999).** The effects of antibiotic usage in food animals on the development of antimicrobial resistance of importance for humans in *Campylobacter* and *Escherichia coli*. *Microbes Infect* **1**, 639-644.
- Akinbowale, O. L., Peng, H. & Barton, M. D. (2006).** Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia. *J Appl Microbiol* **100**, 1103-1113.
- Aoi, Y., Hosogai, M. & Tsuneda, S. (2006).** Real-time quantitative LAMP (loop-mediated isothermal amplification of DNA) as a simple method for monitoring ammonia-oxidizing bacteria. *J Biotechnol* **125**, 484-491.
- Baker-Austin, C., McArthur, J. V., Lindell, A. H., Wright, M. S., Tuckfield, R. C., Gooch, J., Warner, L., Oliver, J. & Stepanauskas, R. (2009).** Multi-site analysis reveals widespread antibiotic resistance in the marine pathogen *Vibrio vulnificus*. *Microb Ecol* **57**, 151-159.
- Barbieri, E., Falzano, L., Fiorentini, C. & other authors (1999).** Occurrence, diversity, and pathogenicity of halophilic *Vibrio* spp. and non-O1 *Vibrio cholerae* from estuarine waters along the Italian Adriatic coast. *Appl Environ Microbiol* **65**, 2748-2753.
- Bates, T. C. & Oliver, J. D. (2004).** The viable but nonculturable state of Kanagawa positive and negative strains of *Vibrio parahaemolyticus*. *J Microbiol* **42**, 74-79.
- Bauer, A., Ostensvik, O., Florvag, M., Ormen, O. & Rorvik, L. M. (2006).** Occurrence of *Vibrio parahaemolyticus*, *V. cholerae*, and *V. vulnificus* in Norwegian Blue Mussels (*Mytilus edulis*). *Appl Environ Microbiol* **72**, 3058-3061.
- Bej, A. K., Patterson, D. P., Brasher, C. W., Vickery, M. C., Jones, D. D. & Kaysner, C. A. (1999).** Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. *J Microbiol Methods* **36**, 215-225.

Brauns, L. A., Hudson, M. C. & Oliver, J. D. (1991). Use of the polymerase chain reaction in detection of culturable and nonculturable *Vibrio vulnificus* cells. *Appl Environ Microbiol* **57**, 2651-2655.

Bryan, P. J., Steffan, R. J., DePaola, A., Foster, J. W. & Bej, A. K. (1999). Adaptive response to cold temperatures in *Vibrio vulnificus*. *Curr Microbiol* **38**, 168-175.

Campbell, M. S. & Wright, A. C. (2003). Real-time PCR analysis of *Vibrio vulnificus* from oysters. *Appl Environ Microbiol* **69**, 7137-7144.

Centers for Disease Control and Prevention (2005). *Vibrio vulnificus*: general information.

Centers for Disease Control and Prevention (2010). Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food--10 states, 2009. *MMWR Morb Mortal Wkly Rep* **58**, 418-422.

Chatzidaki-Livanis, M., Hubbard, M. A., Gordon, K., Harwood, V. J. & Wright, A. C. (2006a). Genetic distinctions among clinical and environmental strains of *Vibrio vulnificus*. *Appl Environ Microbiol* **72**, 6136-6141.

Chatzidaki-Livanis, M., Jones, M. K. & Wright, A. C. (2006b). Genetic variation in the *Vibrio vulnificus* group 1 capsular polysaccharide operon. *J Bacteriol* **188**, 1987-1998.

Chen, S. & Ge, B. (2010). Development of a toxR-based loop-mediated isothermal amplification assay for detecting *Vibrio parahaemolyticus*. *BMC Microbiol* **10**, 41.

Cholera and Other Vibrio Illness Surveillance (2009). Summary of human *Vibrio* isolates reported to CDC 2007. Available at <http://www.cdc.gov/nationalsurveillance/PDFs/CSTEVibrio2007.pdf>. Accessed on April 28, 2010.

Cook, D. W., Bowers, J. C. & DePaola, A. (2002a). Density of total and pathogenic (*tdh*+) *Vibrio parahaemolyticus* in Atlantic and Gulf coast molluscan shellfish at harvest. *J Food Prot* **65**, 1873-1880.

Cook, D. W., O'Leary, P., Hunsucker, J. C., Sloan, E. M., Bowers, J. C., Blodgett, R. J. & Depaola, A. (2002b). *Vibrio vulnificus* and *Vibrio parahaemolyticus* in U.S. retail shell oysters: a national survey from June 1998 to July 1999. *J Food Prot* **65**, 79-87.

Dalmaso, A., Civera, T. & Bottero, M. T. (2009). Multiplex primer-extension assay for identification of six pathogenic vibrios. *Int J Food Microbiol* **129**, 21-25.

Dalsgaard, A., Frimodt-Moller, N., Bruun, B., Hoi, L. & Larsen, J. L. (1996). Clinical manifestations and molecular epidemiology of *Vibrio vulnificus* infections in Denmark. *Eur J Clin Microbiol Infect Dis* **15**, 227-232.

- Datta, S., Janes, M. E. & Simonson, J. G. (2008).** Immunomagnetic separation and coagglutination of *Vibrio parahaemolyticus* with anti-flagellar protein monoclonal antibody. *Clin Vaccine Immunol* **15**, 1541-1546.
- DePaola, A., Capers, G. M. & Alexander, D. (1994).** Densities of *Vibrio vulnificus* in the intestines of fish from the U.S. Gulf Coast. *Appl Environ Microbiol* **60**, 984-988.
- DePaola, A., Kaysner, C. A., Bowers, J. & Cook, D. W. (2000).** Environmental investigations of *Vibrio parahaemolyticus* in oysters after outbreaks in Washington, Texas, and New York (1997 and 1998). *Appl Environ Microbiol* **66**, 4649-4654.
- DePaola, A., Nordstrom, J. L., Bowers, J. C., Wells, J. G. & Cook, D. W. (2003a).** Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. *Appl Environ Microbiol* **69**, 1521-1526.
- DePaola, A., Ulaszek, J., Kaysner, C. A., Tenge, B. J., Nordstrom, J. L., Wells, J., Puhr, N. & Gendel, S. M. (2003b).** Molecular, serological, and virulence characteristics of *Vibrio parahaemolyticus* isolated from environmental, food, and clinical sources in North America and Asia. *Appl Environ Microbiol* **69**, 3999-4005.
- DePaola, A., Jones, J. L., Woods, J. & other authors (2010).** Bacterial and viral pathogens in live oysters: 2007 United States market survey. *Appl Environ Microbiol* **76**, 2754-2768.
- Eiken Genome Site (2000).** Standard procedures using LAMP method. <http://loopamp.eiken.co.jp/e/tech/index.html>.
- Elmore, S. P., Watts, J. A., Simpson, L. M. & Oliver, J. D. (1992).** Reversal of hypotension induced by *Vibrio vulnificus* lipopolysaccharide in the rat by inhibition of nitric oxide synthase. *Microb Pathog* **13**, 391-397.
- Fukuda, S., Sasaki, Y., Kuwayama, M. & Miyazaki, K. (2007).** Simultaneous detection and genogroup-screening test for norovirus genogroups I and II from fecal specimens in single tube by reverse transcription- loop-mediated isothermal amplification assay. *Microbiol Immunol* **51**, 547-550.
- Gopal, S., Otta, S. K., Kumar, S., Karunasagar, I. & Nishibuchi, M. (2005).** The occurrence of *Vibrio* species in tropical shrimp culture environments; implications for food safety. *Int J Food Microbiol* **102**, 151-159.
- Gordon, K. V., Vickery, M. C., DePaola, A., Staley, C. & Harwood, V. J. (2008).** Real-time PCR assays for quantification and differentiation of *Vibrio vulnificus* strains in oysters and water. *Appl Environ Microbiol* **74**, 1704-1709.
- Goto, M., Hayashidani, H., Takatori, K. & Hara-Kudo, Y. (2007).** Rapid detection of enterotoxigenic *Staphylococcus aureus* harbouring genes for four classical enterotoxins, SEA,

SEB, SEC and SED, by loop-mediated isothermal amplification assay. *Lett Appl Microbiol* **45**, 100-107.

Hara-Kudo, Y., Yoshino, M., Kojima, T. & Ikedo, M. (2005). Loop-mediated isothermal amplification for the rapid detection of *Salmonella*. *FEMS Microbiol Lett* **253**, 155-161.

Hara-Kudo, Y., Nemoto, J., Ohtsuka, K., Segawa, Y., Takatori, K., Kojima, T. & Ikedo, M. (2007). Sensitive and rapid detection of Vero toxin-producing *Escherichia coli* using loop-mediated isothermal amplification. *J Med Microbiol* **56**, 398-406.

Hervio-Heath, D., Colwell, R. R., Derrien, A., Robert-Pillot, A., Fournier, J. M. & Pommepuy, M. (2002). Occurrence of pathogenic vibrios in coastal areas of France. *J Appl Microbiol* **92**, 1123-1135.

Hill, W. E., Keasler, S. P., Trucksess, M. W., Feng, P., Kaysner, C. A. & Lampel, K. A. (1991). Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. *Appl Environ Microbiol* **57**, 707-711.

Hoi, L., Larsen, J. L., Dalsgaard, I. & Dalsgaard, A. (1998). Occurrence of *Vibrio vulnificus* biotypes in Danish marine environments. *Appl Environ Microbiol* **64**, 7-13.

Honda, T., Miwatani, T., Yabushita, Y., Koike, N. & Okada, K. (1995). A novel method to chemically immobilize antibody on nylon and its application to the rapid and differential detection of two *Vibrio parahaemolyticus* toxins in a modified enzyme-linked immunosorbent assay. *Clin Diagn Lab Immunol* **2**, 177-181.

Iida, T., Park, K. S. & Honda, T. (2006). *Vibrio parahaemolyticus*, p. 340-348. Edited by F. L. Thompson, B. Austin, and J. Swings (ed.). *The biology of vibrios*.

Johnson, D. E., Calia, F. M., Musher, D. M. & Goree, A. (1984). Resistance of *Vibrio vulnificus* to serum bactericidal and opsonizing factors: relation to virulence in suckling mice and humans. *J Infect Dis* **150**, 413-418.

Joseph, S. W., DeBell, R. M. & Brown, W. P. (1978). In vitro response to chloramphenicol, tetracycline, ampicillin, gentamicin, and beta-lactamase production by halophilic *Vibrios* from human and environmental sources. *Antimicrob Agents Chemother* **13**, 244-248.

Kaneko, H., Kawana, T., Fukushima, E. & Suzutani, T. (2007). Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. *Journal of biochemical and biophysical methods* **70**, 499-501.

Kaspar, C. W. & Tamplin, M. L. (1993). Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. *Appl Environ Microbiol* **59**, 2425-2429.

Kaysner, C. A. & DePaola, A. (2001). *Vibrio*. In *Bacteriological Analytical Manual online*, Chapter 9. Edited by U.S Food and Drug Administration. Rockville, MD.

- Kobayashi, T., Enomoto, S., Sakazaki, R. & Kuwahara, S. (1963).** [a New Selective Isolation Medium for the *Vibrio* Group; on a Modified Nakanishi's Medium (Tcbs Agar Medium)]. *Nippon Saikingaku Zasshi* **18**, 387-392.
- Lee, J. H., Rho, J. B., Park, K. J., Kim, C. B., Han, Y. S., Choi, S. H., Lee, K. H. & Park, S. J. (2004).** Role of flagellum and motility in pathogenesis of *Vibrio vulnificus*. *Infect Immun* **72**, 4905-4910.
- Lesmana, M., Subekti, D., Simanjuntak, C. H., Tjaniadi, P., Campbell, J. R. & Oyoyo, B. A. (2001).** *Vibrio parahaemolyticus* associated with cholera-like diarrhea among patients in North Jakarta, Indonesia. *Diagn Microbiol Infect Dis* **39**, 71-75.
- Linkous, D. A. & Oliver, J. D. (1999).** Pathogenesis of *Vibrio vulnificus*. *FEMS Microbiol Lett* **174**, 207-214.
- Litwin, C. M., Rayback, T. W. & Skinner, J. (1996).** Role of catechol siderophore synthesis in *Vibrio vulnificus* virulence. *Infect Immun* **64**, 2834-2838.
- Luan, X., Chen, J., Liu, Y., Li, Y., Jia, J., Liu, R. & Zhang, X. H. (2008).** Rapid quantitative detection of *Vibrio parahaemolyticus* in seafood by MPN-PCR. *Curr Microbiol* **57**, 218-221.
- Maluping, R. P., Lavilla-Pitogo, C. R., DePaola, A., Janda, J. M., Krovacek, K. & Greko, C. (2005).** Antimicrobial susceptibility of *Aeromonas* spp., *Vibrio* spp. and *Plesiomonas shigelloides* isolated in the Philippines and Thailand. *Int J Antimicrob Agents* **25**, 348-350.
- Massad, G. & Oliver, J. D. (1987).** New selective and differential medium for *Vibrio cholerae* and *Vibrio vulnificus*. *Appl Environ Microbiol* **53**, 2262-2264.
- McPherson, V. L., Watts, J. A., Simpson, L. M. & Oliver, J. D. (1991).** Physiological effects of the lipopolysaccharide of *Vibrio vulnificus* on mice and rats. *Microbios* **67**, 141-149.
- Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M. & Tauxe, R. V. (1999a).** Food-related illness and death in the United States. *Emerg Infect Dis* **5**, 607-625.
- Mead, P. S., Slutsker, L., Griffin, P. M. & Tauxe, R. V. (1999b).** Food-related illness and death in the United States reply to Dr. Hedberg. *Emerg Infect Dis* **5**, 841-842.
- Mekata, T., Kono, T., Savan, R., Sakai, M., Kasornchandra, J., Yoshida, T. & Itami, T. (2006).** Detection of yellow head virus in shrimp by loop-mediated isothermal amplification (LAMP). *J Virol Methods* **135**, 151-156.
- Misawa, Y., Yoshida, A., Saito, R., Yoshida, H., Okuzumi, K., Ito, N., Okada, M., Moriya, K. & Koike, K. (2007).** Application of loop-mediated isothermal amplification technique to

rapid and direct detection of methicillin-resistant *Staphylococcus aureus* (MRSA) in blood cultures. *J Infect Chemother* **13**, 134-140.

Mori, Y., Nagamine, K., Tomita, N. & Notomi, T. (2001). Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem Biophys Res Commun* **289**, 150-154.

Morris, J. G., Jr. & Tenney, J. (1985). Antibiotic therapy for *Vibrio vulnificus* infection. *JAMA* **253**, 1121-1122.

Morris, J. G., Jr., Wright, A. C., Roberts, D. M., Wood, P. K., Simpson, L. M. & Oliver, J. D. (1987). Identification of environmental *Vibrio vulnificus* isolates with a DNA probe for the cytotoxin-hemolysin gene. *Appl Environ Microbiol* **53**, 193-195.

Nagamine, K., Watanabe, K., Ohtsuka, K., Hase, T. & Notomi, T. (2001). Loop-mediated isothermal amplification reaction using a nondenatured template. *Clinical chemistry* **47**, 1742-1743.

Nagamine, K., Hase, T. & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and cellular probes* **16**, 223-229.

Nilsson, W. B., Paranjypte, R. N., DePaola, A. & Strom, M. S. (2003). Sequence polymorphism of the 16S rRNA gene of *Vibrio vulnificus* is a possible indicator of strain virulence. *J Clin Microbiol* **41**, 442-446.

Nishibuchi, M. & Seidler, R. J. (1985). Rapid microimmunodiffusion method with species-specific antiserum raised to purified antigen for identification of *Vibrio vulnificus*. *J Clin Microbiol* **21**, 102-107.

Nordstrom, J. L., Rangdale, R., Vickery, M. C., Phillips, A. M., Murray, S. L., Wagley, S. & DePaola, A. (2006). Evaluation of an alkaline phosphatase-labeled oligonucleotide probe for the detection and enumeration of the thermostable-related hemolysin (*trh*) gene of *Vibrio parahaemolyticus*. *J Food Prot* **69**, 2770-2772.

Nordstrom, J. L., Vickery, M. C., Blackstone, G. M., Murray, S. L. & DePaola, A. (2007). Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. *Appl Environ Microbiol* **73**, 5840-5847.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* **28**, E63.

Ohtsuka, K., Yanagawa, K., Takatori, K. & Hara-Kudo, Y. (2005). Detection of *Salmonella enterica* in naturally contaminated liquid eggs by loop-mediated isothermal amplification, and characterization of *Salmonella* isolates. *Appl Environ Microbiol* **71**, 6730-6735.

Oliver, J. D., Hite, F., McDougald, D., Andon, N. L. & Simpson, L. M. (1995). Entry into, and resuscitation from, the viable but nonculturable state by *Vibrio vulnificus* in an estuarine environment. *Appl Environ Microbiol* **61**, 2624-2630.

Oliver, J. D. (2006). *Vibrio vulnificus*, p. 349-366. Edited by F. L. Thompson, B. Austin, and J. Swings (ed.), *The biology of vibrios*. Washitonton, DC: ASM press.

Panicker, G., Call, D. R., Krug, M. J. & Bej, A. K. (2004a). Detection of pathogenic *Vibrio* spp. in shellfish by using multiplex PCR and DNA microarrays. *Appl Environ Microbiol* **70**, 7436-7444.

Panicker, G., Myers, M. L. & Bej, A. K. (2004b). Rapid detection of *Vibrio vulnificus* in shellfish and Gulf of Mexico water by real-time PCR. *Appl Environ Microbiol* **70**, 498-507.

Panicker, G., Vickery, M. C. & Bej, A. K. (2004c). Multiplex PCR detection of clinical and environmental strains of *Vibrio vulnificus* in shellfish. *Can J Microbiol* **50**, 911-922.

Panicker, G. & Bej, A. K. (2005). Real-time PCR detection of *Vibrio vulnificus* in oysters: comparison of oligonucleotide primers and probes targeting *vhA*. *Appl Environ Microbiol* **71**, 5702-5709.

Parker, R. W. & Lewis, D. H. (1995). Sandwich enzyme-linked immunosorbent assay for *Vibrio vulnificus* hemolysin to detect *V. vulnificus* in environmental specimens. *Appl Environ Microbiol* **61**, 476-480.

Raghunath, P., Pradeep, B. & Karunasagar, I. (2007). Rapid detection and enumeration of trh-carrying *Vibrio parahaemolyticus* with the alkaline phosphatase-labelled oligonucleotide probe. *Environ Microbiol* **9**, 266-270.

Rosche, T. M., Yano, Y. & Oliver, J. D. (2005). A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical or environmental isolation. *Microbiol Immunol* **49**, 381-389.

Strom, M. S. & Paranjpye, R. N. (2000). Epidemiology and pathogenesis of *Vibrio vulnificus*. *Microbes Infect* **2**, 177-188.

Takahashi, A., Sato, Y., Shiomi, Y., Cantarelli, V. V., Iida, T., Lee, M. & Honda, T. (2000). Mechanisms of chloride secretion induced by thermostable direct haemolysin of *Vibrio parahaemolyticus* in human colonic tissue and a human intestinal epithelial cell line. *J Med Microbiol* **49**, 801-810.

Tamplin, M., Rodrick, G. E., Blake, N. J. & Cuba, T. (1982). Isolation and characterization of *Vibrio vulnificus* from two Florida estuaries. *Appl Environ Microbiol* **44**, 1466-1470.

- Tamplin, M. L., Martin, A. L., Ruple, A. D., Cook, D. W. & Kaspar, C. W. (1991).** Enzyme immunoassay for identification of *Vibrio vulnificus* in seawater, sediment, and oysters. *Appl Environ Microbiol* **57**, 1235-1240.
- Tang, H. J., Chang, M. C., Ko, W. C., Huang, K. Y., Lee, C. L. & Chuang, Y. C. (2002).** In vitro and in vivo activities of newer fluoroquinolones against *Vibrio vulnificus*. *Antimicrob Agents Chemother* **46**, 3580-3584.
- Teh, C. S., Chua, K. H. & Thong, K. L. (2009).** Simultaneous differential detection of human pathogenic and nonpathogenic *Vibrio* species using a multiplex PCR based on *gyrB* and *pntA* genes. *J Appl Microbiol*.
- Thomas, A., Straif-Bourgeois, S., Sokol, T. M. & Ratard, R. C. (2007).** *Vibrio* infections in Louisiana: twenty-five years of surveillance 1980-2005. *J La State Med Soc* **159**, 205-208, 210-201.
- Vickery, M. C., Nilsson, W. B., Strom, M. S., Nordstrom, J. L. & DePaola, A. (2007).** A real-time PCR assay for the rapid determination of 16S rRNA genotype in *Vibrio vulnificus*. *J Microbiol Methods* **68**, 376-384.
- Ward, L. N. & Bej, A. K. (2006).** Detection of *Vibrio parahaemolyticus* in shellfish by use of multiplexed real-time PCR with TaqMan fluorescent probes. *Appl Environ Microbiol* **72**, 2031-2042.
- Warner, E. & Oliver, J. D. (2008).** Population structures of two genotypes of *Vibrio vulnificus* in oysters (*Crassostrea virginica*) and seawater. *Appl Environ Microbiol* **74**, 80-85.
- Warner, J. M. & Oliver, J. D. (1999).** Randomly amplified polymorphic DNA analysis of clinical and environmental isolates of *Vibrio vulnificus* and other *vibrio* species. *Appl Environ Microbiol* **65**, 1141-1144.
- Wong, H. C., Ho, C. Y., Kuo, L. P., Wang, T. K., Lee, C. L. & Shih, D. Y. (1999).** Ribotyping of *Vibrio parahaemolyticus* isolates obtained from food poisoning outbreaks in Taiwan. *Microbiol Immunol* **43**, 631-636.
- Wright, A. C., Simpson, L. M. & Oliver, J. D. (1981).** Role of iron in the pathogenesis of *Vibrio vulnificus* infections. *Infect Immun* **34**, 503-507.
- Wright, A. C., Simpson, L. M., Oliver, J. D. & Morris, J. G., Jr. (1990).** Phenotypic evaluation of acapsular transposon mutants of *Vibrio vulnificus*. *Infect Immun* **58**, 1769-1773.
- Wright, A. C., Miceli, G. A., Landry, W. L., Christy, J. B., Watkins, W. D. & Morris, J. G., Jr. (1993).** Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. *Appl Environ Microbiol* **59**, 541-546.

Wright, A. C., Hill, R. T., Johnson, J. A., Roghman, M. C., Colwell, R. R. & Morris, J. G., Jr. (1996). Distribution of *Vibrio vulnificus* in the Chesapeake Bay. *Appl Environ Microbiol* **62**, 717-724.

Yamada, Y., Itoh, M. & Yoshida, M. (2006). Sensitive and rapid diagnosis of human parvovirus B19 infection by loop-mediated isothermal amplification. *Br J Dermatol* **155**, 50-55.

Yamazaki, W., Ishibashi, M., Kawahara, R. & Inoue, K. (2008a). Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of *Vibrio parahaemolyticus*. *BMC Microbiol* **8**, 163.

Yamazaki, W., Seto, K., Taguchi, M., Ishibashi, M. & Inoue, K. (2008b). Sensitive and rapid detection of cholera toxin-producing *Vibrio cholerae* using a loop-mediated isothermal amplification. *BMC Microbiol* **8**, 94.

Yamazaki, W., Taguchi, M., Ishibashi, M., Kitazato, M., Nukina, M., Misawa, N. & Inoue, K. (2008c). Development and evaluation of a loop-mediated isothermal amplification assay for rapid and simple detection of *Campylobacter jejuni* and *Campylobacter coli*. *J Med Microbiol* **57**, 444-451.

Yano, A., Ishimaru, R. & Hujikata, R. (2007). Rapid and sensitive detection of heat-labile I and heat-stable I enterotoxin genes of enterotoxigenic *Escherichia coli* by Loop-Mediated Isothermal Amplification. *J Microbiol Methods* **68**, 414-420.

Yeh, H. Y., Shoemaker, C. A. & Klesius, P. H. (2006). Sensitive and rapid detection of *Flavobacterium columnare* in channel catfish *Ictalurus punctatus* by a loop-mediated isothermal amplification method. *J Appl Microbiol* **100**, 919-925.

Yoshida, S., Ogawa, M. & Mizuguchi, Y. (1985). Relation of capsular materials and colony opacity to virulence of *Vibrio vulnificus*. *Infect Immun* **47**, 446-451.

Zanetti, S., Spanu, T., Deriu, A., Romano, L., Sechi, L. A. & Fadda, G. (2001). In vitro susceptibility of *Vibrio* spp. isolated from the environment. *Int J Antimicrob Agents* **17**, 407-409.

CHAPTER 3: PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITY OF *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS* FROM LOUISIANA GULF AND RETAIL RAW OYSTERS

Part of this chapter is reprinted by permission of “Applied and Environmental Microbiology” (see appendix for permission from publisher)

Citation: Han, F., R. D. Walker, M. E. Janes, W. Prinyawiwatkul, and B. Ge. 2007.

Antimicrobial susceptibilities of *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates from Louisiana Gulf and retail raw oysters. *Applied and Environmental Microbiology*.73:7096-8

Introduction

Members of the genus *Vibrio* are Gram-negative, motile bacteria that inhabit warm coastal and estuarine waters worldwide (Barbieri *et al.*, 1999; Hervio-Heath *et al.*, 2002; Hoi *et al.*, 1998). However, the consumption of raw or undercooked seafood, particularly oysters, may lead to their transmission to humans with clinical manifestations ranging from mild diarrhea to death (Blake *et al.*, 1980). Among pathogenic vibrios, *Vibrio parahaemolyticus* is the leading cause of seafood-associated bacterial gastroenteritis in the United States (Mead *et al.*, 1999) and the most common foodborne pathogen in Asian countries (Joseph *et al.*, 1982); whereas *Vibrio vulnificus* inflicts the highest mortality rate (> 50% in primary septicemia patients and 25% for those with wound infections) among all foodborne pathogens, responsible for more than 95% of seafood-related deaths in the U.S. (Oliver, 2006). Fortunately, the vast majority of environmental *V. parahaemolyticus* strains tested are not pathogenic (being both *tdh* and *trh* negative) (DePaola *et al.*, 2003), and for fatal *V. vulnificus* infections, predisposed risk factors have been identified, which include compromised immune systems, diabetes, and elevated serum iron concentrations due to liver disease or alcohol abuse (Strom & Paranjpye, 2000). Nonetheless, according to a recent FoodNet report from the Centers for Disease Control and Prevention (CDC), amidst the decrease of infections caused by all major foodborne pathogens, the incidence of *V. parahaemolyticus* and *V. vulnificus* infections due to eating raw or undercooked oysters has shown a sustained increase since 2001, indicating further measures are needed to prevent human illness caused by these pathogens in oysters (Centers for Disease Control and Prevention, 2010).

Although most *V. parahaemolyticus* infections are self-limiting, antimicrobial therapy may be necessary in patients with severe or prolonged diarrhea (Centers for Disease Control and Prevention, 2005b). In the case of *V. vulnificus* primary septicemia and wound infection, prompt

antimicrobial treatment can be lifesaving because of the extreme rapidity of disease progress and severity of patient outcomes (Strom & Paranjpye, 2000). Tetracycline has been recommended as the antimicrobial of choice to treat severe *Vibrio* infections (Morris & Tenney, 1985), and alternative treatments are a combination of a third-generation cephalosporin (e.g., ceftazidime) and doxycycline, or a fluoroquinolone alone (Tang *et al.*, 2002). Trimethoprim-sulfamethoxazole plus an aminoglycoside are used to treat children in whom doxycycline and fluoroquinolones are contraindicated (Centers for Disease Control and Prevention, 2005a).

Traditionally, *Vibrio* is considered highly susceptible to virtually all antimicrobials, although ampicillin resistance has been observed in both *V. parahaemolyticus* and *V. vulnificus* (Joseph *et al.*, 1978; Zanetti *et al.*, 2001). During the past decade, however, antimicrobial resistance has emerged and evolved in many species of microorganisms due to the excessive use of antimicrobials in humans, agriculture, and aquaculture (Cabello, 2006; Mazel & Davies, 1999). *Campylobacter* and *Salmonella*, two major foodborne bacterial pathogens of terrestrial sources, are part of the National Antimicrobial Resistance Monitoring System (NARMS) in the U.S. (Centers for Disease Control and Prevention, 2006), and have been studied extensively for the development and dissemination of antimicrobial resistance (Chen *et al.*, 2004; D'Lima C *et al.*, 2007; Ge *et al.*, 2005; Skov *et al.*, 2007). In contrast, the awareness of antimicrobial-resistant bacteria in the aquatic environment is not as well documented (Cabello, 2006). For both clinical and environmental isolates of *Vibrio cholera*, antimicrobial resistance has been investigated extensively (Dalsgaard *et al.*, 2000; Hochhut *et al.*, 2001; Iwanaga *et al.*, 2004), and there are some very recent reports examining the antimicrobial susceptibilities in other aquatic species such as *Aeromonas* (Gordon *et al.*, 2007; Jacobs & Chenia, 2007). But only a few recent studies have investigated the antimicrobial resistance in non-cholera vibrios. These studies were

conducted in Australia, India, the Philippines, and Thailand, and included small numbers of either *V. parahaemolyticus* or *V. vulnificus* (Akinbowale *et al.*, 2006; Maluping *et al.*, 2005; Vaseeharan *et al.*, 2005). Antimicrobial resistance in these two bacterial species has not been reported in the U.S. since the 1990s.

The objectives of this study were to determine the prevalence of *V. parahaemolyticus* and *V. vulnificus* in oysters obtained from the Louisiana Gulf Coast and four retail outlets in Baton Rouge, Louisiana, and more importantly, to determine the antimicrobial susceptibility profiles of these *Vibrio* isolates by a broth microdilution method.

Materials and Methods

Sample Collection and Preparation. From June 2005 to September 2006, a total of 20 oyster samples were harvested, at quarterly intervals, from two sites near the Louisiana Gulf Coast. Due to Hurricanes Katrina and Rita, no sampling trips were made in September 2005. Both sites were in shellfish-growing areas approved by the National Shellfish Sanitation Program and available for commercial harvesting. Another 74 oyster samples were obtained through retail sampling conducted weekly in Baton Rouge, Louisiana from February to August 2006. One grocery store that offered shellstock oysters for sale (February-April) and three seafood restaurants with “oyster bars” where shellstock oysters were opened onsite for raw consumption (April-August) were included in the sampling. On each sampling day, two paired oyster samples (12 oysters/sample) were obtained from each site, transported on ice to the laboratory, and analyzed within 4 h of collection. The oysters collected from both the Gulf and retail outlets were Eastern oysters, *Crassostrea virginica*.

Oysters in the shell were scrubbed, and those belonging to one sample were shucked into a sterile stomacher bag to make 200-250 g per sample. After adding equal volume of alkaline

peptone water (APW; BD Diagnostic Systems, Sparks, MD), samples were homogenized in a food stomacher (Model 400; Tekmar Company, Cincinnati, OH) for 90 seconds to produce 1:1 oyster-APW homogenate.

Bacterial Isolation. Dehydrated media and reagents were purchased from BD Diagnostic Systems. Media formulations and procedures described in the Food and Drug Administration Bacteriological Analytical Manual (BAM) (Kaysner & DePaola, 2001) and a previous study (DePaola *et al.*, 2003) were used to isolate *V. parahaemolyticus* and *V. vulnificus* with a few modifications. Two isolation methods, direct plating and enrichment, were proceeded simultaneously.

For direct plating, 200 µl of the 1:1 oyster-APW homogenate (i.e., 0.1 g homogenized oyster tissue) and 100 µl of a 1:10 dilution of the oyster-APW homogenate in APW (i.e., 0.01 g homogenized oyster tissue) were spread-plated in duplicate onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar and modified cellobiose-polymyxin B-colistin (mCPC) agar for isolating *V. parahaemolyticus* and *V. vulnificus*, respectively. After 24 h of incubation at 35°C and 40°C for TCBS and mCPC, respectively, five to ten presumptive *V. parahaemolyticus* colonies on TCBS (green or bluish green, round, 2-4 mm in diameter) and *V. vulnificus* on mCPC (flat yellow, fried egg shape, 1-2 mm in diameter) were subcultured on trypticase soy agar with 2% NaCl (TSAS) and confirmed by PCR as described below. Following isolation and identification, the isolates were stored at -80°C in brain heart infusion with 50% glycerol.

For enrichment, 50 g of the 1:1 oyster-APW homogenate was added into a sterile flask containing 200 ml of APW and incubated at 35°C with shaking at 100 rpm for 24 h. The enrichment broth was serially diluted in phosphate buffered saline (PBS) and appropriate

dilutions (10^{-2} , 10^{-3} , or 10^{-4}) were plated in duplicate onto either TCBS or mCPC agar. The remaining isolation procedures were the same as those described above for direct plating.

PCR Assays. Presumptive *V. parahaemolyticus* and *V. vulnificus* isolates were confirmed by performing a multiplex PCR assay that targeted both *V. parahaemolyticus* thermolabile hemolysin (encoded by *tl*) and *V. vulnificus* cytolysin (*vvhA*). For confirmed *V. parahaemolyticus* by this PCR, another multiplex PCR targeting the thermostable direct hemolysin (Tdh) encoded by *tdh* and the Tdh-related *trh* gene was conducted. Primer sequences for all four genes were the same as those described in BAM (Kaysner & DePaola, 2001).

Bacterial DNA templates were prepared by heating cell suspensions in TE (10 mM Tris, 1 mM EDTA; pH 8.0) at 95°C for 10 min. Each multiplex PCR mixture contained 1 x PCR buffer, 0.2 mM of deoxynucleotide triphosphate, 2.5 mM of MgCl₂, 0.5 unit of *Taq* DNA polymerase (Applied Biosystems, Foster City, CA), 0.5 μM of each primer, and 5 μl of DNA template in a total volume of 25 μl. PCR was conducted using 30 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, extension at 72°C for 1 min, and a final extension of 72°C for 7 min in a GeneAmp PCR System 2400 (Applied Biosystems). PCR products were analyzed by electrophoresis on 2% agarose gel containing ethidium bromide, and visualized under UV light. Gel images were documented by a Stratagene Eagle Eye II system (La Jolla, CA).

Antimicrobial Susceptibility Testing. Minimal inhibitory concentrations (MICs) for a randomly selected subset of 319 *V. parahaemolyticus* and *V. vulnificus* (Table 3-1, last three columns, numbers in parenthesis) were determined using a broth microdilution testing method as described in a document recently published by the Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS) (Clinical and Laboratory Standards Institute, 2006b). Additionally,

ten reference strains, including six *V. parahaemolyticus* (ATCC33847, ATCC49529, TX-2103, CT-6636, NY-477, and 8332924) and four *V. vulnificus* (VV1007, ATCC29306, VV-WR1, and 515-4C2), provided by Dr. Angelo DePaola from the Gulf Coast Seafood Laboratory, Food and Drug Administration, were tested. Eight antimicrobial agents of clinical relevance to treating vibriosis were tested: ampicillin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, imipenem, and tetracycline. All antimicrobials were purchased from Sigma-Aldrich (St. Louis, MO) except for imipenem (United States Pharmacopeia, Rockville, MD). For preparing stock solutions, antimicrobials were dissolved in distilled water or buffer as recommended by CLSI (Clinical and Laboratory Standards Institute, 2006a). A custom designed panel plate incorporating these antimicrobials at a concentration range of 0.03-64 µg/ml (for chloramphenicol and gentamicin, the test ranges of 1-1024 µg/ml and 0.06-64 µg/ml were used, respectively) was made with cation-adjusted Mueller-Hinton broth, sealed with perforated plate seal (Trek Diagnostic Systems Inc., Cleveland, OH), and stored at -80°C.

Before testing, the susceptibility panels were warmed at room temperature for at least 30 min. *Escherichia coli* ATCC 25922 was used as the quality control organism (Clinical and Laboratory Standards Institute, 2006b). Briefly, suspensions of *Vibrio* were prepared from fresh cultures grown on TSAS plates into 3-4 ml of 0.85% NaCl (physiological saline) and adjusted to a turbidity of 0.5 McFarland. Further dilutions were made in 10 ml of saline. A multichannel pipette was used to inoculate the panels, leaving only the negative control well uninoculated. Colony counts were performed regularly to validate the inoculum density to be around 5×10^5 CFU/ml in the wells. The panels were sealed and incubated at 35°C in ambient air for 16-20 h. MICs were recorded as the lowest concentrations of antimicrobial agents that completely

inhibited bacterial growth in the wells. The interpretive criteria followed CLSI document M45-A (Clinical and Laboratory Standards Institute, 2006b).

Data Analysis. Prevalence data sorted by *Vibrio* species, sampling location, and sampling season were analyzed by using the analysis of variance (ANOVA) (SAS for Windows, Version 9; SAS Institute Inc., Cary, NC). Antimicrobial susceptibility data were organized by each species for MIC₅₀, MIC₉₀, MIC range, and percentages of isolates that were susceptible, intermediate, or resistant to each antimicrobial tested. The MIC values (expressed in log₂ scale) between the two species were also compared using ANOVA. Additionally, resistance plus intermediate rates to ampicillin between species and among different sampling locations were analyzed by Chi-square and Fisher's exact two-tailed tests, and ANOVA. Differences between mean values were considered significant when $P < 0.05$.

Results

Five sampling trips were made to the Louisiana Gulf Coast from June 2005 to September 2006. Four retail outlets, including one grocery store and three seafood restaurants, were visited a total of 38 times between February and August 2006. Ninety-four oyster samples, 20 from the Gulf and 74 at retail, were collected and analyzed for the prevalence of *V. parahaemolyticus* and *V. vulnificus*, followed by susceptibility testing of some of the isolates.

Prevalence of *V. parahaemolyticus* and *V. vulnificus*. The prevalence rates of *V. parahaemolyticus* and *V. vulnificus* in 94 Gulf and retail oyster samples are shown in Table 3-1. A total of 622 *Vibrio* isolates, including 252 *V. parahaemolyticus* and 370 *V. vulnificus*, were recovered from 82 (87.2%) of the oyster samples. Among 53 samples (56.4%) that contained *V. parahaemolyticus*, the grocery store had the highest prevalence rate (85.7%), followed by restaurant A (66.7%), the Gulf (50%), restaurant B (50%), and restaurant C (25%). In contrast,

restaurant B (87.5%) had a significantly higher *V. vulnificus* prevalence rate ($P < 0.05$) in its oyster samples than those of the Gulf (55%) and restaurant C (50%), but not significantly different from that of restaurant A (66.7%) (Table 3-1). Interestingly, not a single *V. vulnificus* was isolated from the grocery store during the period sampled (Table 3-1). Among twenty-five (26.6%) oyster samples from which both *V. parahaemolyticus* and *V. vulnificus* were recovered, nine, nine, five, and two originated from restaurants A, B, the Gulf, and restaurant C, respectively. Overall the prevalence of one or both *Vibrio* species fell between 58.3% (restaurant C) and 100% (restaurant B), with restaurant C having a significantly lower rate than other sampling locations ($P < 0.05$) (Table 3-1). The average prevalence rate among the four retail outlets was calculated to be 89.2%. About half of the 622 *Vibrio* isolates were recovered using the direct plating method, and the other half through enrichment. For paired oyster samples collected from the same collection site, there were nine out of 38 occasions from retail outlets where the presence of *Vibrio* species differed (data not shown).

Seasonality of *V. parahaemolyticus* and *V. vulnificus* Presence in Oysters. The prevalence of *V. parahaemolyticus* and *V. vulnificus* varied greatly during the sampling period (Table 3-2). For example, there was an increase in the prevalence of *V. vulnificus* and simultaneous decrease of *V. parahaemolyticus* as the survey proceeded in 2006. The Gulf and retail samples in February and March were 75-100% culture positive for *V. parahaemolyticus*, but decreased to 0-33% in or between April and September (Table 3-2). In contrast, the prevalence rate of *V. vulnificus* in oysters was low in February/March (0 in the grocery store and 50% in the Gulf), and reached 100% in June and September for the retail and Gulf samples, respectively. At the end of retail sampling in August, the culture positive rate for *V. vulnificus* remained above 75%; whereas *V. parahaemolyticus* rates ranged between 0 to 67%. For the Gulf

Coast sampling in September and December, prevalence rates for *V. vulnificus* were 100% and 25%, respectively; whereas the positive rates for *V. parahaemolyticus* were 0 in September and 25% in December (Table 3-2). Overall a prevalence rate of 100% was observed for oysters from certain collecting sites between February and June for *V. parahaemolyticus* and June to September for *V. vulnificus*.

Due to unavailable or few data points in certain months (Table 3-2), we grouped the months arbitrarily into seasons, with December, February, and March as winter, April to June as spring, and July to September as summer. When pooling the samples from all locations, the prevalence of *V. parahaemolyticus* was significantly higher in winter (80%) and spring (77.8%) than summer (23.7%), where *V. vulnificus* had a higher significantly higher prevalence rate in summer (84.2%) than either spring (52.8%) or winter (15%), with the rates of spring and winter being significantly different from each other as well ($P < 0.05$). However, when examining the prevalence of either *V. parahaemolyticus* or *V. vulnificus* in oysters sampled during the seasons, no significant difference was observed, and the prevalence rates were 91.7%, 86.8%, and 80% for spring, summer, and winter, respectively.

Multiplex PCR Results. The first multiplex PCR confirmed the identity of presumptive *V. parahaemolyticus* and *V. vulnificus* in approximately 60% and 70% of the isolates picked, respectively (data not shown). However, there were a few cases (53 out of 252 *V. parahaemolyticus*) where colonies from mCPC were tested by this PCR to be *V. parahaemolyticus*, and even less frequently, 19 out of 370 *V. vulnificus* were isolated from the TCBS plates and confirmed by this PCR. Based on the second multiplex PCR assay specific for the *tdh* and *trh* genes in *V. parahaemolyticus*, none of the 252 *V. parahaemolyticus* recovered in this study possessed these two virulence-related genes.

Table 3 - 1 Prevalence of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in 94 Louisiana Gulf and Retail Oysters

Oyster site	collecting	Samples collected	No. (%) of samples positive for ^a			Isolates recovered ^b	No. of isolates belonging to ^b	
			V. <i>parahaemolyticus</i>	V. <i>vulnificus</i>	Either species		V. <i>parahaemolyticus</i>	V. <i>vulnificus</i>
Gulf		20	10 (50) ^{BC}	11 (55) ^B	16 (80) ^A	114 (70)	57 (40)	57 (30)
Grocery Store		14	12 (85.7) ^A	0 (0) ^C	12 (85.7) ^A	49 (46)	49 (46)	0 (0)
Restaurant A		24	16 (66.7) ^{AB}	16 (66.7) ^{AB}	23 (95.8) ^A	180 (86)	78 (44)	102 (42)
Restaurant B		24	12 (50) ^{BC}	21 (87.5) ^A	24 (100) ^A	227 (96)	54 (30)	173 (66)
Restaurant C		12	3 (25) ^C	6 (50) ^B	7 (58.3) ^B	52 (21)	14 (8)	38 (13)
Total		94	53 (56.4)	54 (57.4)	82 (87.2)	622 (319)	252 (168)	370 (151)

^a Prevalence rates under the same column followed by different letters are significantly different ($P < 0.05$). The letters A to C were used to indicate a descending order of the mean values of each group.

^b The numbers in parenthesis are the number of isolates included for susceptibility testing.

Table 3 - 2 Prevalence of *Vibrio parahaemolyticus* and *Vibrio vulnificus* by month of isolation, in oyster samples obtained from the Louisiana Gulf Coast and retail outlets in 2005-2006

Site	<i>Vibrio</i> Species	Ratio of positive samples vs. samples purchased at that site ^a											
		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Gulf	<i>parahaemolyticus</i>			3/4			6/6			0/6			1/4
	<i>vulnificus</i>			2/4			2/6			6/6			1/4
Grocery store	<i>parahaemolyticus</i>		2/2	10/10	0/2								
	<i>vulnificus</i>		0/2	0/10	0/2								
Restaurant A	<i>parahaemolyticus</i>				4/4	6/6	4/4	2/8	0/2				
	<i>vulnificus</i>				0/4	4/6	4/4	6/8	2/2				
Restaurant B	<i>parahaemolyticus</i>					4/4	3/6	1/8	4/6				
	<i>vulnificus</i>					1/4	6/6	8/8	6/6				
Restaurant C	<i>parahaemolyticus</i>					1/2	0/2	1/4	1/4				
	<i>vulnificus</i>					0/2	2/2	1/4	3/4				

^a When multiple sampling visits were made at one site during a particular month, the average prevalence rates were used. Blank data point means no sampling visits were made to that site in that month.

Antimicrobial Susceptibility Profiles. The MIC distributions of the 319 *V. parahaemolyticus* and *V. vulnificus* isolates sorted by species are presented in Table 3-3. The MIC₅₀, MIC₉₀, and MIC range for *V. parahaemolyticus* tended to be one or more dilutions higher than those for *V. vulnificus*, except in the case of imipenem where both *V. vulnificus* MIC₅₀ and MIC₉₀ were about four-fold higher. For ampicillin, the differences in MIC distributions between the two species were much greater (32-64 fold in MIC₅₀ and MIC₉₀), with *V. parahaemolyticus* MIC₅₀ falling in the resistance end of the MIC ranges, whereas *V. vulnificus* in the susceptible end.

Based on the statistical comparison of log₂MIC values among *V. parahaemolyticus* and *V. vulnificus* included in the susceptibility testing, significant differences were observed in MICs to ampicillin, cefotaxime, ciprofloxacin, imipenem, and tetracycline ($P < 0.0001$), among which imipenem was the only one that *V. vulnificus* had higher mean log₂MIC value (Table 3-3).

According to breakpoints recommended by the CLSI M45-A document (Clinical and Laboratory Standards Institute, 2006b), the only non-susceptible isolates among the 319 *Vibrio* tested were 95 (29.8%) and 41 (12.9%) isolates, possessing ampicillin MICs in the resistant and intermediate categories, respectively. These isolates were all *V. parahaemolyticus*, resulting in the overall ampicillin resistance and intermediate rates among the 168 *V. parahaemolyticus* isolates tested to be 56.5% and 24.4%, respectively (Table 3-4), i.e., 81% of the *V. parahaemolyticus* tested had an MIC ≥ 16 $\mu\text{g/ml}$. The 151 *V. vulnificus* isolates included in the susceptibility testing, on the other hand, were all susceptible to ampicillin. The distribution of ampicillin-resistant *V. parahaemolyticus* in the oyster collecting sites indicated that restaurant A had the highest ampicillin resistance and intermediate combined rate (90.9%), followed closely by restaurant B (90%), the Gulf (77.5%), the grocery store (74%), and restaurant C (50%).

Table 3 - 3 Antimicrobial susceptibility testing ranges, breakpoints, and MIC distribution for 319 *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates for eight antimicrobials tested

Antimicrobial agent	Test range (µg/ml)	Breakpoint (µg/ml) ^a			MIC (µg/ml) distribution for ^b							
		S	I	R	<i>V. parahaemolyticus</i> (n =168)				<i>V. vulnificus</i> (n =151)			
					MIC ₅₀	MIC ₉₀	Range	Mean ^b	MIC ₅₀	MIC ₉₀	Range	Mean ^b
Ampicillin	0.03-64	≤8	16	≥32	32	32	0.5-64	4.25 ^c	1	1	0.06-8	-0.20 ^c
Cefotaxime	0.03-64	≤8	16-32	≥64	0.25	0.5	≤0.03-4	-1.85 ^c	0.25	0.25	≤0.03-2	-2.27 ^c
Ceftazidime	0.03-64	≤8	16	≥32	0.25	0.5	≤0.03-4	-2.13	0.25	0.5	≤0.03-1	-2.17
Chloramphenicol	1-1024	≤8	16	≥32	1	1	0.06-8	-0.75	1	1	0.06-8	-0.50
Ciprofloxacin	0.03-64	≤1	2	≥4	0.125	0.5	≤0.03-1	-3.28 ^c	≤0.03	0.06	≤0.03-0.25	-5.39 ^c
Gentamicin	0.06-64	≤4	8	≥16	0.5	2	0.125-2	-0.49	0.5	1	≤0.03-2	-0.57
Imipenem	0.03-64	≤4	8	≥16	≤0.03	0.06	≤0.03-2	-5.29 ^c	0.125	0.25	≤0.03-0.5	-3.28 ^c
Tetracycline	0.03-64	≤4	8	≥16	0.5	1	0.06-2	-1.14 ^c	0.25	0.5	0.06-2	-2.21 ^c

^a Breakpoints recommended by the Clinical and Laboratory Standards Institute in M45-A (Clinical and Laboratory Standards Institute, 2006b). S, I, and R stand for susceptible, intermediate, and resistant, respectively.

^b Mean MIC values were expressed in log₂ scale. When MIC was ≤0.03 µg/ml, a -6 value was used as the value for mean calculation.

^c Indicates significantly different mean MIC values (in log₂ scale) between *V. parahaemolyticus* and *V. vulnificus* (*P* < 0.05).

However, except for that of restaurant C, all other ampicillin resistance and intermediate combined rates were not statistically significant (Table 3-4).

For the ten reference strains included in the susceptibility testing, similar to our oyster isolates, these strains were susceptible to the majority of antimicrobials tested except that ampicillin resistance was observed in 3 out of 6 *V. parahaemolyticus* strains and one additional *V. parahaemolyticus* was intermediate to ampicillin.

Table 3 - 4 The presence of ampicillin-resistant and intermediate *Vibrio parahaemolyticus* isolates from Louisiana Gulf and retail oysters among 168 *Vibrio parahaemolyticus* isolates included in the susceptibility testing

Site	No. (% ^a) of <i>V. parahaemolyticus</i> ^b		
	R	I	R+I ^c
Gulf	18 (45)	13 (32.5)	31 (77.5) ^A
Grocery Store	25 (54.3)	9 (19.6)	34 (73.9) ^A
Restaurant A	29 (65.9)	11 (25)	40 (90.9) ^A
Restaurant B	21 (70)	6 (20)	27 (90) ^A
Restaurant C	2 (25)	2 (25)	4 (50) ^B
Total	95 (56.5)	41 (24.4)	136 (81)

^a Denominators indicate numbers of *V. parahaemolyticus* recovered from oyster samples collected in that particular sampling site and were included for susceptibility testing

^b R and I stand for resistant and intermediate to ampicillin, respectively.

^c Percentages in the same column followed by different letters are significantly different ($P < 0.05$).

Discussion

This study represents the first report on both prevalence and antimicrobial susceptibility of *V. parahaemolyticus* and *V. vulnificus* in Louisiana oysters, from the Gulf harvesting sites and consumer ready, since 1998. The study demonstrated that these two *Vibrio* spp. may be frequently isolated from oysters harvested from the Louisiana Gulf Coast (80%) and from retail

outlets (89.2%), particularly during spring to summer (February to June) for *V. parahaemolyticus* and summer months (June to September) for *V. vulnificus*, when a 100% detection rate was recorded at some sampling sites. This observation is supported by previous survey studies indicating generally high prevalence and density of these *Vibrio* spp. in the Gulf Coast oysters and waters (Cook *et al.*, 2002a; Cook *et al.*, 2002b; DePaola *et al.*, 1990; DePaola *et al.*, 2003), and correlates well with human *Vibrio* infections reported to CDC through the Cholera and other *Vibrio* Illness Surveillance (COVIS) System that began in Gulf Coast states (Alabama, Florida, Louisiana, Mississippi, and Texas) in 1988 (Centers for Disease Control and Prevention, 2005c) and through FoodNet.

The prevalence rates (50%-60%) of individual *Vibrio* spp. observed in this study for either Gulf Coast or retail oyster samples combined, however, were lower than those reported by other investigators. In a comprehensive national survey of these two *Vibrio* spp. in U.S. retail shell oysters from June 1998 to July 1999, Cook *et al.* reported that oysters from the Gulf Coast consistently had the highest numbers of both *V. parahaemolyticus* and *V. vulnificus* year-round, and prevalence rates of 97.4% and 96.2% were observed for *V. parahaemolyticus* and *V. vulnificus*, respectively, among 80 retail oyster samples harvested from Louisiana (Cook *et al.*, 2002b). Another study conducted in 1999 and 2000 by the same group, examining *V. parahaemolyticus* in Atlantic and Gulf Coast oysters at harvest, reported the highest detection rate (82%) for the Gulf Coast samples and the lowest (34.6%) for the North Atlantic coast samples (Cook *et al.*, 2002a). A third study testing 156 Alabama oysters during the same period of time for *V. parahaemolyticus* reported the detection of this organism in all samples with the 25 g enrichments (DePaola *et al.*, 2003). Several reasons may account for differences in prevalence rates between the present study and others. First, since the main aim of our study was

to examine the antimicrobial susceptibility of the two vibrios instead of quantifying the levels of their presence in the oysters, our methodology was focused on isolation rather than enumeration. Therefore, we picked 5 to 10 presumptive *Vibrio* colonies from the direct plating or enrichment plates, instead of up to 48 colonies as recommended for quantitative direct-plating (DePaola *et al.*, 2003). Second, oysters sold at retail outlets, although harvested in the same region as the Gulf, may have gone through post-harvest treatments, resulting in significant reduction of the prevalence of *Vibrio* spp., but that information was not made available for the present study. Last, all the studies mentioned above were conducted in or before 2000, and the Louisiana Gulf Coast environment may have since changed, particularly in light of the two hurricanes, Katrina and Rita, which made landfalls in August and September 2005, respectively. These storms may have had an effect on the ecology of *Vibrio* spp. in oysters harvested from the Louisiana Gulf Coast.

The seasonal distribution of *V. parahaemolyticus* and *V. vulnificus* in the coastal waters and oysters, characterized by maximum population abundance during the summer months and subsequent decline to undetectable levels during the cold winter months, has been well documented by microbiological analysis and supported by epidemiological data of human *Vibrio* infections (Centers for Disease Control and Prevention, 2005c; Cook *et al.*, 2002b; DePaola *et al.*, 2003). Much of this seasonal distribution is attributable to the positive correlation between *Vibrio* density and water temperature (DePaola *et al.*, 2003; Pfeffer *et al.*, 2003; Randa *et al.*, 2004). In the present study, a similar seasonal trend in the prevalence rates of both *Vibrio* spp. was observed. Interestingly, our data indicated a marked difference in the seasonal distribution of individual *Vibrio* populations, with the highest prevalence rates for *V. parahaemolyticus* falling between February and June, whereas *V. vulnificus* from June to September, possibly suggesting

that *V. parahaemolyticus* strains are more cold tolerant than *V. vulnificus* strains. However, further characterizations of these *Vibrio* populations are needed to explain this observation.

Among 252 *V. parahaemolyticus* isolated and identified in this study, none possessed Tdh or Trh, thus were not considered to be pathogenic. Previous studies reported that the prevalence of pathogenic *V. parahaemolyticus* in the U.S. is low, ranging from 0-6% (Cook *et al.*, 2002a; Cook *et al.*, 2002b; DePaola *et al.*, 1990); however, a recent study found a much greater rate of detection (21.8%) of pathogenic *V. parahaemolyticus* isolates when many more colonies were tested for pathogenicity, particularly during cold months (DePaola *et al.*, 2003). Similar to previous studies, the present study did not conduct extensive examination of pathogenic *V. parahaemolyticus*, so the zero prevalence of pathogenic *V. parahaemolyticus* in the Louisiana oyster samples reported in this study should be interpreted with caution.

In 2005, according to CDC, COVIS received reports of 578 *Vibrio* isolates from 546 patients (Centers for Disease Control and Prevention, 2005c). Among 218 patients (40%) infected with *V. parahaemolyticus*, 23% were hospitalized and 1% died. On the other hand, *V. vulnificus* was isolated from 121 (22%) patients; 90% were hospitalized and 26% died (Centers for Disease Control and Prevention, 2005c). Despite their public health significance, strains of *V. parahaemolyticus* and *V. vulnificus* were not extensively monitored for antimicrobial resistance, in contrast to enteric pathogens such as *Salmonella* or *Campylobacter*. Aquatic bacteria including *V. parahaemolyticus* and *V. vulnificus* live in the coastal and estuarine waters, an open area particularly subject to environmental contaminations by agricultural runoff or waste water treatment plants, which may contain various levels of antimicrobials and heavy metals, and act as selective pressure for antimicrobial-resistant aquatic bacteria (Gordon *et al.*, 2007; Stepanauskas *et al.*, 2006).

Prior to May 2006, no standardized susceptibility testing method was available for non-cholera *Vibrio* spp. Because of this, it was difficult to compare data from different laboratories due to variables involved in the testing. CLSI recently published the M45-A document which presented the most current information for drug selection, interpretation, and quality control for MIC testing of infrequently isolated or fastidious bacteria, including non-Cholera *Vibrio* spp. (Clinical and Laboratory Standards Institute, 2006b; Jorgensen & Hindler, 2007). The present study closely followed these guidelines for MIC testing using broth microdilution. All eight antimicrobials tested in the present study were in accordance with the guidelines of the M45-A document and represent antimicrobials agents that may be used in the treatment of non-Cholera *Vibrio* spp. infections, particularly tetracycline, cefotaxime, ceftazidime, and fluoroquinolones. Our findings indicated that these first-line drugs remained highly effective against both *V. parahaemolyticus* and *V. vulnificus*; however, the high prevalence of ampicillin-resistant *V. parahaemolyticus* suggested that ampicillin should not be used empirically to treat *V. parahaemolyticus* infection. This is in contrast to recommendations posted by CDC (Centers for Disease Control and Prevention, 2005d). Interestingly, ampicillin resistance in *V. parahaemolyticus* is not a new phenomenon. A study in 1978 in the U.S. reported that over 90% of 160 *V. parahaemolyticus* was resistant to ampicillin, and exhibited β -lactamase activity (Joseph *et al.*, 1978). Another study conducted in Italy in 2001 also reported unexpectedly high frequency (80%) of ampicillin resistance in eight *V. parahaemolyticus* and six *V. vulnificus*, mostly attributable to the production of β -lactamase (Zanetti *et al.*, 2001). A recent report in the Philippines and Thailand found twelve out of fourteen *V. parahaemolyticus* were resistant to ampicillin (Maluping *et al.*, 2005). Similarly, a study in India reported 100% ampicillin resistance among seven *V. vulnificus* and five *V. parahaemolyticus* tested by the disk diffusion

method (Vaseeharan *et al.*, 2005). More recently, a study in Australia reported an ampicillin resistance rate of 40% for *Vibrio* spp., however, only one *V. parahaemolyticus* and no *V. vulnificus* were included in that study (Akinbowale *et al.*, 2006). The findings in the present study are in agreement with results from these earlier studies, which found high prevalence of ampicillin resistance in *V. parahaemolyticus*. Interestingly, none of the *V. vulnificus* isolates tested for antimicrobial susceptibility in this study showed ampicillin resistance.

Our examination of the prevalence and antimicrobial susceptibility of *V. parahaemolyticus* and *V. vulnificus* also revealed discernable differences between these two species, e.g. the higher prevalence of *V. parahaemolyticus* in colder months, the generally reduced susceptibility of *V. parahaemolyticus* to antimicrobials, and the compromised ability of *V. vulnificus* to grow directly on TCBS agar, suggesting different microbial physiology may play a role. Further studies are needed to better explain these phenomena. Those physiological differences may provide a basis to develop better selective and differential media for isolating *V. parahaemolyticus* and *V. vulnificus*, which can dramatically reduce the number of colonies needed to be tested by molecular confirmation methods such as probe hybridization or PCR.

Conclusion

Our findings demonstrated the prevalence of *V. parahaemolyticus* and *V. vulnificus* in oysters from the Louisiana Gulf and at retail outlets, a point of contact closest to human consumption. These results illustrated the need for appropriate food safety practices when consuming these products. Part of the food safety program could be a national oyster surveillance program at the retail level as no such program currently exists. Moreover, the observed high percentage of ampicillin-resistant *V. parahaemolyticus* isolates suggests a potential for low efficiency of ampicillin in empirical treatment of infections caused by this

organism. Continued monitoring of both the prevalence and antimicrobial susceptibility profile is needed to better ensure oyster safety; particularly the retail survey could be expanded to the national level.

References

Akinbowale, O. L., Peng, H. & Batrton, M. D. (2006). Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia. *J Appl Microbiol* **100**, 1103-1113.

Barbieri, E., Falzano, L., Fiorentini, C. & other authors (1999). Occurrence, diversity, and pathogenicity of halophilic *Vibrio* spp. and non-O1 *Vibrio cholerae* from estuarine waters along the Italian Adriatic coast. *Appl Environ Microbiol* **65**, 2748-2753.

Blake, P. A., Weaver, R. E. & Hollis, D. G. (1980). Diseases of humans (other than cholera) caused by vibrios. *Annual review of microbiology* **34**, 341-367.

Cabello, F. C. (2006). Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental microbiology* **8**, 1137-1144.

Centers for Disease Control and Prevention (2005a). *Vibrio vulnificus*: general information.

Centers for Disease Control and Prevention (2005b). *Vibrio* illnesses after Hurricane Katrina--multiple states, August-September 2005. *MMWR Morb Mortal Wkly Rep* **54**, 928-931.

Centers for Disease Control and Prevention (2005c). *Vibrio* outbreak summaries. Atlanta, GA.

Centers for Disease Control and Prevention (2005d). *Vibrio parahaemolyticus*: general information.

Centers for Disease Control and Prevention (2006). National Antimicrobial Resistance Monitoring System for enteric bacteria: annual report, 2003. Atlanta, GA.

Centers for Disease Control and Prevention (2010). Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food--10 states, 2009. *MMWR Morb Mortal Wkly Rep* **58**, 418-422.

Chen, S., Zhao, S., White, D. G., Schroeder, C. M., Lu, R., Yang, H., McDermott, P. F., Ayers, S. & Meng, J. (2004). Characterization of multiple-antimicrobial-resistant *Salmonella* serovars isolated from retail meats. *Applied and environmental microbiology* **70**, 1-7.

Clinical and Laboratory Standards Institute (2006a). Performance Standards for Antimicrobial Susceptibility Testing; Sixteenth Informational Supplement (M100-S16). Wayne, PA.

Clinical and Laboratory Standards Institute (2006b). Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved standard (M45-A). Wayne, PA.

Cook, D. W., Bowers, J. C. & DePaola, A. (2002a). Density of total and pathogenic (*tdh+*) *Vibrio parahaemolyticus* in Atlantic and Gulf coast molluscan shellfish at harvest. *J Food Prot* **65**, 1873-1880.

Cook, D. W., Oleary, P., Hunsucker, J. C., Sloan, E. M., Bowers, J. C., Blodgett, R. J. & Depaola, A. (2002b). *Vibrio vulnificus* and *Vibrio parahaemolyticus* in U.S. retail shell oysters: a national survey from June 1998 to July 1999. *J Food Prot* **65**, 79-87.

D'Lima C, B., Miller, W. G., Mandrell, R. E., Wright, S. L., Siletzky, R. M., Carver, D. K. & Kathariou, S. (2007). Clonal population structure and specific genotypes of multidrug-resistant *Campylobacter coli* from Turkey. *Applied and environmental microbiology* **73**, 2156-2164.

Dalsgaard, A., Forslund, A., Petersen, A. & other authors (2000). Class 1 integron-borne, multiple-antibiotic resistance encoded by a 150-kilobase conjugative plasmid in epidemic *Vibrio cholerae* O1 strains isolated in Guinea-Bissau. *J Clin Microbiol* **38**, 3774-3779.

DePaola, A., Hopkins, L. H., Peeler, J. T., Wentz, B. & McPhearson, R. M. (1990). Incidence of *Vibrio parahaemolyticus* in U.S. coastal waters and oysters. *Appl Environ Microbiol* **56**, 2299-2302.

DePaola, A., Nordstrom, J. L., Bowers, J. C., Wells, J. G. & Cook, D. W. (2003). Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. *Appl Environ Microbiol* **69**, 1521-1526.

Ge, B., McDermott, P. F., White, D. G. & Meng, J. (2005). Role of efflux pumps and topoisomerase mutations in fluoroquinolone resistance in *Campylobacter jejuni* and *Campylobacter coli*. *Antimicrobial agents and chemotherapy* **49**, 3347-3354.

Gordon, L., Giraud, E., Ganiere, J. P., Armand, F., Bouju-Albert, A., de la Cotte, N., Mangion, C. & Le Bris, H. (2007). Antimicrobial resistance survey in a river receiving effluents from freshwater fish farms. *J Appl Microbiol* **102**, 1167-1176.

Hervio-Heath, D., Colwell, R. R., Derrien, A., Robert-Pillot, A., Fournier, J. M. & Pommeuy, M. (2002). Occurrence of pathogenic vibrios in coastal areas of France. *J Appl Microbiol* **92**, 1123-1135.

- Hochhut, B., Lotfi, Y., Mazel, D., Faruque, S. M., Woodgate, R. & Waldor, M. K. (2001).** Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. *Antimicrob Agents Chemother* **45**, 2991-3000.
- Hoi, L., Larsen, J. L., Dalsgaard, I. & Dalsgaard, A. (1998).** Occurrence of *Vibrio vulnificus* biotypes in Danish marine environments. *Appl Environ Microbiol* **64**, 7-13.
- Iwanaga, M., Toma, C., Miyazato, T., Insisiengmay, S., Nakasone, N. & Ehara, M. (2004).** Antibiotic resistance conferred by a class I integron and SXT constin in *Vibrio cholerae* O1 strains isolated in Laos. *Antimicrob Agents Chemother* **48**, 2364-2369.
- Jacobs, L. & Chenia, H. Y. (2007).** Characterization of integrons and tetracycline resistance determinants in *Aeromonas* spp. isolated from South African aquaculture systems. *International journal of food microbiology* **114**, 295-306.
- Jorgensen, J. H. & Hindler, J. F. (2007).** New consensus guidelines from the Clinical and Laboratory Standards Institute for antimicrobial susceptibility testing of infrequently isolated or fastidious bacteria. *Clin Infect Dis* **44**, 280-286.
- Joseph, S. W., DeBell, R. M. & Brown, W. P. (1978).** *In vitro* response to chloramphenicol, tetracycline, ampicillin, gentamicin, and beta-lactamase production by halophilic vibrios from human and environmental sources. *Antimicrob Agents Chemother* **13**, 244-248.
- Joseph, S. W., Colwell, R. R. & Kaper, J. B. (1982).** *Vibrio parahaemolyticus* and related halophilic vibrios. *Critical reviews in microbiology* **10**, 77-124.
- Kaysner, C. A. & DePaola, A. (2001).** *Vibrio*. In *Bacteriological Analytical Manual online*, Chapter 9. Edited by U.S Food and Drug Administration. Rockville, MD.
- Maluping, R. P., Lavilla-Pitogo, C. R., DePaola, A., Janda, J. M., Krovacek, K. & Greko, C. (2005).** Antimicrobial susceptibility of *Aeromonas* spp., *Vibrio* spp. and *Plesiomonas shigelloides* isolated in the Philippines and Thailand. *Int J Antimicrob Agents* **25**, 348-350.
- Mazel, D. & Davies, J. (1999).** Antibiotic resistance in microbes. *Cell Mol Life Sci* **56**, 742-754.
- Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M. & Tauxe, R. V. (1999).** Food-related illness and death in the United States. *Emerg Infect Dis* **5**, 607-625.
- Morris, J. G., Jr. & Tenney, J. (1985).** Antibiotic therapy for *Vibrio vulnificus* infection. *Jama* **253**, 1121-1122.
- Oliver, J. D. (2006).** *Vibrio vulnificus*, p. 349-366. Edited by F. L. Thompson, B. Austin, and J. Swings (ed.), *The biology of vibrios*. Washitonton, DC: ASM press.

- Pfeffer, C. S., Hite, M. F. & Oliver, J. D. (2003).** Ecology of *Vibrio vulnificus* in estuarine waters of eastern North Carolina. *Appl Environ Microbiol* **69**, 3526-3531.
- Randa, M. A., Polz, M. F. & Lim, E. (2004).** Effects of temperature and salinity on *Vibrio vulnificus* population dynamics as assessed by quantitative PCR. *Appl Environ Microbiol* **70**, 5469-5476.
- Skov, M. N., Andersen, J. S., Aabo, S. & other authors (2007).** Antimicrobial Drug Resistance of *Salmonella* Isolates from Meat and Humans, Denmark. *Emerging infectious diseases* **13**, 638-641.
- Stepanauskas, R., Glenn, T. C., Jagoe, C. H., Tuckfield, R. C., Lindell, A. H., King, C. J. & McArthur, J. V. (2006).** Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. *Environmental microbiology* **8**, 1510-1514.
- Strom, M. S. & Paranjpye, R. N. (2000).** Epidemiology and pathogenesis of *Vibrio vulnificus*. *Microbes and infection / Institut Pasteur* **2**, 177-188.
- Tang, H. J., Chang, M. C., Ko, W. C., Huang, K. Y., Lee, C. L. & Chuang, Y. C. (2002).** *In vitro* and *in vivo* activities of newer fluoroquinolones against *Vibrio vulnificus*. *Antimicrob Agents Chemother* **46**, 3580-3584.
- Vaseeharan, B., Ramasamy, P., Murugan, T. & Chen, J. C. (2005).** *In vitro* susceptibility of antibiotics against *Vibrio* spp. and *Aeromonas* spp. isolated from *Penaeus monodon* hatcheries and ponds. *Int J Antimicrob Agents* **26**, 285-291.
- Zanetti, S., Spanu, T., Deriu, A., Romano, L., Sechi, L. A. & Fadda, G. (2001).** *In vitro* susceptibility of *Vibrio* spp. isolated from the environment. *Int J Antimicrob Agents* **17**, 407-409.

CHAPTER 4: GENOTYPIC CHARACTERIZATION OF *VIBRIO VULNIFICUS* ISOLATES
FROM LOUISIANA GULF AND RETAIL RAW OYSTERS

Part I: Characterization of Clinical and Environmental Types of *Vibrio vulnificus* Isolates from Louisiana Oysters

Reprint by permission of “Foodborne Pathogens and Disease” (see appendix for permission from publisher)

Citation: Han, F., S. Pu, A. Hou, and B. Ge. 2009. Characterization of clinical and environmental types of *Vibrio vulnificus* isolates from Louisiana oysters. Foodborne Pathogens and Disease. 6:1251-8

Introduction

Vibrio vulnificus is a Gram-negative halophilic bacterium that inhabits warm coastal and estuarine waters worldwide, and the numbers are especially high in filter-feeding bivalve mollusks such as oysters (Oliver, 2006; Strom & Paranjpye, 2000). Three types of human illnesses can result from contact with *V. vulnificus*. Ingestion of the organism in raw or undercooked oysters can rapidly lead to primary septicemia, a severe and life-threatening illness characterized by fever, nausea, hypotension, and secondary blistering skin lesions (Blake *et al.*, 1979; Morris, 1988). When persons with preexisting wounds come into direct contact with seawaters containing *V. vulnificus*, potentially fatal wound infections may occur (Tacket *et al.*, 1984). Much less frequently, *V. vulnificus* causes gastroenteritis following raw oyster consumption (Strom & Paranjpye, 2000). While no fatality has been reported from *V. vulnificus* gastroenteritis, the reported case-fatality rate for primary septicemia and wound infection combined is around 30%, responsible for approximately 30 deaths annually in the United States (Cholera and Other *Vibrio* Illness Surveillance, 2009).

As an opportunistic human pathogen, *V. vulnificus* causes fatal illness predominantly in at-risk groups, which include people with immunocompromising conditions, diabetes, and elevated serum iron concentrations due to chronic liver disease or alcohol abuse (Strom & Paranjpye, 2000). Besides host susceptibility, epidemiological data also suggest that only a small percentage of *V. vulnificus* strains in oysters are virulent (Cholera and Other *Vibrio* Illness Surveillance, 2009; Jackson *et al.*, 1997; Jones & Oliver, 2009; Warner & Oliver, 2008a)

Several virulence factors have been identified in *V. vulnificus* as reviewed previously (Gulig *et al.*, 2005; Jones & Oliver, 2009). The expression of a capsular polysaccharide (CPS) is considered a major virulence factor for *V. vulnificus* (Chatzidaki-Livanis *et al.*, 2006b; Simpson

et al., 1987). However, both clinical and environmental isolates are generally encapsulated (Wright *et al.*, 1996). The frequencies of occurrence of other putative virulence factors, including lipopolysaccharide (McPherson *et al.*, 1991), flagella (Kim & Rhee, 2003), cytolysin (Gray & Kreger, 1985), and metalloprotease (Kothary & Kreger, 1987) did not differ between environmental and clinical *V. vulnificus* isolates as examined by multiple studies (DePaola *et al.*, 2003; Gulig *et al.*, 2005; Stelma *et al.*, 1992). Using animal models, studies found that both clinical and environmental *V. vulnificus* isolates were virulent to mice (DePaola *et al.*, 2003; Starks *et al.*, 2000; Stelma *et al.*, 1992; Tison & Kelly, 1986); although one study found that higher bacterial inoculum was required for environmental isolates to generate identical frequency and magnitude of infection as clinical ones (Starks *et al.*, 2000). Therefore, there lacks a unique virulence marker that can be used to screen for virulent *V. vulnificus* isolates from oysters. One exception was the siderophores production which was found to differ between a virulent and weakly virulent *V. vulnificus* strain (Simpson & Oliver, 1983). Another study also reported a good correlation between clinical-type *V. vulnificus* isolates in shellfish and the detection of a vulnibactin-encoding *viuB* gene (Panicker *et al.*, 2004b), one of the siderophore genes involved in iron acquisition (Litwin *et al.*, 1996). However, because the reverse primer used in that study extended to the *viuB* downstream intergenic region, technically, the target detected was a *viuB*-associated fragment, not *viuB* per se.

Given the lack of unique virulence markers, bacterial genotyping methods have been used recently to associate certain *V. vulnificus* genotypes with clinical or environmental type strains (Chatzidaki-Livanis *et al.*, 2006a; Gordon *et al.*, 2008; Nilsson *et al.*, 2003; Rosche *et al.*, 2005; Vickery *et al.*, 2007; Warner & Oliver, 2008a; Warner & Oliver, 1999). Firstly, using random amplified polymorphic DNA (RAPD), a virulence-correlated gene (*vcg*) was identified

in *V. vulnificus* (Warner & Oliver, 1999). Among 55 randomly selected *V. vulnificus*, 90% of clinical isolates possessed the *vcgC* sequence variant and 93% of environmental isolates had the *vcgE* sequence variant (Rosche *et al.*, 2005). Secondly, the polymorphic regions (17 bp) of the *V. vulnificus* 16S rRNA gene have been explored to differentiate between clinical and environmental strains (Nilsson *et al.*, 2003; Vickery *et al.*, 2007). Using restriction fragment length polymorphism (RFLP), a study found that the majority (31 out of 33) of nonclinical *V. vulnificus* isolates had 16S rRNA type A, whereas a significant percentage (24 out of 34) of clinical isolates belonged to 16S rRNA type B (Nilsson *et al.*, 2003). Real-time PCR assays have since been designed to differentiate the two 16S rRNA genotypes (Gordon *et al.*, 2008; Vickery *et al.*, 2007). Lastly, genetic variations in the *V. vulnificus* CPS operon have been characterized and two distinct genotypes termed CPS allele 1 and 2 were identified (Chatzidaki-Livanis *et al.*, 2006b). A follow-up study analyzing 68 clinical and environmental *V. vulnificus* isolates indicated a significant association between clinical isolates and CPS allele 1 whereas environmental isolates and CPS allele 2 (Chatzidaki-Livanis *et al.*, 2006a). Additionally, that study also independently evaluated the use of *vcg* and 16S RNA to differentiate clinical and environmental types of *V. vulnificus* isolates. Although significant associations of distinct genotypes with clinical isolates were confirmed, greater diversity (i.e., lower percentages of association) than those previously reported was also observed (Chatzidaki-Livanis *et al.*, 2006a). Therefore, more independent studies are needed to evaluate the usefulness of these biomarkers to predict clinically important *V. vulnificus* isolates from environmental sources such as oysters.

We previously recovered 349 *V. vulnificus* isolates from Louisiana oysters (Han *et al.*, 2007). The objectives of this study were to determine the distribution of different genotypes among these *V. vulnificus* isolates by analyzing the genetic variations in *vcg*, 16S rRNA, and

CPS, and the presence/absence of the *viuB*-associated fragment, and to identify environmental factors associated with an increased prevalence of clinical-type *V. vulnificus* isolates in oysters.

Materials and Methods

Bacterial Strains and Culture Conditions. *V. vulnificus* strains ($n = 356$) used in this study included 7 reference strains (Table 4-1) and 349 isolates previously recovered from Louisiana Gulf and retail oysters (Han *et al.*, 2007). The *V. vulnificus* isolates from oysters were recovered using methods described in the Food and Drug Administration Bacteriological Analytical Manual and PCR was used for confirmation and speciation (Han *et al.*, 2007). The cultures were stored in Luria-Bertani broth (BD Diagnostic Systems, Sparks, MD) containing 30% glycerol at -80°C . *V. vulnificus* strains were routinely cultured on trypticase soy agar (BD Diagnostic Systems) supplemented with 2% NaCl and incubated at 35°C for 24 h.

Table 4 - 1 *Vibrio vulnificus* reference strains used in this study, their source of isolation, and genotypic characteristics based on the virulence-correlated gene (*vcg*), 16S rRNA, capsular polysaccharide operon (CPS), and the vulnibactin gene (*viuB*)-associated fragment

<i>V. vulnificus</i> strain ^a	Source of isolation	Characteristics ^b						
		<i>vcg</i>		16S rRNA		CPS		<i>viuB</i>
		C	E	A	B	1	2	
Clinical strains ($n = 5$)								
ATCC 27562	Blood, Florida	-	+	+	-	-	+	+
ATCC 29306	Corneal ulcer, Virginia	-	+	+	-	-	+	+
ATCC 33815	Leg ulcer, Wisconsin	+	-	-	+	+	-	+
ATCC 33816	Blood, Alaska	+	-	-	+	+	-	+
1007	Blood, Louisiana	+	-	-	+	+	-	+
Environmental strains ($n = 2$)								
515-4C2	Oyster, California	-	+	+	-	-	+	-
WR1	Sea water, Washington	-	+	+	-	-	-	-

^a ATCC, American Type Culture Collection, Manassas, VA.

^b The following genotypes denote clinical-type *V. vulnificus*: *vcgC*, 16S rRNA type B, CPS allele 1, and the presence of *viuB*.

“+” stands for amplification of the gene, “-” stands for no amplification of the gene.

Multilocus PCR Assays. Primer sets used for each locus are listed in Table 4-2. For *vcg*, two pairs of primers (a common reverse primer) previously published were utilized, one targeting the clinical-type *V. vulnificus* strains and the other one amplifying environmental-type strains (Rosche *et al.*, 2005). For 16S rRNA, a Taqman real-time PCR assay designed previously was adopted (Vickery *et al.*, 2007). For CPS, two pairs of primers were designed using the Primer3 software (<http://frodo.wi.mit.edu/>), one targeting the hypothetical protein 1 (HP1) in *V. vulnificus* MO6-24/O (CPS allele 1) and the other one amplifying the hypothetical protein 2 (HP2) in *V. vulnificus* YJ106/O (CPS allele 2) (Chatzidaki-Livanis *et al.*, 2006b). For *viuB*-associated fragment, one pair of primers was used which targeted the *viuB* gene and downstream intergenic region, and the amplification of this fragment indicated clinically important *V. vulnificus* strains (Panicker *et al.*, 2004a; Panicker *et al.*, 2004b).

Chromosomal DNAs of the *V. vulnificus* strains were prepared by using a UltraClean DNA isolation kit (MO BIO Laboratories, Carlsbad, CA). PCR or real-time PCR conditions for *vcg*, 16S rRNA, and the *viuB* fragment followed previously described protocols (Panicker *et al.*, 2004b; Rosche *et al.*, 2005; Vickery *et al.*, 2007). For CPS, each PCR mix in a total volume of 25 μ l consisted of the following: 1 \times PCR buffer, 0.2 mM of each deoxynucleotide triphosphate, 1.5 mM of MgCl₂, 0.5 unit of Go Taq Hot Start Polymerase (Promega, Madison, WI), 0.2 μ M of each primer (Invitrogen, Carlsbad, CA), and 1 μ l of chromosomal DNA template. The PCR reaction was conducted using 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min in a Bio-Rad C1000 Thermal Cycler (Hercules, CA). PCR products were analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide, and visualized under UV light, and documented by a Gel Doc XR system (Bio-Rad).

Table 4 - 2 PCR primers used to characterize *Vibrio vulnificus* genotypes

Target locus	Genotype ^a	Primer name	Sequence (5'-3') ^b	GenBank accession no.	Position	Amplicon size (bp) ^c	Reference
<i>vcg</i>	C	P1	AGCTGCCGATAGCGATCT	AY626575	156-173	278	(Rosche <i>et al.</i> , 2005)
		P3	CGCTTAGGATGATCGGTG		416-433		
	E	P2	CTCAATTGACAATGATCT	AY626579	156-173	278	
		P3	CGCTTAGGATGATCGGTG		416-433		
16S rRNA	Common	Vvu16S51-F	CAAGTCGAGCGGCAGCA	X76333 and X76334	51-67	171	(Vickery <i>et al.</i> , 2007)
		Vvu16S221-R	TCCTGACGCGAGAGGCC		205-221		
	A	Vvu16SA-P	6FAM-TGATAGCTTCGGCTCAA-MGBNFQ	X76333	173-189		
	B	Vvu16SB-P	TET-CCCGTAGGCATCATGC-MGBNFQ		X76334		
CPS	1	HP1F	TTTGGGATTTGAAAGGCTTG	DQ360502	2156-2175	342	This study
		HP1R	GTGCCTTTGCGAATTTTGAT		2478-2497		
	2	HP2F	TTCCATCAAACATCGCAGAA	NC_005139 (VV0338)	125-144	152	
		HP2R	CTTTTGTCCGGCTTCTATCG		257-276		
<i>viuB</i> fragment	clinical	F- <i>viuB</i>	GGTTGGGCACTAAAGGCAGATATA	U32676	1729-1752	505	(Panicker <i>et al.</i> , 2004a)
		R- <i>viuB</i>	CGGCAGTGGACTAATACGCAGC		2212-2233		

^a The following genotypes denote clinical-type *V. vulnificus*: *vcgC*, 16S rRNA type B, CPS allele 1, and the presence of *viuB*-associated fragment.

^b MGBNFQ is minor groove binding non-fluorescent quencher.

^c Differences in amplicon sizes were noted from those originally published after reanalysis of the sequences and primer locations.

Statistical Analysis. The distributions of different genotypes among *V. vulnificus* oyster isolates were sorted by the target locus and the month of isolation and analyzed by using one-way analysis of variance (ANOVA) followed by post-hoc multiple comparisons using the Least Significant Difference (LSD) test (SAS for Windows, version 9; SAS Institute Inc., Cary, NC). Additionally, differences in the presence of the *viuB*-associated fragment among clinical-type (harboring *vcgC*, 16S rRNA B, CPS allele 1, and the *viuB* fragment) and environmental-type (being *vcgE*, 16S rRNA A, CPS allele 2, and lack the *viuB* fragment) *V. vulnificus* isolates as characterized by different markers (*vcg*, 16S rRNA, and CPS) were compared by using the Chi-square test. Differences between the mean values were considered significant when $P < 0.05$.

Results

Distribution of *V. vulnificus* Genotypic Profiles. Genotypic profiles of the seven *V. vulnificus* reference strains are shown in Table 4-1. *V. vulnificus* genotypes were designated using a combination of the strains' *vcg* type, 16S rRNA type, and CPS allele type following that order. Among the five *V. vulnificus* clinical strains, two (ATCC 27562 and ATCC 29306) had genotype EA2, and the rest (ATCC 33815, ATCC 33816, and 1007) CB1. For the two environmental strains, 515-4C2 possessed genotype EA2 whereas WR1 had genotype EANone since no amplification was obtained using either CPS allele 1 or 2 primer sets (Table 4-1).

Table 4-3 shows the distribution of genotypic profiles among 349 *V. vulnificus* oyster isolates. Based on the *vcg* genotypic analysis, the majority (59%) of *V. vulnificus* oyster isolates belonged to environmental-type strains (genotype *vcgE*), and the remainders (41%) were *vcgC* genotype which was associated with clinical-type strains. Similarly, there was also a higher percentage of *V. vulnificus* oyster isolates possessing environmental-type genotypes than that of clinical-type ones based on the analysis of the 16S rRNA and CPS loci (Table 4-3). For 16S

rRNA, there was a small proportion (8%) of the oyster isolates harbored both A and B genotypes, making it difficult to designate them as clinical- or environmental-type strains (Table 4-3). For CPS, the absence of both allele 1 and 2 indicated that the strain was not encapsulated, i.e., an environmental-type strain. Therefore, environmental-type strains were 72.5% based on the CPS analysis, much higher than clinical-type strains (27.5%).

Table 4 - 3 Distributions of genotypic profiles among 349 *Vibrio vulnificus* oyster isolates based on genotypic characteristics of three loci (*vcg*, 16S rRNA, and CPS)

Target locus	Clinical-type		Environmental-type		Atypical ^a	
	Genotype	No. (%)	Genotype	No. (%)	Genotype	No. (%)
<i>vcg</i>	C	143 (41)	E	206 (59)	ND	
16S rRNA	B	141 (40.4)	A	180 (51.6)	AB	28 (8)
CPS	1	96 (27.5)	2	180 (51.6)	ND	
			None	73 (20.9)		
			Subtotal	253 (72.5)		
All three loci	CB1	74 (21.2)	EA2	130 (37.2)	CA1	1 (0.3)
			EANone	31 (8.9)	CA2	3 (0.9)
			Subtotal	161 (46.1)	CAB2	1 (0.3)
					CABNone	1 (0.3)
					CB2	25 (7.2)
					CBNone	38 (10.9)
					EA1	15 (4.3)
					EAB1	6 (1.7)
					EAB2	18 (5.2)
					EABNone	2 (0.6)
					EB2	3 (0.9)
					EBNone	1 (0.3)
					Subtotal	114 (32.7)

^a ND means not detected.

When combining the three loci, the prevalence of environmental-type *V. vulnificus* strains among the 349 oyster isolates was 46.1% compared to 21.2% for clinical-type strains (Table 4-3). A notable percentage (32.7%) of isolates was atypical with various genotypic combinations

of the three loci (Table 4-3). Major atypical genotypes include CBNone (38; 10.9%), CB2 (25; 7.2%), EAB2 (18; 5.2%), and EA1 (15; 4.3%).

Prevalence of the *viuB*-associated Fragment. The presence of the *viuB* fragment among the seven *V. vulnificus* reference strains is also shown in Table 4-1. All five clinical strains possessed the *viuB*-associated fragment, whereas neither of the two environmental *V. vulnificus* strains was positive for this gene, indicating a good correlation between the presence of the *viuB* fragment and the clinical source of the strains as well as the absence of this gene and the environmental source of the strains.

Among 349 *V. vulnificus* oyster isolates, the majority (59%) showed no amplification of the *viuB* fragment. The association between environmental and clinical genotypes based on analysis of each of the three loci (*vcg*, 16S rRNA, and CPS) and the presence/absence of the *viuB* fragment is presented in Table 4-4. Based on either *vcg* or 16S rRNA analysis, over 75% of isolates assigned to the clinical-type *V. vulnificus* strains had the *viuB* fragment, whereas between 83-85% of the environmental-type strains lacked this fragment. For isolates assigned to be clinical-type strains based on the CPS analysis, 63.5% of them were *viuB*-associated fragment positive, while only 32.4% of environmental-type isolates based on CPS were *viuB*-associated fragment positive (Table 4-4). Statistical analysis indicated significantly higher percentages of clinical-type strains than environmental ones possessed the *viuB* fragment regardless of which genotyping analysis was used to assign the clinical- or environmental-type strains ($P < 0.0001$).

Prediction of Clinically Important *V. vulnificus*. Predictions were made using various combinations of the four biomarkers: *vcg* type, 16S rRNA type, CPS allele type, and the presence/absence of the *viuB*-associated fragment. When all four biomarkers were used, three out of five *V. vulnificus* reference strains from clinical sources were shown to be clinically important

because of having CA1 genotype and possessing *viuB*-associated fragment, whereas none of the two environmental strains was presumed to be clinically important due to possessing EA2 or EAnone genotype and lacking the *viuB* fragment.

Table 4 - 4 Associations between environmental and clinical *Vibrio vulnificus* genotypes based on analysis of each of the three loci (*vcg*, 16S rRNA, and CPS) and the presence/absence of the *viuB*-associated fragment among 349 *V. vulnificus* oyster isolates

Target locus ^a	Number (percentage) of <i>V. vulnificus</i> isolates with the presence or absence of the <i>viuB</i> -associated fragment among all isolates of							
	Clinical-type				Environmental-type			
	Genotype	No.	Present	Absent	Genotype	No.	Present	Absent
<i>vcg</i>	C	143	108 (75.5)	35 (24.5)	E	206	35 (17)	171 (83)
16S rRNA	B	141	107 (75.9)	34 (24.1)	A	180	27 (15)	153 (85)
CPS	1	96	61 (63.5)	35 (36.5)	2	180	49 (27.2)	131 (72.8)
					None	73	33 (45.2)	40 (54.8)
					Subtotal	253	82 (32.4)	171 (67.6)

^a Due to the presence of atypical isolates based on the 16S rRNA gene analysis, the total number of isolates did not add up to 349 for the 16S rRNA row.

Table 4-5 shows predictions of clinically important *V. vulnificus* isolates from the oyster samples based on various combinations of the biomarkers used. When using all four biomarkers, 16.3% (57/349) of isolates were predicted to be clinically important due to harboring *vcgC*, 16S rRNA B, CPS allele 1, and the *viuB* fragment. When three biomarkers were used, the predicted percentages of clinical-type *V. vulnificus* strains ranged between 16.3% and 30.7%. When only two biomarkers were used, the range became 17.5-39.3% for clinically important *V. vulnificus* strains. Regardless of two or three biomarkers were used, combinations including *vcg* and 16S rRNA gave higher percentages of clinically important *V. vulnificus* isolates whereas combinations including CPS gave lower percentages. Furthermore, agreements in assigning clinical or environmental type *V. vulnificus* strains based on all the biomarkers used in each combination are presented in Table 4-5. Not surprisingly, the agreement was the lowest when all

four biomarkers were used (55.3%), and among combinations involving two biomarkers, the highest agreement was found in the *vcg*/16S rRNA combination (89.7%) whereas the lowest one CPS/*viuB* (66.5%). When three markers were used, the highest agreement was found for *vcg*/16S rRNA/*viuB* (73.6%) and the lowest for 16S rRNA/CPS/*viuB* (56.2%).

Table 4 - 5 Predictions of clinically important *Vibrio vulnificus* isolates from Louisiana oysters based on various combinations of the four biomarkers used in this study

Biomarker combination ^a	Clinical-type		Environmental-type		Agreement ^b (%)
	Genotype	No. (%)	Genotype	No. (%)	
<i>vcg</i> /16S rRNA/CPS/ <i>viuB</i>	CB1Yes	57 (16.3)	EA2No or EANoneNo	136 (39)	193 (55.3)
<i>vcg</i> /16S rRNA/CPS	CB1	74 (21.2)	EA2 or EANone	161 (46.1)	235 (67.3)
<i>vcg</i> /16S rRNA/ <i>viuB</i>	CBYes	107 (30.7)	EANo	150 (43)	257 (73.6)
<i>vcg</i> /CPS/ <i>viuB</i>	C1Yes	58 (16.6)	E2No or ENoneNo	153 (43.8)	211 (60.5)
16S rRNA/CPS/ <i>viuB</i>	B1Yes	57 (16.3)	A2No or ANoneNo	139 (39.8)	196 (56.2)
<i>vcg</i> /16S rRNA	CB	137 (39.3)	EA	176 (50.4)	313 (89.7)
<i>vcg</i> /CPS	C1	75 (21.5)	E2 or ENone	185 (53)	260 (74.5)
<i>vcg</i> / <i>viuB</i>	CYes	108 (30.9)	ENo	171 (49)	279 (80)
16S rRNA/CPS	B1	74 (21.2)	A2 or ANone	164 (47)	238 (68.2)
16S rRNA/ <i>viuB</i>	BYes	107 (30.7)	ANo	153 (43.8)	260 (74.5)
CPS/ <i>viuB</i>	1Yes	61 (17.5)	2No or NoneNo	171 (49)	232 (66.5)

^a Due to the presence of atypical genotypes, the total number of isolates did not add up to be 349.

^b Percent agreement was calculated by using the total number of isolates assigned to either clinical or environmental-type *V. vulnificus* strains by all the biomarkers used in each combination divided by 349.

Seasonal Distribution of Clinical-type *V. vulnificus*. Figure 4-1 presents the ratios of clinical-type and environmental-type *Vibrio vulnificus* oyster isolates by the month of isolation. An apparent seasonal pattern where warmer months were associated with more clinical-type *V. vulnificus* oyster isolates was observed when analyzing using *vcg*, 16S rRNA, CPS, and the *viuB*-associated fragment individually (Fig. A-D). A combination of the four markers analysis also indicated a similar seasonal distribution (Fig. E), and clinical-type strains had the highest

percentage in September followed by August, July, and June, whereas absent from May and April isolates ($P < 0.05$).

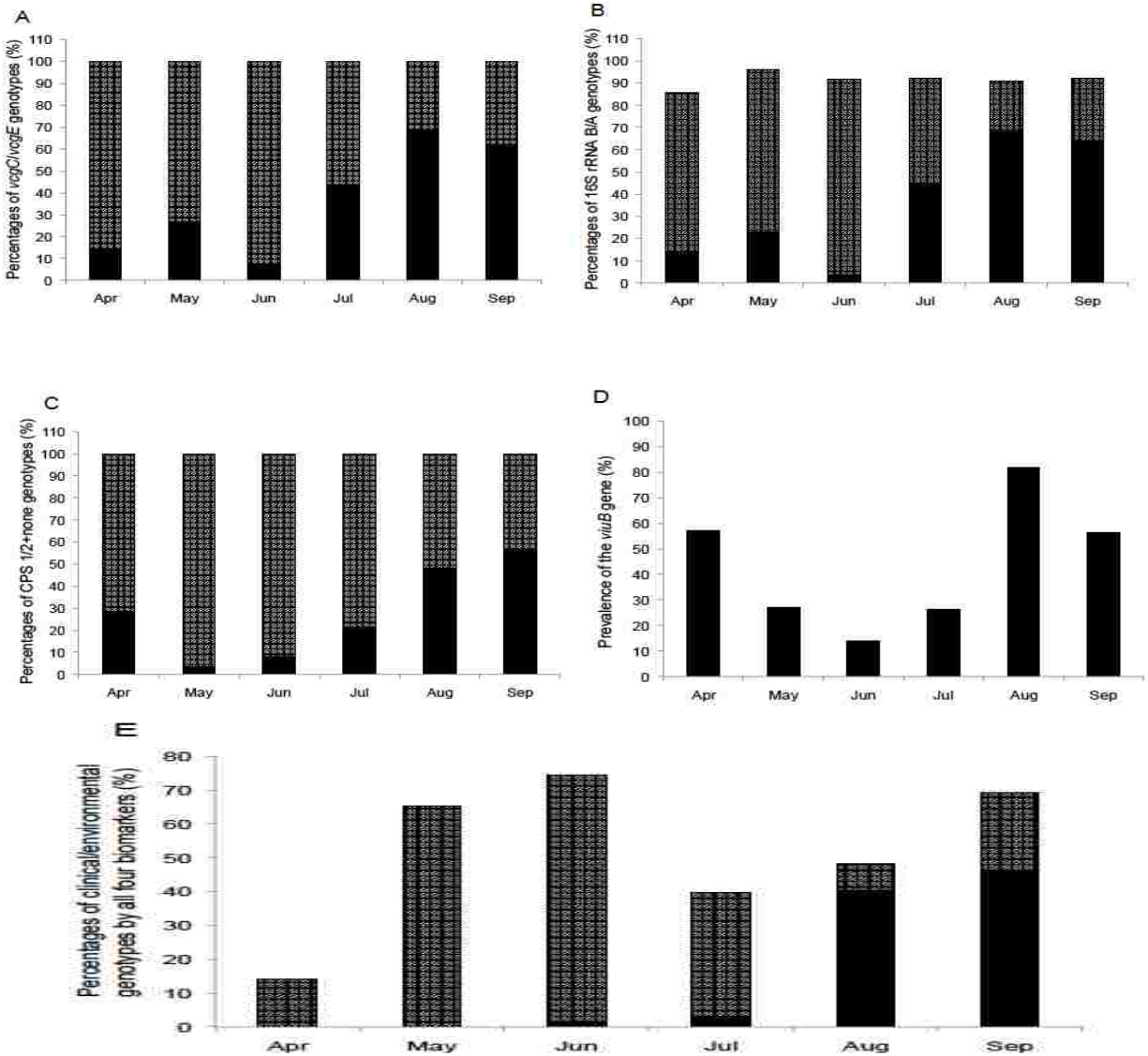


Figure 4 - 1 Ratios of clinical-type and environmental-type of *Vibrio vulnificus* oyster isolates by the month of isolation when analyzed by *vcg* (A), 16S rRNA (B), CPS (C), *viuB*-associated fragment (D), and a combination of all four markers (E).

The solid bars at the bottom indicate percentages of clinical-type strains (*vcgC*, 16S rRNA type B, CPS allele 1, and the presence of *viuB* fragment) and the cross-hatch bars indicate percentages of environmental-type strains. Due to the presence of strains with atypical genotypes by 16S rRNA and combinations of all four biomarkers, the total percentages for some months did not add up to be 100% in B and E.

Discussion

The apparent discrepancy between human exposure to *V. vulnificus* through oyster consumption and the annual number of illness/deaths resulted have prompted many investigators to examine the virulence potential of *V. vulnificus* isolates from oysters and to investigate tools that could be used to differentiate clinical-type *V. vulnificus* strains from environmental-type ones (Aznar *et al.*, 1994; DePaola *et al.*, 2003; Gordon *et al.*, 2008; Jones & Oliver, 2009; Nilsson *et al.*, 2003; Panicker *et al.*, 2004b; Rosche *et al.*, 2005; Sanjuan *et al.*, 2009; Vickery *et al.*, 2007; Warner & Oliver, 2008b; Warner & Oliver, 1999). In the present study, we evaluated 356 *V. vulnificus* strains including 7 reference strains and 349 oyster isolates using four biomarkers, *vcg*, 16S rRNA, CPS, and *viuB*. This is the first study that combinations of these four biomarkers were used to independently evaluate their usefulness in predicting clinically important *V. vulnificus* isolates from oysters.

Genotypic analysis of three biomarkers (*vcg*, 16S rRNA, and CPS) indicated that the majority of 349 *V. vulnificus* oyster isolates belonged to environmental-type strains, i.e., *vcgE*, 16S rRNA type A, and capsule allele 2 or absent, although the actual percentages of environmental-type strains differed dependent upon the biomarkers examined, ranging from 51.6% for 16S rRNA (type A) and 72.5% for the capsular polysaccharide operon (allele 2 or absent). Very few studies have examined *V. vulnificus* populations from natural oyster or seawater by using these markers. One study characterized *vcg* genotypes among *V. vulnificus* oyster and seawater isolates recovered from the eastern coast of North Carolina and found that 84.4% of the 880 oyster isolates had the *vcgE* type, whereas an almost equal distribution of the two *vcg* genotypes (46.9% *vcgE* versus 53.1% *vcgC*) was found among 292 seawater isolates (Warner & Oliver, 2008a). Another study using SYBR-based real-time PCR to detect and

differentiate 16S rRNA A and B types showed that the ratio of A: B was 5:8 (A type 38.5%; $n = 26$) from oysters in poor water quality while for oysters in good water quality, the ratio was 10:1 (A type 90.9%; $n = 22$) (Gordon *et al.*, 2008). In our study, the percentages of oyster isolates possessing *vcgE* and 16S rRNA A genotypes were 59% and 51.6%, respectively, which may be partly explained by the different geographical regions and seasons involved in sampling. On the other hand, when using the three biomarkers to analyze seven reference strains with known source of isolation in this study, two out of five clinical strains showed environmental-type characteristics (*vcgE*, 16S rRNA type A, and CPS allele 2) by all three markers (Table 4-1). This finding is not surprising as indicated by multiple previous studies using larger collections of *V. vulnificus* strains, although significant associations between clinical strains and *vcgC*, 16S rRNA type B, or CPS allele 1 were observed, small percentages of clinical isolates did fall into the opposite genotypes, i.e., EA2 (Chatzidaki-Livanis *et al.*, 2006a; Nilsson *et al.*, 2003; Rosche *et al.*, 2005). Such findings highlight the drawbacks associated with assigning clinical or environmental-type *V. vulnificus* strains based on genotyping studies, rather than based on the presence or absence of a unique virulence marker.

Interestingly, analyzing the 349 oyster isolates by 16S rRNA real-time PCR indicated approximately 8% of the isolates had both A and B types, which confirmed findings from previous studies that the two 16S rRNA sequence variants could be present simultaneously within a single *V. vulnificus* strain (Chatzidaki-Livanis *et al.*, 2006b; Vickery *et al.*, 2007). Type AB strains have been sequenced previously and data suggested that in the genome of most strains, there were more copies of the type A gene present than that of type B (Vickery *et al.*, 2007). Nonetheless, the presence of 16S rRNA AB type made it difficult to designate those strains as either clinical- or environmental-type strains, which was not a desirable feature when

genotyping was conducted primarily to differentiate clinical-type *V. vulnificus* strains from environmental-type ones.

In contrast to the above mentioned three biomarkers, especially *vcg* and 16S rRNA, *viuB* has not been used widely to differentiate clinical and environmental-type *V. vulnificus* strains. Among the seven reference strains used in the study, a 100% correlation between the presence or absence of the *viuB*-associated fragment with strains from clinical or environmental sources, respectively was found, similar to those reported previously (Panicker *et al.*, 2004b). Such findings suggested that the *viuB* fragment could be used as a promising biomarker to identify *V. vulnificus* environmental or food isolates with clinical potentials, although further studies examining a large collection of *V. vulnificus* strains with known clinical or environmental sources coupled with mouse bioassays would be necessary to confirm this possibility.

Among the 349 *V. vulnificus* oyster isolates examined in this study, approximately 41% showed amplification of the *viuB* fragment. A previous study examining the prevalence of *viuB* among natural oyster samples reported 5 out of 33 (15%) *V. vulnificus*-positive oyster samples contained *viuB* (Panicker *et al.*, 2004b). Again, different regions and seasons of oyster sample collection may explain the difference in the prevalence of *viuB* fragment observed. It is noteworthy that when combining *viuB* analysis with three marker genotyping data, our data showed that over 75% of clinical-type strains assigned by *vcg* and 16S rRNA possessed the *viuB*-associated fragment whereas approximately 83% of environmental-type strains lacked this fragment (Table 4-4). Such significant association ($P < 0.0001$) further suggested the potential usefulness of the *viuB*-associated fragment as a biomarker for identifying clinical-type *V. vulnificus* strains.

Based on the agreement analysis, among the four biomarkers used in this study, the best

agreement was found between *vcg* and 16S rRNA (Table 4-5). Additionally, our data indicated that CPS tended to give lower estimate of potentially clinical important *V. vulnificus* strains compared to *vcg*, 16S rRNA, or *viuB*. However, findings based on a single study are not conclusive and future studies involving a large collection of *V. vulnificus* strains would be needed to better assess the usefulness of these biomarkers in predicting clinically important *V. vulnificus* strains from oysters or the environment. Nonetheless, the fact that there were many atypical strains when combinations of biomarkers were used suggest that fundamentally it is difficult to appropriately assign clinical or environmental types to these *V. vulnificus* strains.

Interestingly, a seasonal presence of clinical-type *V. vulnificus* among oyster isolates was observed, corroborating findings from previous studies (Panicker *et al.*, 2004b; Warner & Oliver, 2008a). Such finding is alarming, as it is well established that *V. vulnificus* resides in high numbers (10^3 to 10^4 per gram) in oysters during summer months and temperature is a major parameter that strongly correlates with *V. vulnificus* density (Randa *et al.*, 2004; Wright *et al.*, 1996). Therefore, future control measures need to target more specifically on seasons that tend to accumulate high-density clinical-type *V. vulnificus*.

Finally, besides markers examined in this study, other markers such as *vvhA* (encodes *V. vulnificus* hemolysin) has been used for the similar purpose. In a very recent study evaluating multiple genotypic (ribotyping, DNA polymorphism at *vcg*, 16S rRNA, and *vvhA*) and phenotypic (API 20E, API 20 NE, and BIOLOG) methods to distinguish clinical from environmental *V. vulnificus* strains, the authors concluded that profile 1 strains were *vcgC*, 16S rRNA B, and *vvhA* type 1, and included most (75%) of the biotype 1 human septicemic isolates from blood (Sanjuan *et al.*, 2009). In contrast to our findings, 75% of oyster isolates also belonged to profile 1, suggesting of clinical-type strains. It is noteworthy that the isolates used in

that study were collected from multiple continents over extended time periods, and may not represent isolates typical in the United States Gulf region. Our findings, although supported the usefulness of utilizing biomarkers in characterizing *V. vulnificus* strains, also highlighted the drawbacks of using biomarker genotypes to predict *V. vulnificus* strain virulence and called for more virulence mechanism studies to identify unique virulence markers in this organism to be used as unequivocal ways to screen for virulent *V. vulnificus* strains from oysters or the environment.

Conclusion

In this study, we used multiple biomarkers to characterize 349 *V. vulnificus* oyster isolates previously isolated from Louisiana oysters. The majority of *V. vulnificus* isolates were of environmental type, and there existed a seasonal variation in the *V. vulnificus* genotypes identified, which may have important implications for future control measures. Among the biomarkers used, *vcg* and 16S rRNA had the best agreement, whereas using all four biomarkers was the most discriminatory typing method. However, predicting *V. vulnificus* strain virulence using biomarker genotyping had drawbacks and unique virulence markers in this organism would be necessary to facilitate future screening of virulent *V. vulnificus* strains from oysters or the environment.

References

Aznar, R., Ludwig, W., Amann, R. I. & Schleifer, K. H. (1994). Sequence determination of rRNA genes of pathogenic *Vibrio* species and whole-cell identification of *Vibrio vulnificus* with rRNA-targeted oligonucleotide probes. *Int J Syst Bacteriol* **44**, 330-337.

Blake, P. A., Merson, M. H., Weaver, R. E., Hollis, D. G. & Heublein, P. C. (1979). Disease caused by a marine *Vibrio*. Clinical characteristics and epidemiology. *N Engl J Med* **300**, 1-5.

Chatzidaki-Livanis, M., Hubbard, M. A., Gordon, K., Harwood, V. J. & Wright, A. C. (2006a). Genetic distinctions among clinical and environmental strains of *Vibrio vulnificus*. *Appl Environ Microbiol* **72**, 6136-6141.

Chatzidaki-Livanis, M., Jones, M. K. & Wright, A. C. (2006b). Genetic variation in the *Vibrio vulnificus* group 1 capsular polysaccharide operon. *Journal of bacteriology* **188**, 1987-1998.

Cholera and Other *Vibrio* Illness Surveillance (2009). Summary of human *Vibrio* isolates reported to CDC 2007. Available at <http://www.cdc.gov/nationalsurveillance/PDFs/CSTEVIbrio2007.pdf>. Accessed on April 28, 2010.

DePaola, A., Nordstrom, J. L., Dalsgaard, A., Forslund, A., Oliver, J., Bates, T., Bourdage, K. L. & Gulig, P. A. (2003). Analysis of *Vibrio vulnificus* from market oysters and septicemia cases for virulence markers. *Appl Environ Microbiol* **69**, 4006-4011.

Gordon, K. V., Vickery, M. C., DePaola, A., Staley, C. & Harwood, V. J. (2008). Real-time PCR assays for quantification and differentiation of *Vibrio vulnificus* strains in oysters and water. *Appl Environ Microbiol* **74**, 1704-1709.

Gray, L. D. & Kreger, A. S. (1985). Purification and characterization of an extracellular cytolysin produced by *Vibrio vulnificus*. *Infect Immun* **48**, 62-72.

Gulig, P. A., Bourdage, K. L. & Starks, A. M. (2005). Molecular Pathogenesis of *Vibrio vulnificus*. *Journal of microbiology (Seoul, Korea)* **43 Spec No**, 118-131.

Han, F., Walker, R. D., Janes, M. E., Prinyawiwatkul, W. & Ge, B. (2007). Antimicrobial susceptibilities of *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates from Louisiana Gulf and retail raw oysters. *Appl Environ Microbiol* **73**, 7096-7098.

Jackson, J. K., Murphree, R. L. & Tamplin, M. L. (1997). Evidence that mortality from *Vibrio vulnificus* infection results from single strains among heterogeneous populations in shellfish. *J Clin Microbiol* **35**, 2098-2101.

Jones, M. K. & Oliver, J. D. (2009). *Vibrio vulnificus*: disease and pathogenesis. *Infect Immun* **77**, 1723-1733.

Kim, Y. R. & Rhee, J. H. (2003). Flagellar basal body *flg* operon as a virulence determinant of *Vibrio vulnificus*. *Biochem Biophys Res Commun* **304**, 405-410.

Kothary, M. H. & Kreger, A. S. (1987). Purification and characterization of an elastolytic protease of *Vibrio vulnificus*. *Journal of general microbiology* **133**, 1783-1791.

Litwin, C. M., Rayback, T. W. & Skinner, J. (1996). Role of catechol siderophore synthesis in *Vibrio vulnificus* virulence. *Infect Immun* **64**, 2834-2838.

McPherson, V. L., Watts, J. A., Simpson, L. M. & Oliver, J. D. (1991). Physiological effects of the lipopolysaccharide of *Vibrio vulnificus* on mice and rats. *Microbios* **67**, 141-149.

- Morris, J. G., Jr. (1988).** *Vibrio vulnificus*--a new monster of the deep? *Ann Intern Med* **109**, 261-263.
- Nilsson, W. B., Paranjypte, R. N., DePaola, A. & Strom, M. S. (2003).** Sequence polymorphism of the 16S rRNA gene of *Vibrio vulnificus* is a possible indicator of strain virulence. *J Clin Microbiol* **41**, 442-446.
- Oliver, J. D. (2006).** *Vibrio vulnificus*, p. 349-366. Edited by F. L. Thompson, B. Austin, and J. Swings (ed.), *The biology of vibrios*. Washitonton, DC: ASM press.
- Panicker, G., Call, D. R., Krug, M. J. & Bej, A. K. (2004a).** Detection of pathogenic *Vibrio* spp. in shellfish by using multiplex PCR and DNA microarrays. *Appl Environ Microbiol* **70**, 7436-7444.
- Panicker, G., Vickery, M. C. & Bej, A. K. (2004b).** Multiplex PCR detection of clinical and environmental strains of *Vibrio vulnificus* in shellfish. *Can J Microbiol* **50**, 911-922.
- Randa, M. A., Polz, M. F. & Lim, E. (2004).** Effects of temperature and salinity on *Vibrio vulnificus* population dynamics as assessed by quantitative PCR. *Appl Environ Microbiol* **70**, 5469-5476.
- Rosche, T. M., Yano, Y. & Oliver, J. D. (2005).** A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical or environmental isolation. *Microbiol Immunol* **49**, 381-389.
- Sanjuan, E., Fouz, B., Oliver, J. D. & Amaro, C. (2009).** Evaluation of genotypic and phenotypic methods to distinguish clinical from environmental *Vibrio vulnificus* strains. *Appl Environ Microbiol* **75**, 1604-1613.
- Simpson, L. M. & Oliver, J. D. (1983).** Siderophore production by *Vibrio vulnificus*. *Infect Immun* **41**, 644-649.
- Simpson, L. M., White, V. K., Zane, S. F. & Oliver, J. D. (1987).** Correlation between virulence and colony morphology in *Vibrio vulnificus*. *Infect Immun* **55**, 269-272.
- Starks, A. M., Schoeb, T. R., Tamplin, M. L., Parveen, S., Doyle, T. J., Bomeisl, P. E., Escudero, G. M. & Gulig, P. A. (2000).** Pathogenesis of infection by clinical and environmental strains of *Vibrio vulnificus* in iron-dextran-treated mice. *Infect Immun* **68**, 5785-5793.
- Stelma, G. N., Jr., Reyes, A. L., Peeler, J. T., Johnson, C. H. & Spaulding, P. L. (1992).** Virulence characteristics of clinical and environmental isolates of *Vibrio vulnificus*. *Appl Environ Microbiol* **58**, 2776-2782.
- Strom, M. S. & Paranjypte, R. N. (2000).** Epidemiology and pathogenesis of *Vibrio vulnificus*. *Microbes Infect* **2**, 177-188.

- Tacket, C. O., Barrett, T. J., Mann, J. M., Roberts, M. A. & Blake, P. A. (1984).** Wound infections caused by *Vibrio vulnificus*, a marine vibrio, in inland areas of the United States. *J Clin Microbiol* **19**, 197-199.
- Tison, D. L. & Kelly, M. T. (1986).** Virulence of *Vibrio vulnificus* strains from marine environments. *Appl Environ Microbiol* **51**, 1004-1006.
- Vickery, M. C., Nilsson, W. B., Strom, M. S., Nordstrom, J. L. & DePaola, A. (2007).** A real-time PCR assay for the rapid determination of 16S rRNA genotype in *Vibrio vulnificus*. *J Microbiol Methods* **68**, 376-384.
- Warner, E. & Oliver, J. D. (2008a).** Population structures of two genotypes of *Vibrio vulnificus* in oysters (*Crassostrea virginica*) and seawater. *Appl Environ Microbiol* **74**, 80-85.
- Warner, E. B. & Oliver, J. D. (2008b).** Multiplex PCR assay for detection and simultaneous differentiation of genotypes of *Vibrio vulnificus* biotype 1. *Foodborne Pathog Dis* **5**, 691-693.
- Warner, J. M. & Oliver, J. D. (1999).** Randomly amplified polymorphic DNA analysis of clinical and environmental isolates of *Vibrio vulnificus* and other *vibrio* species. *Appl Environ Microbiol* **65**, 1141-1144.
- Wright, A. C., Hill, R. T., Johnson, J. A., Roghman, M. C., Colwell, R. R. & Morris, J. G., Jr. (1996).** Distribution of *Vibrio vulnificus* in the Chesapeake Bay. *Appl Environ Microbiol* **62**, 717-724.

Part II: Multiplex PCR Assays for Simultaneous Detection and Characterization of *Vibrio vulnificus* Strains

Reprint by permission of “Letters in Applied Microbiology” (see appendix for permission from publisher)

Citation: Han, F. and B. Ge. 2010. Multiplex PCR assays for simultaneous detection and characterization of *Vibrio vulnificus* strains. Letters in Applied Microbiology (in press)

Introduction

Vibrio vulnificus, a Gram-negative halophilic bacterium, is a natural inhabitant of the estuarine and coastal waters worldwide (Oliver, 2006). This organism is capable of causing fatal illness such as primary septicemia and wound infection, especially among immunocompromised persons and those with increased serum iron level due to diabetes or chronic liver disease (Strom & Paranjpye, 2000). In the United States, *V. vulnificus* is responsible for over 95% of seafood-related deaths (Oliver, 2006). One major source of *V. vulnificus* infection is the consumption of raw or undercooked seafood, particularly oysters (Cholera and Other *Vibrio* Illness Surveillance, 2009). To better control *V. vulnificus* risks in oysters, rapid and specific detection methods are imperative, especially if information on the virulence potential of the strain could be obtained simultaneously.

A number of molecular-based detection methods, primarily PCR and real-time PCR targeting the *V. vulnificus* cytolysin/hemolysin gene (*vvhA*) have been described (Coleman *et al.*, 1996; Hill *et al.*, 1991; Panicker & Bej, 2005; Wright *et al.*, 2007). Although widely used and highly specific to *V. vulnificus*, this species-specific gene is not capable of predicating the virulence potential of *V. vulnificus* strains. Given that unique virulence markers, which present exclusively in virulent *V. vulnificus* strains have not been identified (Han *et al.*, 2009), alternative strategies are sought. Recently, several biomarkers have been explored to differentiate virulent- (i.e., clinical-) from non-virulent- (i.e., environmental-) type *V. vulnificus* strains with varied degree of success. The first biomarker is the virulence-correlated gene (*vcg*), which was identified using randomly amplified polymorphic DNA (Warner & Oliver, 1999). A follow-up study showed that 90% of clinical strains had the *vcgC* sequence variant whereas 93% of environmental isolates possessed the *vcgE* sequence variant (Rosche *et al.*, 2005). Secondly,

polymorphism in 17 nucleotides of the *V. vulnificus* 16S rRNA gene was used to differentiate clinical- from environmental-type strains using restriction fragment length polymorphism (Nilsson *et al.*, 2003). By real-time PCR, the majority of clinical isolates were determined to be 16S rRNA type B, while most environmental strains belonged to 16S rRNA type A (Gordon *et al.*, 2008; Vickery *et al.*, 2007). Thirdly, the capsular polysaccharide (CPS) operon was examined and significant associations were identified between clinical isolates and CPS allele 1, as well as between environmental isolates and CPS allele 2 (Chatzidaki-Livanis *et al.*, 2006a; Chatzidaki-Livanis *et al.*, 2006b). Therefore, it now seems technically feasible to design molecular detection assays that specifically target polymorphic regions of these biomarkers to differentiate virulent- from non-virulent-type *V. vulnificus* strains.

In most studies using biomarkers to differentiate *V. vulnificus* strains, single PCR or real-time PCR assays have been used. Frequently, two PCR reactions were needed for one biomarker and a high-fidelity DNA polymerase was required (Chatzidaki-Livanis *et al.*, 2006a; Rosche *et al.*, 2005; Sanjuan *et al.*, 2009), rendering the process both labor-intensive and costly. In addition, species detection and strain characterization were commonly done in sequential steps, rather than simultaneously. Recently, a multiplex PCR assay was developed which targeted *vcgC*, *vcgE*, and *vvhA*, that allowed for both species-level identification and determination of the *vcg* genotype (Warner & Oliver, 2008b). Nonetheless, only one biomarker was targeted in this multiplex PCR and several studies have pointed out the benefit of using multiple biomarkers in characterizing *V. vulnificus* strains (Han *et al.*, 2009; Sanjuan *et al.*, 2009).

The objective of this study was to develop multiplex PCR assays that targeted *vvhA* and a combination of several potential virulence biomarkers (*vcg*, 16S rRNA, and CPS) to

simultaneously detect and characterize *V. vulnificus* strains, either virulent type or non-virulent type.

Materials and Methods

Bacterial Strains and Culture Conditions. *V. vulnificus* strains ($n = 90$) used in this study included 10 reference strains (Table 4-6) and 80 isolates (Table 4-7) previously recovered from Louisiana Gulf and retail oysters (Han *et al.*, 2007). The strains were confirmed using the *vvhA*-PCR (Kaysner & DePaola, 2001) and characterized by single PCR or real-time PCR for the three biomarkers, *vcg*, 16S rRNA, and CPS (Han *et al.*, 2009). The cultures were stored in Luria-Bertani broth (BD Diagnostic Systems, Sparks, MD) containing 30% glycerol at -80°C . *V. vulnificus* strains were routinely cultured on trypticase soy agar (BD Diagnostic Systems) supplemented with 2% NaCl and incubated at 35°C for 24 h.

Multiplex PCR Assays. PCR primers used for each biomarker are listed in Table 4-8. Two pairs of multiplex PCR assays were developed, with one pair targeted four genes (*vcg*, 16S rRNA, CPS and *vvhA*) and the other pair three genes (*vcg*, 16S rRNA, and *vvhA*). For virulent-type *V. vulnificus* assays, a combination of *vcgC*, 16S rRNA type B, CPS allele 1, and *vvhA* was used, whereas for non-virulent-type assays, a combination of *vcgE*, 16S rRNA type A, CPS allele 2, and *vvhA* was used. Two sets of *vcgC* primers were used, one in the 4-target virulent-type multiplex PCR and the other one in 3-target virulent-type multiplex PCR, amplifying PCR bands of 99 bp and 278 bp, respectively (Table 4-8).

V. vulnificus genomic DNAs were prepared by using an UltraClean Microbial DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA). Each prototype PCR mix in a total volume of 25 μl consisted of the following: 1 \times PCR buffer, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 1.5 mM of MgCl_2 , 0.5 U of GoTaq Hot Start polymerase (Promega,

Madison, WI), 0.2 μ M of each primer (Invitrogen, Carlsbad, CA), and 1 μ l of genomic DNA template. The PCR reaction was conducted using 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min in a Bio-Rad C1000 Thermal Cycler (Hercules, CA).

PCR products were analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide, visualized under UV light, and documented by a Gel Doc XR system (Bio-Rad).

Optimization of Multiplex PCR Assays. Annealing temperature (50 to 70°C in 2°C increments) and primer concentration (0.1 to 0.8 μ M in 0.1 μ M increments and one pair at a time) were optimized. Annealing temperature was decreased when the bands intensity was too low, and increased when it was too high. In contrast, primer concentration was increased when the corresponding band intensity was too low, and decreased it was too high. *V. vulnificus* ATCC 33815 was used for optimizing the 4-target or 3-target virulent-type multiplex PCR assays whereas *V. vulnificus* 515-4C2 for non-virulent-type assays. Each optimization experiment was repeated twice.

Specificity of Multiplex PCR Assays. Ten *V. vulnificus* reference strains and eighty *V. vulnificus* oyster isolates with known strain characteristics for the three biomarker genes (Han *et al.*, 2009) were used. False positive and false negative rates, if any, were calculated by using the numbers of false positive or false negative strains divided by respective negative or positive strains included in the testing.

Results

Effect of Annealing Temperature on Multiplex PCR. To allow for high-efficiency PCR amplifications of all of the target genes, the annealing temperature was optimized in 2°C increments and repeated twice.

Table 4 - 6 Ten *Vibrio vulnificus* reference strains used for multiplex PCR

<i>V. vulnificus</i> strain ID ^a	Source and origin	Characteristics of target gene ^b			Multiplex PCR results							
					virulent-type				non-virulent-type			
		<i>vcg</i>	16S	CPS	<i>vcgC</i>	16S B	CPS 1	<i>vvhA</i>	<i>vcgE</i>	16S A	CPS 2	<i>vvhA</i>
ATCC 27562	Blood, Florida	E	A	2	-	-	-	+	+	+	+	+
ATCC 29306	Corneal ulcer, Virginia	E	A	2	-	-	-	+	+	+	+	+
ATCC 33815	Leg ulcer, Wisconsin	C	B	1	+	+	+	+	-	-	-	+
ATCC 33816	Blood, Alaska	C	B	1	+	+	+	+	-	-	-	+
1003	Wound, Louisiana	C	B	1	+	+	+	+	-	-	-	+
1004	Stool, Louisiana	C	B	1	+	+	+	+	-	-	-	+
1006	Blood, Louisiana	C	B	2	+	+	-	+	-	-	+	+
1007	Blood, Louisiana	C	B	1	+	+	+	+	-	-	-	+
WR1	Seawater, Washington	E	A	none	-	-	-	+	+	+	-	+
515-4C2	Oyster, California	E	A	2	-	-	-	+	+	+	+	+

^a ATCC, American Type Culture Collection, Manassas, VA.

^b Obtained using single PCR methods described in our previous study (Han *et al.*, 2009). The 16S was a short form of 16S rRNA. “+” stands for amplification of the gene, “-” stands for no amplification of the gene.

Table 4 - 7 *Vibrio vulnificus* oyster isolates used in the specificity test of multiplex PCR

<i>V. vulnificus</i> strain type and ID ^a	No.	Characteristics of target gene ^a			Multiplex PCR results							
					virulent-type				non-virulent-type			
		<i>vcg</i>	16S	CPS	<i>vcgC</i>	16S B	CPS 1	<i>vhA</i>	<i>vcgE</i>	16S A	CPS 2	<i>vhA</i>
Virulent-type	19	C	B	1	+	+	+	+	-	-	-	+
V332, V358, V360, V363, V388, V419, V443, V444, V469, V499, V501, V504, V513, V534, V544, V545, V560, V601, V616		C	B	1	+	+	+	+	-	-	-	+
Non-virulent-type	34	E	A	2 or none	-	-	-	+	+	+	varies	+
V209, V213, V225, V240, V242, V248, V252, V261, V280, V292, V324, V327, V333, V350, V372, V381, V383, V392, V416, V471, V484, V546, V574, V631	24	E	A	2	-	-	-	+	+	+	+	+
V223, V238, V260, V274, V299, V304, V308, V354, V364, V477	10	E	A	None	-	-	-	+	+	+	-	+
Atypical	27		varies						varies			
V246	1	C	A	1	+	-	+	+	-	+	-	+
V241, V457	2	C	A	2	+	-	-	+	-	+	+	+
V214	1	C	AB	2	+	+	-	+	-	+	+	+
V414	1	C	AB	None	+	+	-	+	-	+	-	+
V371, V385, V389, V447	4	C	B	2	+	+	-	+	-	-	+	+
V377, V465, V552, V576	4	C	B	None	+	+	-	+	-	-	-	+
V239, V297, V387, V438, V490	5	E	A	1	-	-	+	+	+	+	-	+
V337, V598	2	E	AB	1	-	+	+	+	+	+	-	+
V328, V584	2	E	AB	2	-	+	-	+	+	+	+	+
V470, V474	2	E	AB	None	-	+	-	+	+	+	-	+
V432, V433	2	E	B	2	-	+	-	+	+	-	+	+
V606	1	E	B	None	-	+	-	+	+	-	-	+

^a Obtained using single PCR methods described in our previous study (Han *et al.*, 2009). The 16S was a short form of 16S rRNA.

“+” stands for amplification of the gene, “-” stands for no amplification of the gene.

Table 4 - 8 Multiplex PCR primers to simultaneously detect and characterize *Vibrio vulnificus* strains

Assay type	Target gene	Primer name	Sequence (5'-3')	GenBank accession no.	Position	Amplicon size (bp)	Reference		
Both	<i>vvhA</i>	Vvh-785F	CCGCGGTACAGGTTGGCGCA	M34670	785-804	519	(Kaysner & DePaola, 2001)		
		Vvh-1303R	CGCCACCCACTTTCGGGCC		1285-1303				
Virulent	<i>vcgC</i> ^a	<i>vcgC</i> F	AGCTGCCGATAGCGATCT	AY626575	156-173	99 ^b	(Warner & Oliver, 2008b)		
		<i>vcgC</i> R	TGAGCTAACGCGAGTAGTGAG		234-254				
		P1	AGCTGCCGATAGCGATCT		156-173	278	(Rosche <i>et al.</i> , 2005)		
		P3	CGCTTAGGATGATCGGTG		416-433				
		16S B	B F1		GCCTACGGGCCAAAGAGG	X76334		177-194	839
			B R1		CCTGCGTCTCCGCTGGCT		998-1015		
			CPS 1		HP1F	TTTGGGATTTGAAAGGCTTG	DQ360502	2156-2175	342
		HP1R	GTGCCTTTGCGAATTTGAT	2478-2497					
Non-virulent	<i>vcgE</i>	P2	CTCAATTGACAATGATCT	AY626579	156-173	278	(Rosche <i>et al.</i> , 2005)		
		P3	CGCTTAGGATGATCGGTG		416-433				
		16S A	A F2		AGCTTCGGCTCAAAGAGG	X76333	177-194	839	This study
			A R2		CCAGCGTCTCCGCTAGAT		998-1015		
			CPS 2		HP2F	TTCCATCAAACATCGCAGAA	NC_005139 (VV0338)	125-144	152
		HP2R	CTTTGTCCGGCTTCTATCG	257-276					

^a Two sets of *vcgC* primers were used in this study, i.e., *vcgC* F/*vcgC* R and P1/P3 for the 4-target and 3-target virulent-type multiplex PCR, respectively.

^b In the original paper, 97 bp was used.

The optimized annealing temperature for 4-target multiplex PCR assays was 56°C for virulent-type *V. vulnificus* and 50°C for non-virulent-type *V. vulnificus*. After excluding CPS, the optimized annealing temperature for 3-target multiplex PCR assays was 60°C for virulent-type *V. vulnificus* and 50°C for non-virulent-type *V. vulnificus*.

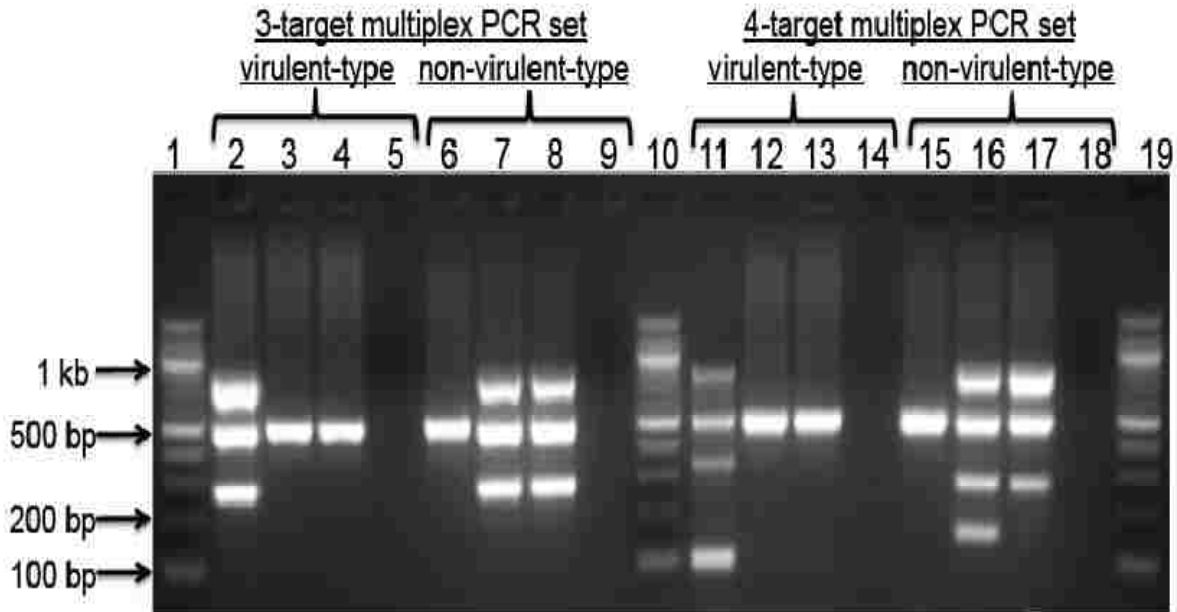


Figure 4 - 2 An agarose gel showing multiplex PCR amplification products using typical virulent- and non-virulent-type *Vibrio vulnificus* strains. Lanes 1, 10, 19: molecular weight marker (New England Biolabs, Ipswich, MA); lanes 2-5, obtained using 3-target multiplex PCR (60°C annealing temperature) for virulent-type *V. vulnificus*; lanes 6-9, using 3-target multiplex PCR (50°C annealing temperature) for non-virulent-type *V. vulnificus*; lanes 11-14, using 4-target multiplex PCR (56°C annealing temperature) for virulent-type *V. vulnificus*; lanes 15-18, using 4-target multiplex PCR (50°C annealing temperature) for non-virulent-type *V. vulnificus*. DNA templates used for multiplex PCR amplifications: *V. vulnificus* ATCC 33815 (*vcgC*, 16S rRNA B type, CPS allele 1, and *vvhA* +) in lanes 2, 6, 11, 15; *V. vulnificus* 515-4C2 (*vcgE*, 16S rRNA A type, CPS allele 2, and *vvhA* +) in lanes 3, 7, 12, 16; *V. vulnificus* V223 (*vcgE*, 16S rRNA A type, CPS none, and *vvhA* +) in lanes 4, 8, 13, 17; negative control in lanes 5, 9, 14, 18. Target gene amplification products as shown in the descending order by size in the 3-target multiplex PCR pair are: 16S rRNA B type (839 bp), *vvhA* (519 bp), and *vcgC* (278 bp) for virulent-type *V. vulnificus*, and 16S rRNA A type (839 bp), *vvhA* (519 bp), and *vcgE* (278 bp) for non-virulent-type *V. vulnificus*. In the 4-target multiplex PCR pair, the amplicon sizes are: 16S rRNA B type (839 bp), *vvhA* (519 bp), CPS allele 1 (342 bp), and *vcgC* (99 bp) for the multiplex PCR assays for virulent-type *V. vulnificus*, and 16S rRNA A type (839 bp), *vvhA* (519 bp), *vcgE* (278 bp), and CPS allele 2 (152 bp) for the multiplex PCR assays for non-virulent-type *V. vulnificus*.

Effect of Primer Concentration on Multiplex PCR. In order to amplify all of the target genes with equal efficiency, i.e., same band intensity, the concentrations of individual primer pairs were optimized. The optimized primer concentrations for the 4-target virulent-type multiplex PCR were: 0.3 μM of *vcgC* primers (*vcgC* F/*vcgC* R), 0.2 μM of 16S rRNA type B primers (B F1/B R1), 0.7 μM of CPS allele 1 primers (HP1F/HP1R), and 0.1 μM of *vvhA* primers (Vvh-785F/Vvh-1303R). For the 4-target non-virulent-type assay, the optimized primer concentrations were: 0.3 μM of *vcgE* primers (P2/P3), 0.3 μM of 16S rRNA type A primers (A F2/A R2), 0.2 μM of CPS allele 2 primers (HP2F/HP2R), and 0.1 μM of *vvhA* primers (Vvh-785F/Vvh-1303R) (data not shown). After excluding CPS, the optimum primer concentrations remained the same as in the corresponding 4-target multiplex PCR pair.

Specificity of Multiplex PCR Assays. Among 10 *V. vulnificus* reference strains and 80 oyster isolates (Tables 4-6 & 4-7), false-positive or false-negative multiplex PCR results were not observed for any of the four target genes, i.e., 100% match with known strain characteristics obtained previously using single PCR or real-time PCR assay (Han *et al.*, 2009). Figure 4-2 shows a representative gel of multiplex PCR amplification products using typical virulent- and non-virulent-type *V. vulnificus* strains.

Discussion

In order to simultaneously detect and differentiate *V. vulnificus* strains, we combined the species-specific *vvhA* gene with three *V. vulnificus* potential virulence biomarkers (*vcg*, 16S rRNA, and CPS) in two pairs of multiplex PCR assays. The first multiplex PCR pair amplified all of the four target genes whereas the second pair amplified three genes except for CPS. For each multiplex pair, one assay was designed for virulent-type *V. vulnificus* strains while the other one for non-virulent-type *V. vulnificus* strains. Although these biomarkers have been used

previously in single PCR or real-time PCR format to distinguish between clinical- (i.e., virulent-) and environmental- (i.e., non-virulent) type *V. vulnificus* strains (Chatzidaki-Livanis *et al.*, 2006a; Gordon *et al.*, 2008; Rosche *et al.*, 2005; Vickery *et al.*, 2007), this is the first study that designed assays targeting multiple *V. vulnificus* biomarkers and *vvhA* to detect and characterize either virulent-type or non-virulent-type *V. vulnificus* strains.

The *vvhA* gene was included as a species control so that only *V. vulnificus* strains were detected and characterized by these multiplex PCR assays. Additionally, BLAST searches (<http://www.ncbi.nih.gov/BLAST>) indicated both *vcg* and CPS are highly specific to *V. vulnificus*. Therefore, we did not include non-*V. vulnificus* strains in the specificity testing. Application of these multiplex PCR assays in 10 *V. vulnificus* reference strains and 80 *V. vulnificus* oyster isolates suggested that the assays were highly specific and accurate, with results matching exactly with those obtained previously by using single PCR or real-time PCR (Han *et al.*, 2009). Therefore, by running one multiplex PCR reaction, e.g., the 4-target virulent-type assay, information on the strain genus/species and the presence or absence of the *vcgC* sequence variant, 16S rRNA type B, and CPS allele 1 would be obtained simultaneously. Similarly, the 4-target non-virulent-type assay would specifically characterize the strain genus/species and the presence or absence of the *vcgE* sequence variant, 16S rRNA type A, and CPS allele 2.

In addition, results for some biomarkers from the paired multiplex PCR assays agreed well with each other, e.g., a strain positive for *vcgC* by using the virulent-type multiplex PCR assay would show negative amplification for *vcgE* by using the non-virulent-type assay, and vice versa. This mutual exclusivity of *vcgC* and *vcgE* sequence variants in *V. vulnificus* agreed with several previous studies (Chatzidaki-Livanis *et al.*, 2006a; Drake *et al.*, 2010; Han *et al.*, 2009;

Rosche *et al.*, 2005; Sanjuan *et al.*, 2009). However, another study (Warner & Oliver, 2008a) reported the isolation of *V. vulnificus* strains from oyster and water that amplified both *vcgC* and *vcgE* sequences. For 16S rRNA types, the majority of *V. vulnificus* strains were mutually exclusive, reflected by the amplification of either 16S rRNA B type in the virulent-type multiplex PCR assay or A type in the non-virulent-type assay. Occasionally, strains amplifying both 16S rRNA A and B types occurred as shown in Table 4-7 under atypical strains. This phenomenon has been frequently reported in the literature (Chatzidaki-Livanis *et al.*, 2006a; Drake *et al.*, 2010; Gordon *et al.*, 2008; Han *et al.*, 2009; Sanjuan *et al.*, 2009; Senoh *et al.*, 2005; Vickery *et al.*, 2007; Warner & Oliver, 2008a), possibly to meet *V. vulnificus* survival needs under different environmental conditions. For CPS, there has been no reports of *V. vulnificus* strain possessing both allele 1 and 2; however, strains possessing neither allele have been reported (Chatzidaki-Livanis *et al.*, 2006a; Han *et al.*, 2009). In that scenario, those strains would be negative for CPS allele 1 by the virulent-type multiplex PCR as well as CPS allele 2 negative by the non-virulent-type assay. Regardless of the strains being virulent-type, non-virulent-type, or atypical, evaluating 90 *V. vulnificus* confirmed the accuracy of the multiplex PCR results when compared with single PCR or real-time PCR results (Tables 4-6 & 4-7), suggesting reliable multiplex PCR assays.

In the present study, besides a pair of 4-target multiple PCR assays, we also designed a pair of 3-target multiplex PCR assays which excluded CPS. There are a few explanations. Firstly, in our previous study, CPS tended to give a low estimate of potentially clinical important *V. vulnificus* strains compared to *vcg*, 16S rRNA, or *viuB*, and the agreement between CPS and other biomarkers was poor (Han *et al.*, 2009). Secondly, there was an ambiguity associated with CPS as mentioned above, i.e., non-virulent-type *V. vulnificus* strains possessed either CPS allele

2 or none. Thirdly, CPS has not been used widely as a potential virulence biomarker for *V. vulnificus* as *vcg* or 16S rRNA. Therefore, in parallel to the 4-target multiplex PCR pair, we also designed the 3-target multiplex PCR pair, which could be used when the determination of CPS allele types is not desirable.

Practically, when applying these multiplex PCR assays in microbial ecology or epidemiology studies, it is acceptable to run one multiplex PCR assay, either the virulent-type or non-virulent type. For the 3-target or 4-target virulent-type multiplex PCR, a typical virulent-type *V. vulnificus* strain would generate 3 or 4 bands, respectively, whereas a typical non-virulent strain would generate only one *vvhA* band (Figure 4-2), and the opposite holds true for non-virulent-type multiplex PCR assays. However, given the ambiguity associated with 16S rRNA and CPS mentioned above and the presence of atypical strains, it is preferable that the multiplex PCR assay pair be performed so that the definite strain characteristics for these biomarkers could be obtained. On the other hand, for oyster and water monitoring programs, the virulent-type multiplex PCR would be a preferred assay and the generation of more than one *vvhA* band would suggest virulent-type strains and warrant further characterization of strain genotypes.

For the 4-target virulent-type multiplex PCR, the optimized annealing temperature was 56°C since the CPS allele 1 target could not be amplified at annealing temperatures greater than 56°C. In the corresponding 3-target virulent-type multiplex PCR, which excluded CPS, the optimized annealing temperature was increased to 60°C. For the non-virulent-type multiplex PCR assays, regardless of the number of targets included, *vcgE* could not be amplified at annealing temperatures greater than 50°C, therefore the optimized annealing temperature for was 50°C for both 3-target and 4-target non-virulent-type multiplex PCRs. In a single PCR format, CPS allele 1 and *vcgE* could be amplified at >60°C and 56°C, respectively. While in a multiplex

PCR setting, due to the competition of primers and PCR reagents, several primers such as *vcgE* or CPS 1 may not amplify in certain stringent condition (i.e. higher annealing temperature).

Besides the three potential virulence biomarkers used in this study, markers such as *viuB* (Han *et al.*, 2009; Panicker *et al.*, 2004a; Panicker *et al.*, 2004b) and *vvhA* (Sanjuan *et al.*, 2009; Senoh *et al.*, 2005) have been used to differentiate *V. vulnificus* strain types. Recently, a phenotypic trait mannitol fermentation has been reported to correlate with these virulence-associated genotypic characteristics (Drake *et al.*, 2010). However, none of these biomarkers are confirmed unique virulent markers for *V. vulnificus*, and the assignment of strains to virulent-type based on these biomarkers only suggests their potential to cause clinical infections.

Conclusion

The multiplex PCR assays developed in the present study were capable of detecting and characterizing multiple *V. vulnificus* potential virulence biomarkers simultaneously. Because of the multiplex format, these assays obviate the need to run multiple single PCR reactions and the need for high-fidelity enzyme. The assays would be valuable tools in microbial ecology and epidemiology studies as well as facilitating regulatory agencies and the oyster industry in controlling *V. vulnificus* risks in oysters by specifically detecting virulent-type strains, which has the potential to cause clinical infections.

References

Chatzidaki-Livanis, M., Hubbard, M. A., Gordon, K., Harwood, V. J. & Wright, A. C. (2006a). Genetic distinctions among clinical and environmental strains of *Vibrio vulnificus*. *Appl Environ Microbiol* **72**, 6136-6141.

Chatzidaki-Livanis, M., Jones, M. K. & Wright, A. C. (2006b). Genetic variation in the *Vibrio vulnificus* group 1 capsular polysaccharide operon. *J Bacteriol* **188**, 1987-1998.

Cholera and Other *Vibrio* Illness Surveillance (2009). Summary of human *Vibrio* isolates reported to CDC 2007. Available at

<http://www.cdc.gov/national-surveillance/PDFs/CSTEVibrio2007.pdf>. Accessed on April 28, 2010.

Coleman, S. S., Melanson, D. M., Biosca, E. G. & Oliver, J. D. (1996). Detection of *Vibrio vulnificus* biotypes 1 and 2 in eels and oysters by PCR amplification. *Appl Environ Microbiol* **62**, 1378-1382.

Drake, S. L., Whitney, B., Levine, J. F., DePaola, A. & Jaykus, L. A. (2010). Correlation of mannitol fermentation with virulence-associated genotypic characteristics in *Vibrio vulnificus* isolates from oysters and water samples in the Gulf of Mexico. *Foodborne Pathog Dis* **7**, 97-101.

Gordon, K. V., Vickery, M. C., DePaola, A., Staley, C. & Harwood, V. J. (2008). Real-time PCR assays for quantification and differentiation of *Vibrio vulnificus* strains in oysters and water. *Appl Environ Microbiol* **74**, 1704-1709.

Han, F., Walker, R. D., Janes, M. E., Prinyawiwatkul, W. & Ge, B. (2007). Antimicrobial susceptibilities of *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates from Louisiana Gulf and retail raw oysters. *Appl Environ Microbiol* **73**, 7096-7098.

Han, F., Pu, S., Hou, A. & Ge, B. (2009). Characterization of clinical and environmental types of *Vibrio vulnificus* isolates from Louisiana oysters. *Foodborne Pathog Dis* **6**, 1251-1258.

Hill, W. E., Keasler, S. P., Trucksess, M. W., Feng, P., Kaysner, C. A. & Lampel, K. A. (1991). Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. *Appl Environ Microbiol* **57**, 707-711.

Kaysner, C. A. & DePaola, A. (2001). *Vibrio*. In *Bacteriological Analytical Manual online*, Chapter 9. Edited by U.S Food and Drug Administration. Rockville, MD.

Nilsson, W. B., Paranjypte, R. N., DePaola, A. & Strom, M. S. (2003). Sequence polymorphism of the 16S rRNA gene of *Vibrio vulnificus* is a possible indicator of strain virulence. *J Clin Microbiol* **41**, 442-446.

Oliver, J. D. (2006). *Vibrio vulnificus*, p. 349-366. Edited by F. L. Thompson, B. Austin, and J. Swings (ed.), *The biology of vibrios*. Washitonton, DC: ASM press.

Panicker, G., Call, D. R., Krug, M. J. & Bej, A. K. (2004a). Detection of pathogenic *Vibrio* spp. in shellfish by using multiplex PCR and DNA microarrays. *Appl Environ Microbiol* **70**, 7436-7444.

Panicker, G., Vickery, M. C. & Bej, A. K. (2004b). Multiplex PCR detection of clinical and environmental strains of *Vibrio vulnificus* in shellfish. *Can J Microbiol* **50**, 911-922.

Panicker, G. & Bej, A. K. (2005). Real-time PCR detection of *Vibrio vulnificus* in oysters: comparison of oligonucleotide primers and probes targeting *vhA*. *Appl Environ Microbiol* **71**, 5702-5709.

Rosche, T. M., Yano, Y. & Oliver, J. D. (2005). A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical or environmental isolation. *Microbiol Immunol* **49**, 381-389.

Sanjuan, E., Fouz, B., Oliver, J. D. & Amaro, C. (2009). Evaluation of genotypic and phenotypic methods to distinguish clinical from environmental *Vibrio vulnificus* strains. *Appl Environ Microbiol* **75**, 1604-1613.

Senoh, M., Miyoshi, S., Okamoto, K., Fouz, B., Amaro, C. & Shinoda, S. (2005). The cytotoxin-hemolysin genes of human and eel pathogenic *Vibrio vulnificus* strains: comparison of nucleotide sequences and application to the genetic grouping. *Microbiol Immunol* **49**, 513-519.

Strom, M. S. & Paranjpye, R. N. (2000). Epidemiology and pathogenesis of *Vibrio vulnificus*. *Microbes and infection / Institut Pasteur* **2**, 177-188.

Vickery, M. C., Nilsson, W. B., Strom, M. S., Nordstrom, J. L. & DePaola, A. (2007). A real-time PCR assay for the rapid determination of 16S rRNA genotype in *Vibrio vulnificus*. *J Microbiol Methods* **68**, 376-384.

Warner, E. & Oliver, J. D. (2008a). Population structures of two genotypes of *Vibrio vulnificus* in oysters (*Crassostrea virginica*) and seawater. *Appl Environ Microbiol* **74**, 80-85.

Warner, E. B. & Oliver, J. D. (2008b). Multiplex PCR assay for detection and simultaneous differentiation of genotypes of *Vibrio vulnificus* biotype 1. *Foodborne Pathog Dis* **5**, 691-693.

Warner, J. M. & Oliver, J. D. (1999). Randomly amplified polymorphic DNA analysis of clinical and environmental isolates of *Vibrio vulnificus* and other *vibrio* species. *Appl Environ Microbiol* **65**, 1141-1144.

Wright, A. C., Garrido, V., Debuex, G., Farrell-Evans, M., Mudbidri, A. A. & Otwell, W. S. (2007). Evaluation of postharvest-processed oysters by using PCR-based most-probable-number enumeration of *Vibrio vulnificus* bacteria. *Appl Environ Microbiol* **73**, 7477-7481.

CHAPTER 5: MOLECULAR DETECTION OF *VIBRIO VULNIFICUS* ISOLATES FROM
LOUISIANA GULF AND RETAIL RAW OYSTERS

Part I: Evaluation of a Loop-Mediated Isothermal Amplification Assay for Detecting *Vibrio vulnificus* in Retail Raw Oysters

Reprint by permission of “Foodborne Pathogens and Disease” (see appendix for permission from publisher)

Citation: Han, F. and B. Ge. 2008. Evaluation of a loop-mediated isothermal amplification assay for detecting *Vibrio vulnificus* in raw oysters. Foodborne Pathogens and Disease. 5:311-20

Introduction

First reported in 1979 as a foodborne pathogen associated with raw oysters (Blake *et al.*, 1979), the Gram-negative, halophilic bacterium *Vibrio vulnificus* is now recognized as the leading cause of seafood-related deaths in the United States, responsible for over 95% of such fatal incidences (Oliver, 2006). *V. vulnificus* causes three quite different human disease symptoms, gastroenteritis, primary septicemia, and wound infection. And the mortality rates run over 50% in primary septicemia and 25% in wound infections, the highest among those caused by foodborne pathogens (Oliver, 2006). In 2004 and 2005, approximately 200 *V. vulnificus* illnesses were reported to the Centers for Disease Control and Prevention (CDC), resulting in around 60 deaths. The majority of patients with known food histories reported oyster consumption prior to *V. vulnificus* infection (Centers for Disease Control and Prevention, 2005). Moreover, according to a recent CDC's FoodNet report, the incidence of *Vibrio parahaemolyticus* and *V. vulnificus* infections in the U.S. due to eating raw or undercooked oysters has shown a sustained increase since 2001, indicating further measures are needed to prevent human illness caused by these pathogens in oysters (Centers for Disease Control and Prevention, 2007). In particular, there is a need for rapid, sensitive, specific, and cost-effective detection assays that can be readily employed in the field to better ensure the safety of oysters from harvest, post-harvest processing (PHP), to retail.

Currently, the Interstate Shellfish Sanitation Conference (ISSC) recommends that all PHP oysters contain no more than 30 *V. vulnificus* colony forming units per gram of oyster (CFU/g) by the most probable number (MPN) analysis (Food and Drug Administration/National Shellfish Sanitation Program, 2005). Such traditional microbiological culturing methods followed by biochemical tests for species identification, however, are labor-intensive and time-consuming

(Kaysner & DePaola, 2001). Besides, reliable biochemical tests/media for *Vibrio* species confirmation are lacking (Warner & Oliver, 2007). DNA probe hybridization has been used in conjunction with direct plating, offering a more rapid (< 24 h) and specific alternative, but is still cumbersome (Kaysner & DePaola, 2001; Morris *et al.*, 1987; Wright *et al.*, 1993). The reported detection limits using DNA probe hybridization fell between 10^2 and 10^4 CFU/g (Kaysner & DePaola, 2001; Morris *et al.*, 1987; Wright *et al.*, 1993). Additionally, specific immunological tests for *V. vulnificus* have been developed with a reported detection limit of 2×10^3 cells and a total detection time of about 2-3 days (Simonson & Siebeling, 1986; Tamplin *et al.*, 1991). However, the required *V. vulnificus*-specific antibodies are no longer commercially available.

Molecular-based DNA amplification techniques, e.g., polymerase chain reaction (PCR) (Arias *et al.*, 1995; Brauns *et al.*, 1991; Coleman *et al.*, 1996; Hill *et al.*, 1991; Lee *et al.*, 2003), reverse-transcription PCR (Fischer-Le Saux *et al.*, 2002), and real-time PCR in recent years (Campbell & Wright, 2003; Panicker *et al.*, 2004; Panicker & Bej, 2005; Takahashi *et al.*, 2005; Wright *et al.*, 2007), have been described for the rapid and sensitive detection of *V. vulnificus* in oysters. Primarily targeting the *vvhA* gene that codes for *V. vulnificus* cytolysin/hemolysin (Wright *et al.*, 1985), these assays reached a detection limit of 10^2 CFU/g oyster without enrichment by real-time PCR or nested PCR (Arias *et al.*, 1995; Campbell & Wright, 2003; Panicker *et al.*, 2004), whereas a 24 h enrichment was required to achieve the same level of sensitivity by PCR (Hill *et al.*, 1991). After 5 h enrichment, a sensitivity of 1 CFU/g was reported again by real-time PCR (Panicker *et al.*, 2004; Panicker & Bej, 2005). The concerns associated with these assays include expensive thermal cycling instrument, particularly the high cost associated with a real-time PCR machine, and the inability to differentiate dead from live cells.

Recently, a novel loop-mediated isothermal amplification (LAMP) technique was described (Notomi *et al.*, 2000) and has shown promise in both bacterial and viral detections (Hara-Kudo *et al.*, 2005; Song *et al.*, 2005; Yeh *et al.*, 2006; Yoda *et al.*, 2007). The assay employs a set of four or six primers that create a target-specific stem-loop DNA structure during initial amplification steps, and subsequent LAMP auto-cycling is achieved by the strand-displacing *Bst* DNA polymerase large fragment (Notomi *et al.*, 2000). Advantages of LAMP relevant to *V. vulnificus* detection include: 1) isothermal (60-65°C), no special thermal cycling instrument is required; 2) rapid, the assay can be completed in 15-60 min (Nagamine *et al.*, 2002); 3) specific, the assay targets 6-8 regions of the target gene sequence; 4) sensitive, extremely large amount of DNA can be amplified from a few target cells; 5) detection by the naked eye due to the formation of large quantities of a by-product, magnesium pyrophosphate, which turns positive reaction tubes turbid (Mori *et al.*, 2001); 6) direct amplification, there is no need to denature DNA templates before amplification (Nagamine *et al.*, 2001); and 7) robust, LAMP is less subjective to inhibition by biological substances (Kaneko *et al.*, 2007).

Given these advantages, we hypothesized that LAMP could potentially be a useful tool for the field detection of *V. vulnificus* in oysters and growing waters. The objective of the present study is to develop a LAMP assay suitable for the rapid and sensitive detection of *V. vulnificus* in raw oysters by targeting the *vvhA* gene.

Materials and Methods

Target and LAMP Primer Design. The *V. vulnificus* cytolysin/hemolysin gene (*vvhA*) (GenBank accession number M34670) was selected as the target for LAMP primer design. A set of four primers, two outer and two inner, that recognize six distinct regions of the target sequence (Figure 5-1) was designed using the PrimerExplorer software (V4, Fujitsu Limited,

Japan; <http://primerexplorer.jp/e>). Forward inner primer (FIP) consisted of a complementary sequence of F1 and a sense sequence of F2, without any linker sequence in between. Similarly, backward inner primer (BIP) was a direct combination of a complementary sequence of B1 and a sense sequence of B2. The two outer primers, forward outer primer and backward outer primer, were F3 and B3, respectively (Figure 5-1). These primers were selected based on criteria described previously by Notomi et al. (Notomi *et al.*, 2000).

Bacterial Strains, Culture Conditions, and DNA Preparation. Bacterial strains used in this study (Table 5-1) were selected from our culture collection at the Department of Food Science, Louisiana State University. *V. vulnificus* 1007, a clinical strain originally isolated by the Louisiana Department of Health and Hospitals, was used for assay optimization and sensitivity testing with pure culture and seeded raw oysters. Additional 19 *V. vulnificus* and 30 non-*V. vulnificus* strains were used to evaluate the assay specificity. All *Vibrio* strains were cultured using tryptic soy agar (TSA; BD Diagnostic Systems, Sparks, MD) or broth containing 2% NaCl at 35°C overnight. Non-*Vibrio* strains were grown on Luria-Bertani agar or blood agar (BD Diagnostic Systems). In addition, modified cellobiose-polymyxin B-colistin (mCPC) agar as recommended in Food and Drug Administration's Bacteriological Analytical Manual (Kaysner & DePaola, 2001) was used to quantify *V. vulnificus* levels in seeded oyster samples.

To prepare DNA templates, a single bacterial colony grown on appropriate agar plates was selected and suspended in 500 µl of TE (10 mM Tris, pH 8.0; 1 mM EDTA). The bacterial suspension was heated for 10 min at 95°C in a dry heating block and stored at -30°C until use.

PCR. As a comparison, two sets of PCR reactions were performed, using LAMP outer primers F3/B3, and the widely used Vvh-785F/Vvh-1303R primers specific for the *V. vulnificus* *vvhA* gene (Kaysner & DePaola, 2001).

Table 5 - 1 Bacterial strains used in this study, their source, and specificity of the *Vibrio vulnificus* cytolysin/hemolysin (*vvhA*) gene amplification by LAMP and PCR

Strain ID, species and group ^a	Source and origin ^b	<i>vvhA</i> amplification	
		LAMP	PCR
<i>V. vulnificus</i> clinical strain (<i>n</i> = 5)			
ATCC 27562	Blood, Florida	+	+
ATCC 29306	Corneal ulcer	+	+
ATCC 33815	Leg ulcer, Wisconsin	+	+
ATCC 33816	Blood, Alaska	+	+
1007	Clinical, Louisiana	+	+
<i>V. vulnificus</i> environmental strain (<i>n</i> = 15)			
WR1	Sea Water, Washington	+	+
515-4C2	Oyster, California	+	+
541-0-84c	Oyster, Gulf, Louisiana	+	+
0106-0-14	Oyster, Gulf, Louisiana	+	+
V97	Oyster, Gulf, Louisiana	+	+
V156	Oyster, Gulf, Louisiana	+	+
V195	Oyster, Gulf, Louisiana	+	+
V196	Oyster, Retail, Louisiana	+	+
V206	Oyster, Retail, Louisiana	+	+
V240	Oyster, Retail, Louisiana	+	+
V261	Oyster, Retail, Louisiana	+	+
V276	Oyster, Retail, Louisiana	+	+
V314	Oyster, Retail, Louisiana	+	+
V325	Oyster, Retail, Louisiana	+	+
V350	Oyster, Retail, Louisiana	+	+
<i>V. parahaemolyticus</i> O3:K6 group (<i>n</i> = 2)			
CT-6636, O3:K6	Clinical, Connecticut	-	-
TX-2103, O3:K6	Clinical, Texas	-	-
<i>V. parahaemolyticus</i> non-O3:K6 group (some with unknown serotypes, <i>n</i> = 11)			
8332924, O1:K56	Oyster, Gulf of Mexico	-	-
NY477, O4:K8	Oyster, New York	-	-
ATCC 49529, O4:K12	Feces, California	-	-
M350A, O5	Oyster, Washington	-	-
ATCC 17802	Shirasu food poisoning, Japan	-	-
ATCC 27969	Blue crab, Maryland	-	-
ATCC 33847	Gastroenteritis, Maryland	-	-
916i	Oyster, Gulf, Louisiana	-	-
541-0-44c	Oyster, Gulf, Louisiana	-	-
V50	Oyster, Retail, Louisiana	-	-
V150	Oyster, Retail, Louisiana	-	-

Table continued

Other <i>Vibrio</i> spp. (n = 5)			
<i>V. alginolyticus</i> ATCC 17749	Spoiled horse mackerel, Japan	-	-
<i>V. cholera</i> ATCC 14035; O:1	NCTC, United Kingdom	-	-
<i>V. cincinnatiensis</i> ATCC 35912	Blood/cerebrospinal fluid, Ohio	-	-
<i>V. harveyi</i> ATCC 35084	Brown shark, Maryland	-	-
<i>V. mimicus</i> ATCC 33655	Feces, Tennessee	-	-
Non- <i>Vibrio</i> spp. (n = 12)			
<i>Campylobacter jejuni</i> 81-176	Human	-	-
<i>Enterobacter aerogenes</i> ATCC 13048	Sputum, South Carolina	-	-
<i>Enterococcus faecalis</i> ATCC 29212	Urine	-	-
<i>Escherichia coli</i> ATCC 25922	Human	-	-
<i>Listeria monocytogenes</i> ATCC 13932, 4b	Spinal fluid, Germany	-	-
<i>Listonella anguillarum</i> ATCC 19264	Ulcerous lesion in cod, UK	-	-
<i>Pseudomonas aeruginosa</i> ATCC 27853	Human blood	-	-
<i>Salmonella enteria</i> Typhimurium LT2	Unknown	-	-
<i>Shigella flexneri</i> ATCC 12022	Unknown	-	-
<i>Shigella sonnei</i> ATCC 25931	Human feces, Panama	-	-
<i>Staphylococcus aureus</i> ATCC 29213	Wound	-	-
<i>Streptococcus pneumoniae</i> ATCC 49619	Sputum, Arizona	-	-

^a ATCC, American Type Culture Collection, Manassas, VA.

^b NCTC, the National Collection of Type Cultures, London, United Kingdom.

“+” stands for amplification of the gene, “-” stands for no amplification of the gene.

The PCR mixture contained 1 × PCR buffer, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 1.5 mM of MgCl₂, 0.5 μM of each primer (synthesized by Invitrogen, Carlsbad, CA), 0.5 U of *Taq* DNA polymerase (Applied Biosystems, Foster City, CA), and 2 μl of DNA template in a total volume of 25 μl. The PCR reaction was conducted using 30 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min in a GeneAmp PCR System 2400 (Applied Biosystems). Aliquots (10 μl) of PCR products were analyzed by electrophoresis on 2% agarose gel, stained with ethidium bromide, and visualized under UV light. Gel images were documented by a Bio-Rad Gel Doc XR system (Hercules, CA).

LAMP. The LAMP mixture in 25 μl total volume consisted of the following: 1 × Thermo buffer (containing 2 mM MgSO₄), 6 mM of MgSO₄, 0.8 M of betaine (Sigma-Aldrich, St. Louis,

MO), 1.4 mM of dNTP, 0.2 μ M of each outer primer, 1.6 μ M of each inner primer, 8 U of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA), and 2 μ l of DNA template. The LAMP reaction was carried out at 63°C for 1 h and terminated at 80°C for 2 min in a water bath. Aliquots (2 μ l) of LAMP amplicons were analyzed similarly by gel electrophoresis.

LAMP Specificity and Sensitivity. The 50 bacterial strains in Table 5-1 were used to determine the LAMP specificity in a blinded manner. DNA templates were made from fresh overnight bacterial cultures and subjected to both LAMP and PCR amplification. False positive and false negative rates, if any, were calculated. Specificity tests were repeated twice.

To determine the sensitivity of the optimized LAMP assay, tenfold serial dilutions of an overnight *V. vulnificus* 1007 culture were prepared in sterile saline solution, quantified using standard plate counts on TSA with 2% NaCl, and aliquots (2 μ l) were subjected to both LAMP and PCR amplification. Sensitivity tests were repeated five times and the data were presented as the lower limits of detection (CFU/reaction).

Detection of *V. vulnificus* in Seeded Oyster Tissue Homogenate. *V. vulnificus* strain 1007 was used to evaluate the capability of LAMP to detect the bacterium in oyster tissue homogenates. All oyster samples (12 oysters per sample) were collected from local seafood restaurants between February and August 2006, shucked, and homogenized with equal volume of alkaline peptone water (APW; BD Diagnostic Systems) in a food stomacher (Model 400; Tekmar Company, Cincinnati, OH). Aliquots of the 1:1 oyster homogenate were tested for the presence of *V. vulnificus* by direct plating and enrichment following procedures described in our previous study (Han *et al.*, 2007), and the remainders were stored at -80°C until use. Only oyster homogenates that were tested negative for *V. vulnificus* were used in the following seeded oyster experiments, which were repeated five times.

Tenfold serial dilutions of a fresh overnight *V. vulnificus* 1007 culture were made in sterile saline, and 1 ml of each dilution was added into 1 ml (i.e., 0.5 g) of thawed oyster tissue homogenate, resulting in bacterial concentrations ranging from approximately 10^8 CFU/g to 1 CFU/g of oyster tissue. Negative oyster control sample without inoculation was also included. For direct detection, the seeded oyster samples were mixed well and aliquots (1 ml) were centrifuged at 200 g for 5 min to remove oyster tissues. Supernatants were transferred to a fresh tube, centrifuged at 12,000 g for 10 min and the pellets were resuspended in 200 μ l TE. After heating at 95°C for 10 min to release the DNA, the solution was centrifuged again at 12,000 g for 10 min, and an aliquot (2 μ l) of the supernatant was used for both LAMP and PCR amplification. Alternatively, a dilution step was added right after inoculation by adding 1 ml of the seeded oyster sample into 50 ml of APW, and processed similarly as above.

For enrichment, 1 ml of each dilution of the overnight *V. vulnificus* 1007 culture was added into a flask containing 48 ml of sterile APW and 1 ml (i.e., 0.5 g) of thawed oyster tissue homogenate, and enriched at 35°C for 5 h. After enrichment, aliquots (1 ml) were taken and processed similarly as above. The *V. vulnificus* levels after enrichment were quantified by standard plate counts on both TSA with 2% NaCl and mCPC agar.

Visualization of LAMP Amplicons by the Naked Eye. To facilitate future field applications of the LAMP assay, detection of LAMP amplicons was also carried out by inspection with naked eyes as described by Parida et al. (Parida *et al.*, 2007). Briefly, after LAMP amplification, each reaction tube was inspected first for white turbidity, then observed immediately for color change (from orange to green or greenish yellow) after adding 0.5 μ l of SYBR Green I dye (Invitrogen). The SYBR Green I color change was observed under both natural light and UV.

Results

Figure 5-1 indicates that six specific locations on the *V. vulnificus* cytolysin/hemolysin gene (*vvhA*) were recognized by the LAMP primers, along with detailed information on each LAMP primer. The detection of LAMP amplicons was done by conventional gel electrophoresis, turbidity inspection, and color observation with the aid of SYBR Green I fluorescent dye.

```

211 CGCTCGGTACAATCGGCAACGTCAGATGGTTTTACTCCTGACGCCAAAAT 260
           F3 →                               F2 →
261 TGTCCGTTTCACCGTCGATGCCGACAAGCCTGGCAGGGTATTCAATTGG 310
                               TTCGGACCGTGCCATAAGTA
                                           ←F1c
311 TTAACGAGCTACAGCAAGATCACAGCTGGTTCAGAGTTGGGCAAACCGC 360
                               B1c →
361 CGCACTTACATTGGTCCATTGCGCCAGCAGTTACGACCTTTGGGTGAAACC 410
                               TGGAAACCCACTTTGG
411 CGTTTCTGGTTACACACCGAAAAAAGCCCGTGACCTACCGCAGAATGAGA 460
    GC          ATGTGTGGCTTTTTTCGGGC
    ←B2                ←B3

F3 (5'-3'): CGGCAACGTCAGATGGT
B3 (5'-3'): CGGGCTTTTTTCGGTGTGTA
FIP (F1c + F2; 5'-3'): ATGAATACCCGTGCCAGGCTTTCCTGACGCCAAAATTGICC
BIP (B1c + B2; 5'-3'): CACAGCTGGTTCAGAGTTGGGCCGGGTTTCACCCAAAGGT

```

Figure 5 - 1 Partial nucleotide sequence of the cytolysin/hemolysin (*vvhA*) gene of *Vibrio vulnificus* EDL174 (accession number M34670), six target regions, and four primers used in the LAMP assay.

Underlined sequences are the six target regions. Oligonucleotide sequences in bold and also listed at the bottom were used as LAMP primers. F3 and B3 are the forward outer primer and backward outer primer, respectively. FIP and BIP are the forward inner primer and the backward inner primer, respectively.

Specificity and Sensitivity of the LAMP Assay. Among 50 bacterial strains used to determine the LAMP specificity, all of the 20 *V. vulnificus* clinical and environmental strains generated the typical ladder-like LAMP banding pattern, and none of the 30 non-*V. vulnificus* strains were positive for LAMP (Table 5-1, second to the last column). There was no false positive or false negative reactions detected, indicating the LAMP assay was highly specific.

Similarly, two sets of PCR assays using both F3/B3 and Vvh-785F/Vvh-1303R did not produce any false positive or false negative results when testing the 50 bacterial strains (Table 5-1, the last column).

The sensitivity of the LAMP assay was determined by testing tenfold serial dilutions of *V. vulnificus* 1007 and comparing it with that of the two conventional PCRs. LAMP was found to be 10-fold more sensitive than PCR, with a detection limit of approximately 20 CFU versus 202 CFU for PCR (Figure 5-2, Table 5-2), whereas the two PCR assays using either F3/B3 or Vvh-785F/Vvh-1303R primers possessed the same level of sensitivity (data not shown).

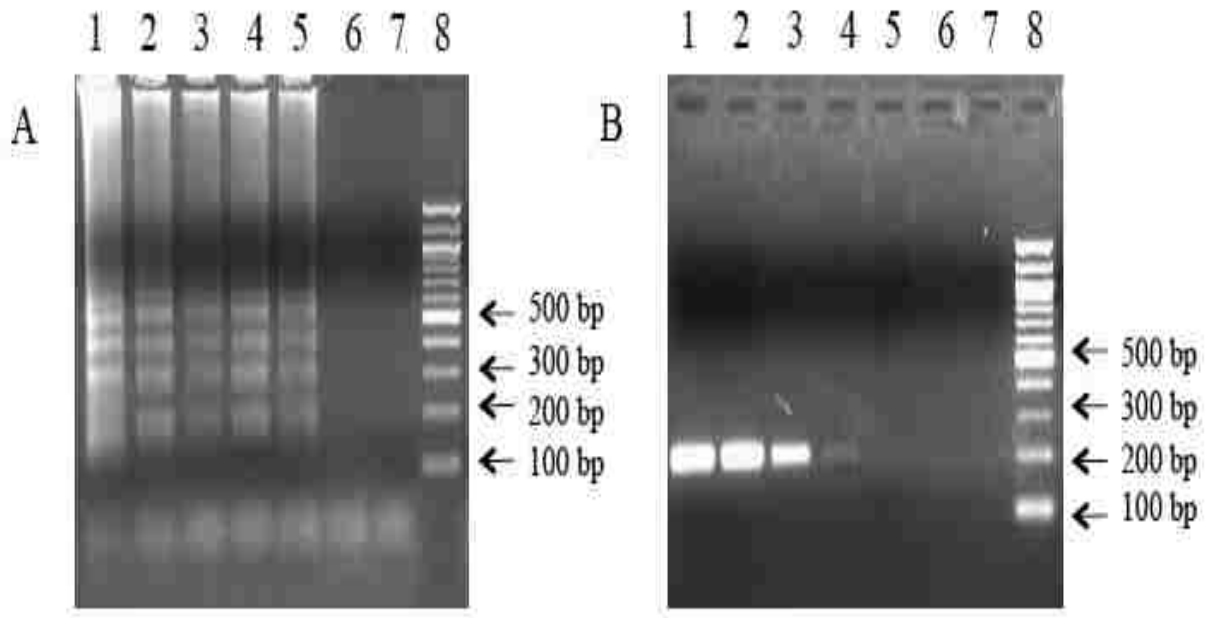


Figure 5 - 2 Sensitivity of *vvhA* LAMP (A) and PCR (B) for detecting *Vibrio vulnificus* 1007 in pure cultures.

For PCR products shown here, primers used were F3 and B3, and the expected size was 217 bp. In both gel, lanes 1-7 are amplicons from serial tenfold dilutions of a bacterial culture (1.01×10^8 CFU/ml). Two μ l of boiled bacterial dilutions were added into the reaction tube, resulting in the total CFU numbers in individual tube for lanes 1-7 were 2.02×10^5 , 2.02×10^4 , 2.02×10^3 , 2.02×10^2 , 20.2, 2.02, and 2.02×10^{-1} , respectively. Lane 8 is 100 bp molecular weight marker (New England Biolabs). Negative controls used were water (not shown). In gel A, 2 μ l of LAMP amplicons were loaded per lane, whereas in gel B, 10 μ l of PCR amplicons were loaded per lane.

Table 5 - 2 Comparison of sensitivities of LAMP and PCR in detecting *Vibrio vulnificus* 1007 in pure cultures and seeded oyster samples

Samples and preparations	Methods	Detection limit (CFU or CFU/g) ^a	Cells in the reaction tube (CFU) ^b
Pure culture	LAMP	20	20
	PCR	202	202
Seeded oyster (no enrichment, no dilution)	LAMP	4×10 ⁸	4×10 ⁶
	PCR	ND	N/A
Seeded oyster (no enrichment, with dilution)	LAMP	4×10 ⁷	8×10 ³
	PCR	ND	N/A
Seeded oyster (5 h enrichment)	LAMP	7	80
	PCR	7×10 ³	4×10 ⁵

^a For pure culture, the unit for detection limit was colony forming unit (CFU), whereas the units for those in seeded oysters were CFU/g of oyster tissue.

^b The formula used to calculate the number of cells in the reaction tubes were: cell concentration (CFU/ml)×2×10⁻³ for pure culture, CFU/ml×10⁻² for seeded oyster with no enrichment or dilution, CFU/ml×2×10⁻⁴ for seeded oyster with no enrichment but with 1:50 dilution before detection, CFU/ml after enrichment×10⁻² for seeded oysters with 5 h enrichment.

ND means not detected. N/A means not applicable.

LAMP Detection of *V. vulnificus* in Experimentally Seeded Oysters. When applying LAMP and PCR for the direct detection of *V. vulnificus* in experimentally seeded raw oysters without enrichment, LAMP was able to detect *V. vulnificus* only at the highest inoculum level tested, i.e. 4×10⁸ CFU/g. When the seeded oyster samples were diluted 1:50 in APW before detection, the LAMP detection limit without enrichment increased tenfold to 4×10⁷ CFU/g (Table 5-2). PCR, however, failed to detect any of the seeded oyster samples without enrichment.

After 5 h enrichment in APW, the LAMP assay was able to detect an initial *V. vulnificus* inoculum of 7 CFU/g of oyster tissue, when the actual *V. vulnificus* level in the enrichment broth reached approximately 8×10³ CFU/ml based on cellobiose-positive colony counts on mCPC agar. The detection limit of PCR for seeded oyster samples after enrichment was 1,000-fold higher than that of LAMP, i.e., 7×10³ CFU/g in the case of PCR, which had an actual cell concentration of 4×10⁷ CFU/ml after 5 h enrichment.

Direct Visualization of LAMP Amplicons by the Naked Eye. Following LAMP amplification at 63°C for 1 h in a water bath, white turbidity could be observed in LAMP-positive samples with the naked eye (Figure 5-3). After adding 0.5 µl of SYBR Green I fluorescent dye, the LAMP-positive samples clearly showed color change from original orange color to green or greenish yellow under normal light, whereas for LAMP-negative samples, the tubes remained the original orange color of the dye (Figure 5-3A). The color difference was more obvious when both positive and negative tubes were exposed to UV light, as positive tubes fluoresced bright green (Figure 5-3B). No turbidity change or color change after adding SYBR Green I was observed in PCR-positive samples.

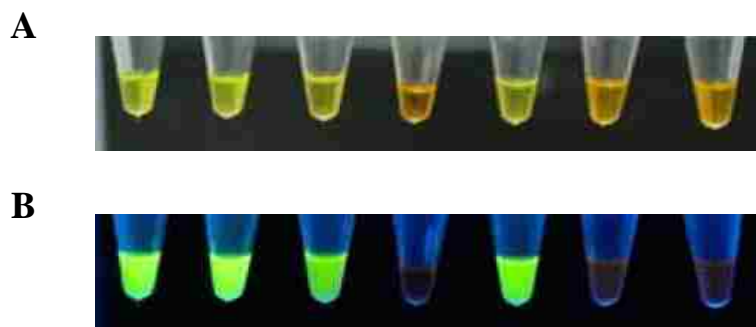


Figure 5 - 3 SYBR Green I fluorescent dye-mediated visualization of LAMP amplification products by the naked eye under normal light (A) and UV (B). After 0.5 µl of SYBR Green I was added, positive wells immediately turned green or greenish-yellow, whereas negative wells remained the orange color of the dye. This color difference was more obvious under UV as positive wells fluoresced bright green. Both photos were taken by John Wozniak of the LSU Agricultural Center.

Discussion

As the cause of over 95% of seafood-related deaths (Oliver, 2006), *V. vulnificus* presents an imminent threat to public health as well as the Gulf oyster industry. To meet the regulatory guideline of less than 30 CFU/g of *V. vulnificus* in PHP oysters, a rapid, sensitive, specific, and

cost-effective detection assay is desirable, particularly if the assay has the potential to be applied in the field. In this study, we applied a novel molecular detection assay, LAMP, for the rapid and sensitive detection of this pathogen in oyster tissue homogenate by targeting the *vvhA* gene. In this initial effort, the LAMP assay was developed and optimized, assay characteristics were evaluated, and its applicability in oyster samples were tested.

The specificity of the LAMP assay was ensured by designing primers targeting unique regions of the *vvhA* gene, and was confirmed by testing 50 *V. vulnificus* and non-*V. vulnificus* strains. This is not surprising since LAMP assays inherently carry a high level of specificity due to the fact that at least six regions on the target DNA sequence were targeted (Notomi *et al.*, 2000). Similarly, PCR using two LAMP outer primers also demonstrated high levels of specificity, further confirming that the region of *vvhA* used for primer design is unique to *V. vulnificus*.

When testing *V. vulnificus* in pure cultures, the LAMP assay was found to be 10-fold more sensitive than the two conventional PCRs included for comparison, capable of detecting 20 CFU of *V. vulnificus* per reaction tube. This greater sensitivity of LAMP compared to PCR has been reported previously (Hara-Kudo *et al.*, 2005; Hara-Kudo *et al.*, 2007; Yano *et al.*, 2007), however, PCR was found to be one order more sensitive than LAMP in detecting *Flavobacterium columnare* when 40 cycles of PCR reaction was used rather than 30 (Yeh *et al.*, 2006). In addition, the LAMP sensitivity observed in this study was comparable to previously reported real-time PCR assays for detecting *V. vulnificus*, ranging from 6 CFU to 100 CFU (Campbell & Wright, 2003; Panicker *et al.*, 2004).

In addition to its specificity and sensitivity, LAMP was demonstrated to be a faster assay than PCR, taking 1 h rather than > 2 h for PCR. Moreover, during LAMP amplification, very

large amount of amplicons were generated rapidly, which could result in white turbidity as well as SYBR Green I fluorescent dye color change. PCR, on the other hand, did not generate sufficient amount of amplicons to be detected by these simple visual observations. According to Mori et al., LAMP synthesized DNA in the range of 10-20 µg/25 µl reaction mix, whereas DNA yield by PCR was about 0.2 µg/25 µl (Mori *et al.*, 2001). This discrepancy accounted for the different reactions observed when adding SYBR Green I dye. This unique yet simple detection method for LAMP could facilitate future field applications of the assay.

When the optimized LAMP assay was used to detect *V. vulnificus* in experimentally seeded Louisiana raw oysters, LAMP was demonstrated to be much less susceptible to certain inhibitors present in oyster samples, resulting in 1,000 fold greater sensitivity than PCR for detection following enrichment (Table 5-2). Nonetheless, although an internal amplification control was not included in our assay development, the inhibitory effects of certain biological substances in the oyster samples were obvious, as evidenced by much hampered sensitivity compared to that in pure cultures (Table 5-2). This observation was in contrast to a previous report that conducted inhibition control study in Alabama oysters and concluded that the oyster tissue matrix did not affect the sensitivity of a real-time PCR (Panicker & Bej, 2005). This discrepancy could be due to that oyster samples from different regions were assessed. Diluting the seeded oyster samples in APW (1:50) before detection was tested, and it lowered the detection limit by 10-fold. However, as indicated in Table 5-2, the actual number of cells in the reaction tube after this dilution step was 8×10^3 compared to 4×10^6 without dilution, suggesting that the dilution procedure did greatly reduce the oyster matrix effect.

Our data clearly indicated that when DNA amplification assays such as LAMP were applied to the detection of *Vibrio* in oysters, direct detection without enrichment lacked the

needed sensitivity (30 CFU/g). Based on theoretical calculations, given a LAMP detection limit of 20 cells/reaction (2 μ l) in pure cultures, it needs at least 1×10^4 CFU/ml bacteria in oyster homogenate not counting oyster matrix effects. There was also a dilution factor (1:50) to reduce inhibitor concentrations (1 ml of bacterial suspension into 50 ml APW) and a concentrating factor (5:1) during sample preparation (1 ml into 200 μ l TE). Taken together, a cell concentration of at least 1×10^5 CFU/ml (i.e., 1×10^5 CFU/g) is needed. Therefore, enrichment seems an inevitable step in terms of *V. vulnificus* detection in oysters, concurring with findings in the literature (Panicker *et al.*, 2004; Panicker & Bej, 2005). Very recently, two studies using real-time PCR to detect pathogenic *V. parahaemolyticus* (Nordstrom *et al.*, 2007) or *V. vulnificus* (Wright *et al.*, 2007) also sought to use a combination of MPN enrichment and real-time PCR approach to achieve the needed sensitivity. In this regard, the LAMP assay could readily pair with MPN but further evaluation is needed.

During the assay development and application in oyster samples, several simple centrifugation steps were used to remove oyster liquid and tissue, which were quite efficient and achieved a detection limit of 7 CFU/g after enrichment. This level of detection was comparable to that of previously reported assays by real-time PCR (Panicker *et al.*, 2004; Panicker & Bej, 2005), and well met the current ISSC recommended 30 CFU/g guidelines (Food and Drug Administration/National Shellfish Sanitation Program, 2005). This simple oyster sample processing method obviated the need for lengthy nucleic acid purification steps, therefore reducing the cost and turnaround time for detection.

Conclusion

A rapid, specific, and inexpensive LAMP assay for detecting *V. vulnificus* in raw oysters was developed and evaluated in this study. The lower limit of detection was 20 CFU of *V.*

vulnificus in pure cultures. After 5 h enrichment, the assay was capable of detecting 7 CFU/g *V. vulnificus* in experimentally inoculated Louisiana oyster samples. Because of its isothermal format and unique amplicon detection technique, this rapid and sensitive LAMP assay holds potential for future field application. Further optimization of the oyster sample processing procedure and large-scale testing on oyster samples obtained from various regions of the U.S. are needed to bring this assay a step closer to the field.

References

- Arias, C. R., Garay, E. & Aznar, R. (1995).** Nested PCR method for rapid and sensitive detection of *Vibrio vulnificus* in fish, sediments, and water. *Appl Environ Microbiol* **61**, 3476-3478.
- Blake, P. A., Merson, M. H., Weaver, R. E., Hollis, D. G. & Heublein, P. C. (1979).** Disease caused by a marine *Vibrio*. Clinical characteristics and epidemiology. *N Engl J Med* **300**, 1-5.
- Brauns, L. A., Hudson, M. C. & Oliver, J. D. (1991).** Use of the polymerase chain reaction in detection of culturable and nonculturable *Vibrio vulnificus* cells. *Appl Environ Microbiol* **57**, 2651-2655.
- Campbell, M. S. & Wright, A. C. (2003).** Real-time PCR analysis of *Vibrio vulnificus* from oysters. *Appl Environ Microbiol* **69**, 7137-7144.
- Centers for Disease Control and Prevention (2005).** *Vibrio vulnificus*: general information.
- Centers for Disease Control and Prevention (2007).** Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food--10 states, 2006. . *MMWR Morb Mortal Wkly Rep* **56**, 336-339.
- Coleman, S. S., Melanson, D. M., Biosca, E. G. & Oliver, J. D. (1996).** Detection of *Vibrio vulnificus* biotypes 1 and 2 in eels and oysters by PCR amplification. *Appl Environ Microbiol* **62**, 1378-1382.
- Fischer-Le Saux, M., Hervio-Heath, D., Loaec, S., Colwell, R. R. & Pommepeuy, M. (2002).** Detection of cytotoxin-hemolysin mRNA in nonculturable populations of environmental and clinical *Vibrio vulnificus* strains in artificial seawater. *Appl Environ Microbiol* **68**, 5641-5646.
- Food and Drug Administration/National Shellfish Sanitation Program (2005).** Guide for the Control of Molluscan Shellfish 2005.

- Han, F., Walker, R. D., Janes, M. E., Prinyawiwatkul, W. & Ge, B. (2007).** Antimicrobial susceptibilities of *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates from Louisiana Gulf and retail raw oysters. *Appl Environ Microbiol* **73**, 7096-7098.
- Hara-Kudo, Y., Yoshino, M., Kojima, T. & Ikedo, M. (2005).** Loop-mediated isothermal amplification for the rapid detection of *Salmonella*. *FEMS microbiology letters* **253**, 155-161.
- Hara-Kudo, Y., Nemoto, J., Ohtsuka, K., Segawa, Y., Takatori, K., Kojima, T. & Ikedo, M. (2007).** Sensitive and rapid detection of Vero toxin-producing *Escherichia coli* using loop-mediated isothermal amplification. *Journal of medical microbiology* **56**, 398-406.
- Hill, W. E., Keasler, S. P., Trucksess, M. W., Feng, P., Kaysner, C. A. & Lampel, K. A. (1991).** Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. *Appl Environ Microbiol* **57**, 707-711.
- Kaneko, H., Kawana, T., Fukushima, E. & Suzutani, T. (2007).** Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. *Journal of biochemical and biophysical methods* **70**, 499-501.
- Kaysner, C. A. & DePaola, A. (2001).** *Vibrio*. In *Bacteriological Analytical Manual online*, Chapter 9. Edited by U.S Food and Drug Administration. Rockville, MD.
- Lee, C. Y., Panicker, G. & Bej, A. K. (2003).** Detection of pathogenic bacteria in shellfish using multiplex PCR followed by CovaLink NH microwell plate sandwich hybridization. *J Microbiol Methods* **53**, 199-209.
- Mori, Y., Nagamine, K., Tomita, N. & Notomi, T. (2001).** Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochemical and biophysical research communications* **289**, 150-154.
- Morris, J. G., Jr., Wright, A. C., Roberts, D. M., Wood, P. K., Simpson, L. M. & Oliver, J. D. (1987).** Identification of environmental *Vibrio vulnificus* isolates with a DNA probe for the cytotoxin-hemolysin gene. *Appl Environ Microbiol* **53**, 193-195.
- Nagamine, K., Watanabe, K., Ohtsuka, K., Hase, T. & Notomi, T. (2001).** Loop-mediated isothermal amplification reaction using a nondenatured template. *Clinical chemistry* **47**, 1742-1743.
- Nagamine, K., Hase, T. & Notomi, T. (2002).** Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and cellular probes* **16**, 223-229.
- Nordstrom, J. L., Vickery, M. C., Blackstone, G. M., Murray, S. L. & DePaola, A. (2007).** Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. *Appl Environ Microbiol* **73**, 5840-5847.

- Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. (2000).** Loop-mediated isothermal amplification of DNA. *Nucleic acids research* **28**, E63.
- Oliver, J. D. (2006).** *Vibrio vulnificus*, p. 349-366. Edited by F. L. Thompson, B. Austin, and J. Swings (ed.), *The biology of vibrios*. Washitonton, DC: ASM press.
- Panicker, G., Myers, M. L. & Bej, A. K. (2004).** Rapid detection of *Vibrio vulnificus* in shellfish and Gulf of Mexico water by real-time PCR. *Appl Environ Microbiol* **70**, 498-507.
- Panicker, G. & Bej, A. K. (2005).** Real-time PCR detection of *Vibrio vulnificus* in oysters: comparison of oligonucleotide primers and probes targeting *vhA*. *Appl Environ Microbiol* **71**, 5702-5709.
- Parida, M. M., Santhosh, S. R., Dash, P. K. & other authors (2007).** Rapid and real-time detection of Chikungunya virus by reverse transcription loop-mediated isothermal amplification assay. *J Clin Microbiol* **45**, 351-357.
- Simonson, J. & Siebeling, R. J. (1986).** Rapid serological identification of *Vibrio vulnificus* by anti-H coagglutination. *Appl Environ Microbiol* **52**, 1299-1304.
- Song, T., Toma, C., Nakasone, N. & Iwanaga, M. (2005).** Sensitive and rapid detection of *Shigella* and enteroinvasive *Escherichia coli* by a loop-mediated isothermal amplification method. *FEMS microbiology letters* **243**, 259-263.
- Takahashi, H., Hara-Kudo, Y., Miyasaka, J., Kumagai, S. & Konuma, H. (2005).** Development of a quantitative real-time polymerase chain reaction targeted to the *toxR* for detection of *Vibrio vulnificus*. *Journal of microbiological methods* **61**, 77-85.
- Tamplin, M. L., Martin, A. L., Ruple, A. D., Cook, D. W. & Kaspar, C. W. (1991).** Enzyme immunoassay for identification of *Vibrio vulnificus* in seawater, sediment, and oysters. *Appl Environ Microbiol* **57**, 1235-1240.
- Warner, E. & Oliver, J. D. (2007).** Refined medium for direct isolation of *Vibrio vulnificus* from oyster tissue and seawater. *Appl Environ Microbiol* **73**, 3098-3100.
- Wright, A. C., Morris, J. G., Jr., Maneval, D. R., Jr., Richardson, K. & Kaper, J. B. (1985).** Cloning of the cytotoxin-hemolysin gene of *Vibrio vulnificus*. *Infect Immun* **50**, 922-924.
- Wright, A. C., Miceli, G. A., Landry, W. L., Christy, J. B., Watkins, W. D. & Morris, J. G., Jr. (1993).** Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. *Appl Environ Microbiol* **59**, 541-546.
- Wright, A. C., Garrido, V., Debuex, G., Farrell-Evans, M., Mudbidri, A. A. & Otwell, W. S. (2007).** Evaluation of postharvest-processed oysters by using PCR-based most-probable-number enumeration of *Vibrio vulnificus* bacteria. *Appl Environ Microbiol* **73**, 7477-7481.

Yano, A., Ishimaru, R. & Hujikata, R. (2007). Rapid and sensitive detection of heat-labile I and heat-stable I enterotoxin genes of enterotoxigenic *Escherichia coli* by Loop-Mediated Isothermal Amplification. *Journal of microbiological methods* **68**, 414-420.

Yeh, H. Y., Shoemaker, C. A. & Klesius, P. H. (2006). Sensitive and rapid detection of *Flavobacterium columnare* in channel catfish *Ictalurus punctatus* by a loop-mediated isothermal amplification method. *Journal of applied microbiology* **100**, 919-925.

Yoda, T., Suzuki, Y., Yamazaki, K., Sakon, N., Kanki, M., Aoyama, I. & Tsukamoto, T. (2007). Evaluation and application of reverse transcription loop-mediated isothermal amplification for detection of noroviruses. *Journal of medical virology* **79**, 326-334.

Part II: Quantitative Detection of *Vibrio vulnificus* in Raw Oysters by Real-Time Loop-Mediated Isothermal Amplification

Reprint by permission of “International Journal of Food Microbiology” (see appendix for permission from publisher)

Citation: Han, F. and B. Ge. 2010. Quantitative detection of *Vibrio vulnificus* in raw oysters by real-time loop-mediated isothermal amplification. International Journal of Food Microbiology (in press)

Introduction

Vibrio vulnificus is a Gram-negative, halophilic bacterium that inhabits warm coastal and estuarine waters throughout the world (Oliver, 2006). Most human infections result from the consumption of raw or undercooked seafood, particularly oysters (Cholera and Other *Vibrio* Illness Surveillance, 2009). *V. vulnificus* is capable of causing fatal diseases such as primary septicemia and wound infections, with reported mortality rates over 50% and 25%, respectively (Oliver, 2006). At-risk groups for fatal *V. vulnificus* infections include people with immunocompromising conditions, diabetes, and elevated serum iron concentrations due to chronic liver disease or alcohol abuse (Strom & Paranjpye, 2000). Annually, there are approximately 30 *V. vulnificus*-associated deaths in the United States (Centers for Disease Control and Prevention, 2010). Therefore, rapid and sensitive detection assays are needed to facilitate better control of potential *V. vulnificus* infections from seafood consumption.

Conventional culture-based methods for the detection and quantification of *V. vulnificus* include the most probable number (MPN) method and DNA hybridization, which are time-consuming and labor-intensive (Kaysner & DePaola, 2001; Wright *et al.*, 1993). Molecular-based assays such as PCR and real-time PCR have been widely employed for the rapid, specific, and sensitive detection and quantification (in the case of real-time PCR) of *V. vulnificus* (Campbell & Wright, 2003; Coleman *et al.*, 1996; Hill *et al.*, 1991; Panicker *et al.*, 2004; Panicker & Bej, 2005; Takahashi *et al.*, 2005; Wright *et al.*, 2007). A previous study reported that a real-time Taqman PCR assay was able to detect 1 CFU of *V. vulnificus* per gram of oyster tissue homogenate after 5 h enrichment (Panicker & Bej, 2005). However, for both PCR and real-time PCR, a dedicated thermal cycler is needed, which is rather expensive especially for real-time PCR, and hinders the wide application of such assays.

A newer DNA amplification technique, loop-mediated isothermal amplification (LAMP) was developed in 2000 (Notomi *et al.*, 2000). LAMP utilizes four to six primers that specifically recognize six to eight regions of the target DNA sequence and amplifies millions of DNA copies under isothermal conditions (60-65°C) within an hour (Notomi *et al.*, 2000). Since its invention, LAMP has been applied to detect multiple bacterial and viral agents, including those of major food safety concerns, such as *Campylobacter*, pathogenic, *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, and noroviruses (Goto *et al.*, 2007; Hara-Kudo *et al.*, 2005; Hara-Kudo *et al.*, 2007; Yamazaki *et al.*, 2008c; Yoda *et al.*, 2007). Very recently, our research group and two others independently developed LAMP assays for *V. vulnificus* detection, of which two targeted the *V. vulnificus* hemolysin (*vvhA*) (Han & Ge, 2008; Ren *et al.*, 2009) and one targeted the *V. vulnificus* *toxR* gene (Nemoto *et al.*, 2008). These assays were reported to be specific and sensitive; however, none of them applied real-time LAMP for *V. vulnificus* quantification. One way to quantify LAMP products is by using a fluorescent DNA-intercalating dye as in a real-time PCR format (Aoi *et al.*, 2006; Hara-Kudo *et al.*, 2005; Monis *et al.*, 2005; Njiru *et al.*, 2008). Additionally, the formation of a by-product (magnesium pyrophosphate) during LAMP amplification causes turbidity change, which correlates with the amount of amplified DNA and could be monitored by a real-time turbidimeter (Mori *et al.*, 2001; Mori *et al.*, 2004).

Built upon our previous report (Han & Ge, 2008), the present study aimed to develop real-time LAMP assays suitable for the quantitative detection of *V. vulnificus* in raw oysters by utilizing two real-time platforms, one was fluorescence-based and the other one turbidity-based.

Materials and Methods

Bacterial Strains and DNA Templates Preparation. *Vibrio vulnificus* clinical strain ATCC 27562 was used for sensitivity testing. An additional 37 *V. vulnificus* clinical and

environmental strains and 42 non- *V. vulnificus* strains were used to evaluate assay specificity (Table 5-3). All *Vibrio* strains were grown overnight at 35°C on trypticase soy agar or in broth (TSA or TSB; BD Diagnostic Systems, Sparks, MD) supplemented with 2% NaCl. Non- *Vibrio* strains were grown on Luria-Bertani agar or blood agar (BD Diagnostic Systems).

Table 5 - 3 Bacterial strains used in real-time LAMP study to detect total *Vibrio vulnificus*

Strain group	Strain ID and serotype ^a	Source and reference
<i>V. vulnificus</i> , clinical (n = 9)	ATCC 27562	Blood, Florida
	ATCC 29306	Corneal ulcer, Virginia
	ATCC 33815	Leg ulcer, Wisconsin
	ATCC 33816	Blood, Alaska
	C7184	Thumb drainage, Texas (Oliver <i>et al.</i> , 1982)
	1003	Wound, Louisiana (Martin & Siebeling, 1991)
	1004	Stool, Louisiana (Martin & Siebeling, 1991)
<i>V. vulnificus</i> , environmental (n = 29)	1006, 1007	Blood, Louisiana (Martin & Siebeling, 1991)
	WR1	Sea water, Washington
	V195	Gulf oyster, Louisiana (Han <i>et al.</i> , 2007)
	V244, V262, V350, V353, V398, V420, V463, V531, V560, V606	Retail oyster, Louisiana (Han <i>et al.</i> , 2007)
<i>V. parahaemolyticus</i> (n = 20)	132 (A1, B5, B8, T5, Z2), 212 (B6, E12, F14, F15, F18, S7, S8, Y10), 342 (E3, E4, E6, E9)	Gulf oyster, Louisiana ^b
	ATCC 17802; O1:K1	Shirasu food poisoning, Japan
	ATCC 27969	Blue crab, Maryland
	ATCC 33847	Gastroenteritis, Maryland
	ATCC 49529; O4:K12	Feces, California
	CT-6636; O3:K6	Clinical, Connecticut
	M350A; O5	Oyster, Washington
	NY477; O4:K8	Oyster, New York
	TX-2103; O3:K6	Clinical, Texas
	8332924; O1:K56	Oyster, Gulf of Mexico
	83AO8757	Clinical, feces
	83AO9148	Clinical, feces
	83AO9756; O4:K12	Clinical, feces
	84AO1516; O4:K12	Clinical, feces
84AO4226	Clinical, feces	
916i, 541-0-44c, V68, V69, V155	Oyster, Gulf, Louisiana (Han <i>et al.</i> , 2007)	

Table continued

	V86	Oyster, Retail, Louisiana (Han <i>et al.</i> , 2007)
Other <i>Vibrio</i> spp. (<i>n</i> = 10)		
<i>Vibrio alginolyticus</i>	ATCC 17749	Spoiled horse mackerel, Japan
	ATCC 33787	Seawater, Hawaii
<i>Vibrio cholerae</i>	ATCC 14035; O:1	NCTC, United Kingdom
<i>Vibrio cincinnatiensis</i>	ATCC 35912	Blood/cerebrospinal fluid, Ohio
<i>Vibrio fluvialis</i>	ATCC 33809	Human feces, Bangladesh
<i>Vibrio harveyi</i>	ATCC 14126	Dead amphipod, Massachusetts
	ATCC 35084	Brown shark, Maryland
<i>Vibrio mimicus</i>	ATCC 33653	Human ear, North Carolina
	ATCC 33655	Feces, Tennessee
<i>Vibrio natriegens</i>	ATCC 14048	Salt marsh mud, Georgia
Non- <i>Vibrio</i> spp. (<i>n</i> = 12)		
<i>Campylobacter jejuni</i>	81-176	Human
<i>Enterobacter aerogenes</i>	ATCC 13048	Sputum, South Carolina
<i>Enterococcus faecalis</i>	ATCC 29212	Urine
<i>Escherichia coli</i>	ATCC 25922	Human
<i>Listeria monocytogenes</i>	ATCC 13932; 4b	Spinal fluid, Germany
<i>Litonella anguillarum</i>	ATCC 19264	Ulcerous lesion in cod, UK
<i>Pseudomonas aeruginosa</i>	ATCC 27853	Human blood
<i>Salmonella enterica</i>	LT2; Typhimurium	Unknown
<i>Shigella flexneri</i>	ATCC 12022; 2b	Unknown
<i>Shigella sonnei</i>	ATCC 25931	Human feces, Panama
<i>Staphylococcus aureus</i>	ATCC 29213	Wound
<i>Streptococcus pneumoniae</i>	ATCC 49619; type 59	Sputum, Arizona

^a ATCC, American Type Culture Collection, Manassas, VA; NCTC, the National Collection of Type Cultures, London, United Kingdom.

^b Isolated from three Louisiana coastal locations (designated as 132, 212, and 342) between 2006-2007.

DNA templates were prepared by suspending a single bacterial colony grown on appropriate agar plates in 500 µl of TE (10 mM Tris, pH 8.0; 1 mM EDTA; Sigma-Aldrich, St. Louis, MO), followed by heated at 95°C for 10 min in a dry heating block. After centrifuge at 12,000 *g* for 2 min, the supernatants were stored at -30°C until use. To prepare templates for sensitivity testing, an overnight *V. vulnificus* ATCC 27562 culture was diluted 50 fold in TSB and incubated at 35°C for 5 h with shaking at 100 rpm. Serial 10-fold dilutions of the mid-log phase culture were made in phosphate-buffered saline (PBS; Sigma-Aldrich) and aliquots of each

dilution were used to prepare DNA templates similarly by the boiling method. The exact cell counts in the templates were obtained by standard plate counting on TSA with 2% NaCl.

Fluorescence-based Real-time LAMP. Four LAMP primers (F3, B3, FIP, and BIP) targeting the *V. vulnificus* cytolysin/hemolysin gene (*vvhA*) as described in our previous report (Han & Ge, 2008) were adopted. Additionally, a loop primer (5'-TCCATTCGCCAGCAGTTACG-3') was designed using the PrimerExplorer software version 4 (Fujitsu Limited, Japan; <http://primerexplorer.jp/e>). All primers were synthesized by Invitrogen (Carlsbad, CA). The LAMP reaction mix (25 μ l) consisted of the following: 1 \times Thermo buffer (New England Biolabs, Ipswich, MA), 6 mM of MgSO₄, 0.8 M of betaine (Sigma-Aldrich), 1.4 mM of deoxynucleotide triphosphate (dNTP), 0.2 μ M of each outer primer (F3 and B3), 1.6 μ M of each inner primer (FIP and BIP), 0.8 μ M of the loop primer, 0.4 μ M of SYTO-9 green fluorescent dye (Invitrogen), 8 U of *Bst* DNA polymerase (New England Biolabs), and 2 μ l of DNA template. A positive control and a negative control were included in each LAMP run.

For the fluorescence-based platform, the LAMP reaction was conducted in a SmartCycler II System (Cepheid, Sunnyvale, CA) at 63°C for 1 h. Fluorescence readings were obtained every 60 s using the FAM channel (excitation at 450-495 nm and detection at 510-527 nm), followed by melting curve analysis from 63°C to 96°C with an increment of 0.2°C per second. The fluorescence threshold unit was set at 30 for background noise deduction.

Turbidity-based Real-time LAMP. The reaction mix was essentially the same as described above for the fluorescence-based platform except that SYTO-9 was omitted. The reaction was carried out at 63°C for 1 h and terminated at 80°C for 5 min in a real-time turbidimeter (LA-320C; Teramecs, Kyoto, Japan), which acquired turbidity readings of the LAMP reaction mix at 650 nm every 6 second. A turbidity threshold value of 0.1 was used.

PCR. As a comparison, two sets of PCR assays targeting the *vvhA* gene were performed, one using the LAMP outer primers (F3/B3) (Han & Ge, 2008) and the other one using the Vvh-785F/Vvh-1303R primers described in the U.S. Food and Drug Administration Bacteriological Analytical Manual (BAM) (Kaysner & DePaola, 2001). PCR conditions followed those described previously (Han & Ge, 2008; Kaysner & DePaola, 2001).

Assay Specificity and Sensitivity. Methods published previously were followed (Chen & Ge, 2010). Briefly, eighty bacterial strains (Table 5-3) were used to determine the real-time LAMP specificity. Aliquots (2 μ l) of each DNA template were subjected to both real-time LAMP and PCR amplifications. Specificity tests were repeated twice.

To determine LAMP sensitivity, aliquots (2 μ l) of the 10-fold serial dilutions of sensitivity templates prepared above were subjected to both real-time LAMP and PCR amplifications. Sensitivity tests were repeated five times.

Quantification of *V. vulnificus* Cells in Spiked Oysters. Oyster samples were obtained from local seafood restaurants and determined to be *V. vulnificus*-free as described previously (Han *et al.*, 2007). Oyster samples were processed following a previous study (Yamazaki *et al.*, 2008a) with slight modifications. Briefly, 25 g of oyster sample was mixed with 225 ml of alkaline peptone water (APW; BD Diagnostic Systems) and homogenized in a food stomacher (Model 400; Tekmar Company, Cincinnati, OH) for 90 s to generate 1:10 oyster in APW homogenate. After homogenization, aliquots (100 μ l) of serial 10-fold dilutions of a mid-log phase *V. vulnificus* ATCC 27562 culture were inoculated into 900 μ l of the 1:10 oyster in APW homogenate. The spiked oyster samples were mixed well and centrifuged at 900 g for 1 min to remove oyster tissues. The supernatants were transferred to a fresh tube and centrifuged at 10,000 g for 5 min to pellet bacterial cells. After removing the supernatants, pellets were

resuspended in 100 μ l of TE and boiled for templates as described above. Aliquots (2 μ l) of the supernatant were used for both real-time LAMP and PCR amplifications. Three sets of independent spiking experiments were performed, and the LAMP reactions were repeated two times for each set of inoculation.

Data Analysis. For specificity data, means and standard deviations of Ct (cycle threshold; for the fluorescence-based platform) or Tt (time threshold; for the turbidity-based platform) values were calculated by using the Microsoft Excel software (Microsoft, Seattle, WA). For sensitivity data, means and standard deviations of Ct or Tt values for detecting 10-fold serial dilutions of *V. vulnificus* ATCC 27562 in pure culture and spiked oyster homogenates were calculated similarly using Microsoft Excel. The limits of detection (CFU/reaction in pure culture or CFU/g in spiked oysters) were determined. In spiked oyster samples, CFU/reaction was calculated by using $\text{CFU/g} \times 0.09 \text{ g/ml} \times 10 \times 2 \times 10^{-3}$, i.e., $\text{CFU/g} \times 1.8 \times 10^{-3}$.

Standard curves to quantify *V. vulnificus* in pure culture and spiked oysters were generated by plotting Ct or Tt values against log CFU/reaction or log CFU/g and linear regression was calculated using Microsoft Excel. Amplification efficiency (E; %) was obtained by using the formula $[10^{(-1/\text{slope})} - 1] \times 100$.

Results

Specificity of Real-time LAMP Assays. Among 80 bacterial strains used to determine LAMP specificity on two real-time platforms, no false positive or false negative results were observed. On the fluorescence-based platform performed in a SmartCycler II system, mean Ct values for 38 *V. vulnificus* clinical and environmental strains ranged between 15.01 and 24.37 min, with an average of 19.35 ± 2.89 min. The melting temperatures for the 38 *V. vulnificus* strains consistently fell between 83.97 and 85.21°C, with an average of $84.79 \pm 0.33^\circ\text{C}$. For 42

non- *V. vulnificus* strains, no C_t value was obtained, with melting curve analysis showing either no peak or a melting temperature at around 63°C, suggesting possible primer-dimer formations. Using the turbidity-based platform in a real-time turbidimeter, T_t values for the 38 *V. vulnificus* clinical and environmental strains ranged from 27.4 to 37.05 min with an average of 32.55 ± 2.92 min. For the 42 non-*V. vulnificus* strains, no T_t value was obtained, indicating negative results for LAMP.

Similarly, no false positive or false negative results for the 80 bacterial strains were observed by PCR using the two primer sets, F3/B3 and BAM primers, indicating good specificity.

Sensitivity and Quantitative Capability of Real-time LAMP Assays. Figure 5-4 presents sensitivity of the fluorescence-based real-time LAMP assay when testing 10-fold serial dilutions of *V. vulnificus* ATCC 27562 DNA templates.

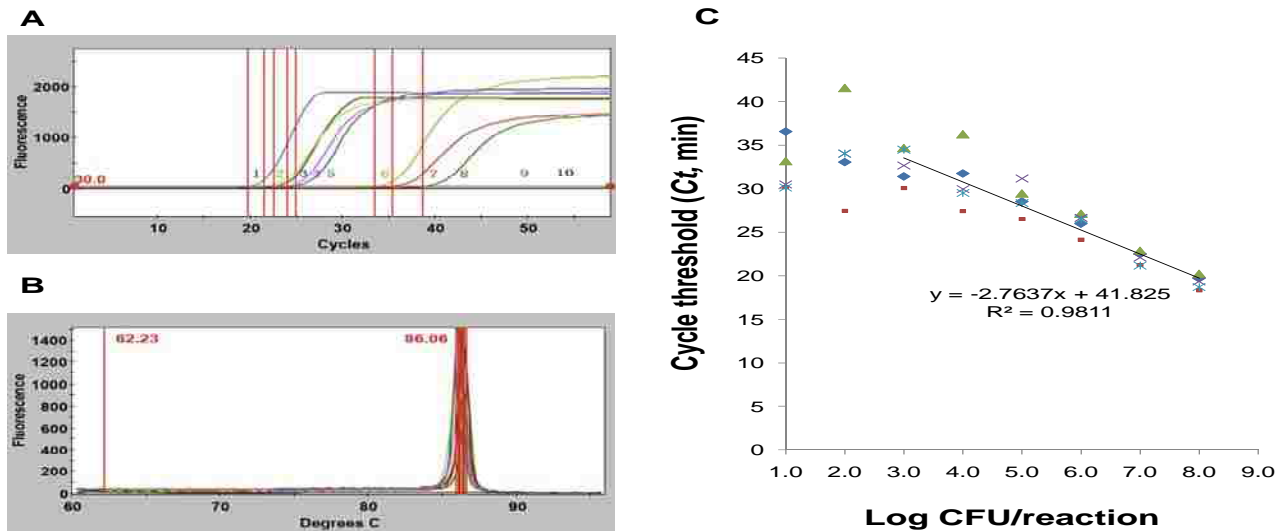


Figure 5 - 4 Sensitivity of the fluorescence-based real-time LAMP when detecting *Vibrio vulnificus* ATCC 27562 in pure culture. (A) A representative optic graph; (B) Corresponding melting curve analysis for samples shown in (A); (C) A standard curve generated based on five independent repeats. Samples (1-9) correspond to 10-fold serial dilutions of *V. vulnificus* ATCC 27562 cells ranging from 1×10^8 to 1 CFU/reaction; sample 10 is water.

A representative optic graph, corresponding melting curve analysis, and a standard curve are shown in Figure 5-4A-C, respectively. For templates ranging in concentration from 1×10^8 to 10 CFU/reaction, the average C_t values of five repeats ranged from 19.26 to 39.79 min, with melting temperatures consistently falling at around 86°C. For the 1 CFU template, in three out of five repeats, amplification occurred. Therefore, the detection limit of the fluorescence-based real-time LAMP assay was 1-10 CFU/reaction. From the standard curve (Figure 5-4C) generated by the fluorescence-based real-time LAMP assay, the correlation coefficient (r^2) was calculated to be 0.98 and the amplification efficiency (E) was 130%.

Figure 5-5 shows the sensitivity of real-time LAMP on the turbidity-based platform when testing the same set of DNA templates. A representative turbidity judgment graph and a standard curve are shown in Figure 5-5A and 5-5B, respectively.

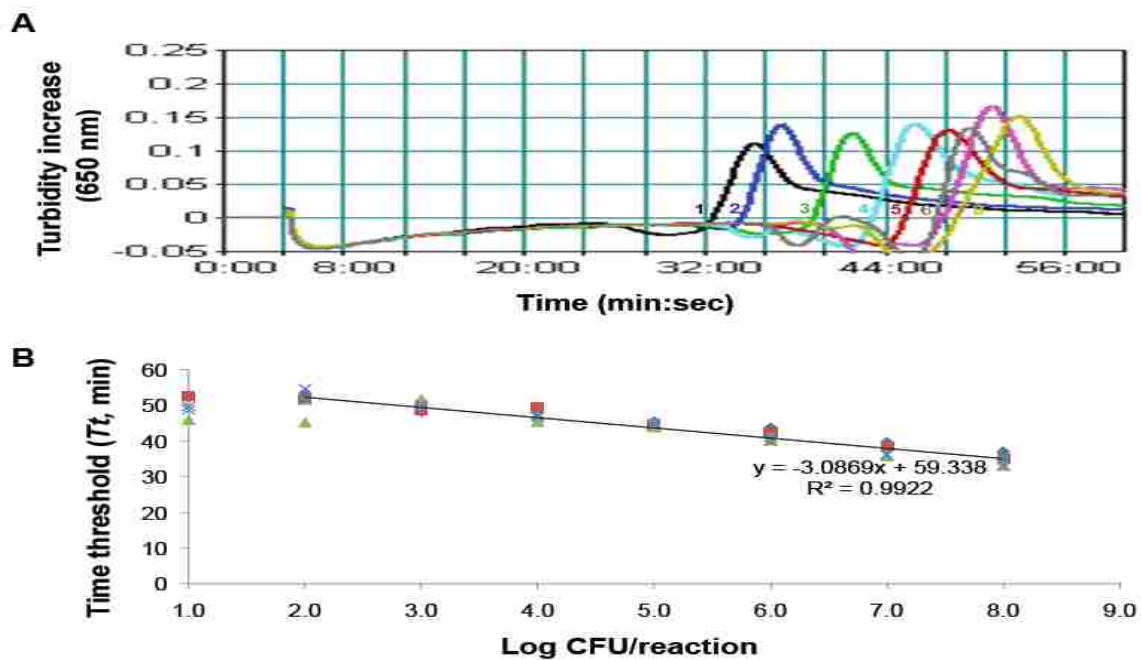


Figure 5 - 5 Sensitivity of the turbidity-based real-time LAMP when testing the same set of *Vibrio vulnificus* ATCC 27562 DNA templates.

(A) A representative turbidity judgment graph; (B) A standard curve generated based on five independent repeats. Samples 1-8 are the same as those shown in Figure 5-4, 10-fold serial dilutions of *V. vulnificus* ATCC 27562 cells ranging from 1×10^8 to 10 CFU/reaction

For templates ranging from 1×10^8 to 10 CFU/reaction, based on five repeats, the average *Tt* values fell between 34.5 and 51.1 min. In two out of five repeats, amplification of the 1 CFU template occurred. Therefore, the limit of detection for the turbidity-based real-time LAMP assay was 1-10 CFU/reaction. Based on the standard curve (Figure 5-5B), the turbidity-based real-time LAMP assay had an r^2 value of 0.99 and an E value of 111%.

When testing the same set of *V. vulnificus* ATCC 27562 templates by PCR using F3/B3 and BAM primers, both primer sets detected 100 CFU/reaction, which were up to 100-fold less sensitive than real-time LAMP assays.

Detection of *V. vulnificus* Cells in Spiked Oysters. In three independent spiking experiments, the average *Ct* values ranged from 25.26 to 34.10 min, whereas the average *Tt* values ranged from 39.83 to 54.80 min. The limit of detection by real-time LAMP assays using the two platforms was 116 CFU/reaction (i.e., 6.4×10^4 CFU/g) of *V. vulnificus* ATCC 27562 in spiked oyster samples without enrichment. However, for PCR assays using either F3/B3 or BAM primers, the limit of detection in spiked oysters was 6.4×10^7 CFU/g (data not shown), 1,000-fold less sensitive than that of real-time LAMP assays. Standard curves (Figure 5-6) generated for the quantitative detection of *V. vulnificus* cells in spiked oyster samples had an r^2 value of 0.99 for both real-time LAMP platforms.

Discussion

In this study, we used our previously designed LAMP inner and outer primers (Han & Ge, 2008) and added a loop primer to specifically target the *V. vulnificus* *vvhA* gene. We further developed the LAMP assay by running on two real-time platforms, one was fluorescence-based and the other one turbidity-based, to quantitatively detect *V. vulnificus* in pure culture and spiked

oyster samples. This is the first report examining the quantitative capability of real-time LAMP for detecting *V. vulnificus* in oysters by targeting the *vvhA* gene.

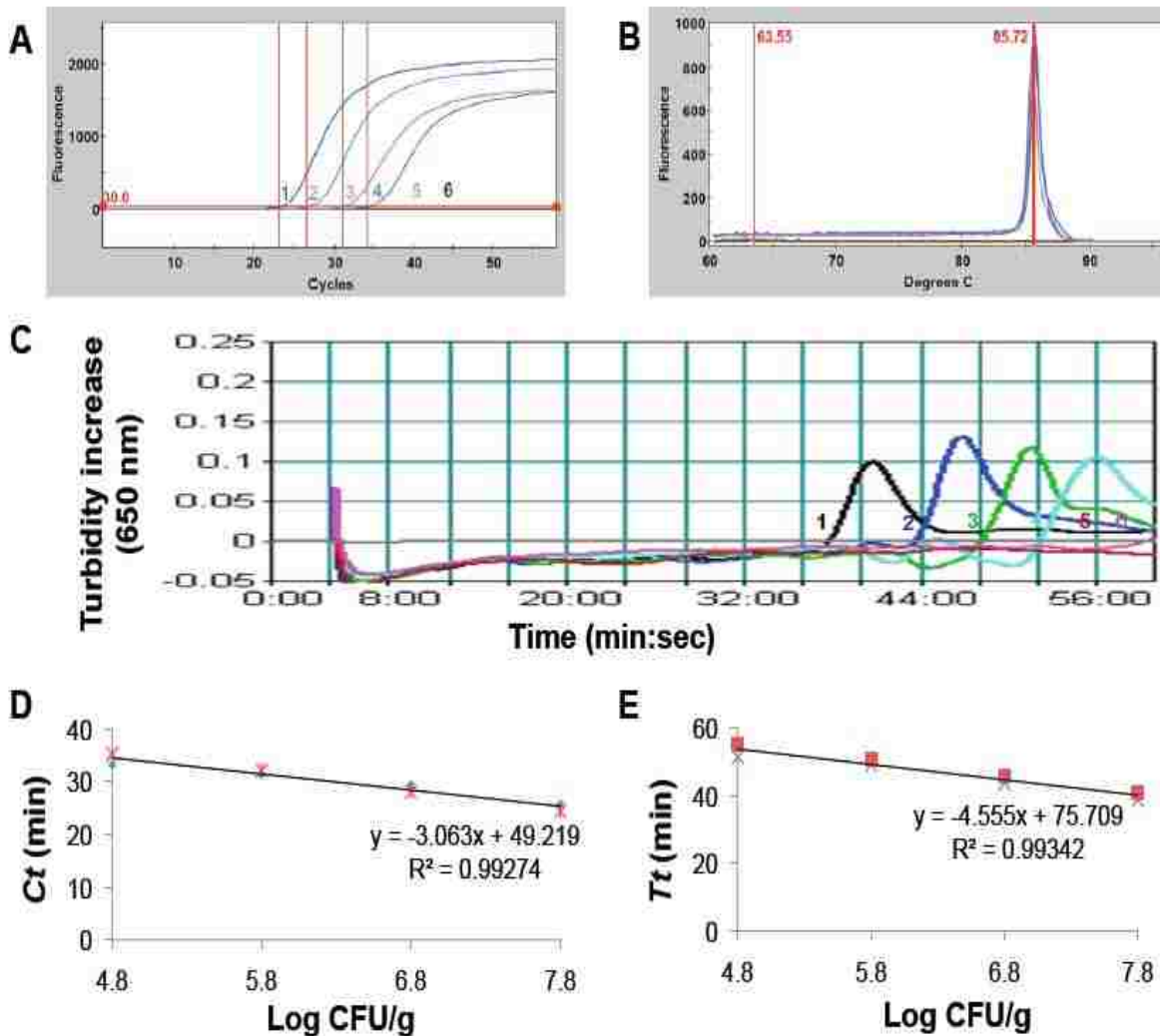


Figure 5 - 6 Quantitative detection of *Vibrio vulnificus* ATCC 27562 in spiked oysters by using fluorescence-based and turbidity-based real-time LAMP. Three sets of independent spiking experiments were performed, and the LAMP reactions were repeated two times for each set of inoculation. (A) A representative optic graph; (B) Corresponding melting curve analysis for samples shown in (A); (C) A representative turbidity judgment graph; (D) A standard curve generated for the fluorescence-based real-time LAMP; (E) A standard curve generated for the turbidity-based real-time LAMP. Samples 1-5 correspond to 10-fold spiked oyster sample ranging from 6.4×10^7 to 6.4×10^3 CFU/g, sample 6 is water.

Among a total of 38 *V. vulnificus* and 42 non- *V. vulnificus* strains tested, the real-time LAMP assay run on both platforms achieved 100% inclusivity and 100% exclusivity. This level of specificity was the same as that of two PCR assays tested simultaneously in this study and several LAMP assays for *V. vulnificus* reported recently (Han & Ge, 2008; Nemoto *et al.*, 2008; Ren *et al.*, 2009).

In addition to high specificity, the real-time LAMP assays were able to detect 1-10 CFU/reaction of *V. vulnificus*, in contrast to 100 CFU/reaction by the two PCR assays. We previously reported a LAMP sensitivity of 20 CFU/reaction and a PCR sensitivity of 202 CFU/reaction (Han & Ge, 2008), which are comparable to findings of this study. A *toxR*-based LAMP assay for *V. vulnificus* reported a minimum detection level of 1 CFU per test (Nemoto *et al.*, 2008), whereas another *vvhA*-based LAMP assay found it to be 10-fold more sensitive than conventional PCR, although the exact cell number was not reported (Ren *et al.*, 2009). Additionally, increased sensitivity (at least 10-fold) of LAMP compared to PCR was reported in studies on the detection of other *Vibrio* spp. (Fall *et al.*, 2008; Yamazaki *et al.*, 2008a; Yamazaki *et al.*, 2008b) or other foodborne pathogens (Hara-Kudo *et al.*, 2005; Hara-Kudo *et al.*, 2007; Yano *et al.*, 2007).

The addition of loop primers generally accelerates LAMP assay by hybridizing to stem-loops formed during LAMP reaction and facilitating DNA strand displacement and amplification (Nagamine *et al.*, 2002). In our previous study, we found that at least 45 min was required to reach a positive signal without the loop primer (Han & Ge, 2008). In this study, the time to positive results was shortened after adding the loop primer on both fluorescence- and turbidity-based platforms, confirming the assay accelerating effect of the loop primer. Noticeably, when testing 38 *V. vulnificus* strains, the average time to positive results for the fluorescence-based

platform as indicated by Ct (19.35 min) was markedly shorter than that of the turbidity-based platform as indicated by Tt (32.55 min). Using YO-PRO-1 as the intercalating dye, a previous study also found that fluorescence-based real-time LAMP was faster than a turbidimetry real-time LAMP (Aoi *et al.*, 2006). In addition, that study reported that the fluorescence-based LAMP assay generated anomalous and irreproducible results in low-concentration templates (less than 1×10^3 copies), which could be due to the effect of the intercalating dye on DNA amplification efficiency (Aoi *et al.*, 2006). Similarly, in our study, the standard curve generated for the fluorescence-based real-time LAMP (Figure 5-4C) showed less reproducible data when the templates concentrations were below 3 log CFU/reaction.

Previously, SYBR Green I has been used as a way to visually observe LAMP results after amplification (Han & Ge, 2008; Parida *et al.*, 2007). Since LAMP reactions generate a large amount of DNA, this open-tube procedure after amplification potentially acts as a significant source of cross-contamination, so close-tube endpoint detection has been suggested (Mori *et al.*, 2006). Real-time LAMP assays run on either platform are advantageous in this regard since the DNA-intercalating dye was added in the reagent mix before amplification on the fluorescence-based platform or omitted on the turbidity-based platform.

For the fluorescence-based real-time LAMP, SYTO-9 was used in this study as the intercalating dye instead of the commonly used SYBR Green I for real-time PCR. A previous study found that SYTO-9 had lower inhibitory effect on the amplification and higher melting curve reproducibility over a broader range of dye concentrations than SYBR Green I (Monis *et al.*, 2005). Additionally, EvaGreen (Biotium, Hayward, CA) was tested but showed high inhibition for LAMP amplification (data not shown). After optimizing SYTO-9 concentration, 0.4 μ M SYTO-9 was used.

The strong linear correlation ($r^2 = 0.98-0.99$) between the number of *V. vulnificus* cells in the LAMP reaction and the associated *Ct* or *Tt* values over a dynamic range of template concentrations (10^8 to 10^3 CFU/reaction in pure culture and 10^5 to 10^2 CFU/reaction in spiked oyster extract) illustrates the quantitative capability of the real-time LAMP assays when detecting this organism in both pure culture and spiked oysters. Very few reports have examined the quantitative ability of LAMP. One study monitoring ammonia-oxidizing bacteria using LAMP also reported it to possess good quantitative capability between 1×10^4 to 1×10^{10} DNA copies (Aoi *et al.*, 2006).

In spiked oyster samples, a detection limit of 116 *V. vulnificus* CFU/reaction was found for real-time LAMP assays run on both platforms, which translates to 6.4×10^4 CFU/g of oyster sample. In contrast, the detection limit of two PCR assays was 6.4×10^7 CFU/g, indicating LAMP was less prone to inhibitor effects in oyster samples compared to PCR. In a most recent survey of the U.S. market oysters, approximately 44% of live oyster samples harvested from Louisiana exceeded 10,000 MPN/g for *V. vulnificus* whereas other states had non-detectable levels of this organism (DePaola *et al.*, 2010). Nonetheless, given the severe disease symptoms and low infectious dose (less than 100) of this organism in immunocompromised persons (U.S. Food and Drug Administration, 2009), the Interstate Shellfish Sanitation Conference recommends that all postharvest-processed oysters contain lower than 30 MPN/g of *V. vulnificus* (U. S. Food and Drug Administration, 2007). Therefore, without enrichment, DNA amplification assays such as LAMP, although potentially quantitative, lack the needed sensitivity when applied in oyster samples (Han & Ge, 2008). Combining MPN overnight enrichment (Nordstrom *et al.*, 2007) or pre-enrichment for 6 h (Nemoto *et al.*, 2009) with LAMP or other DNA amplification

assays is a desirable approach to achieve the needed sensitivity, although in that situation, the quantitative capability of real-time LAMP would not be possible.

The detection limit for *V. vulnificus* was higher in oyster samples (116 CFU/reaction) vs. in pure culture (1-10 CFU/reaction). However, since no extensive sample preparation other than homogenization and two simple centrifugation steps was required, the total assay time was significantly reduced. Adding the 1 h required for the real-time LAMP run, the complete LAMP detection system from sample preparation to quantitative result was markedly faster than either PCR or conventional culture-based methods.

Conclusion

In conclusion, the real-time LAMP assays developed in this study was a highly specific, sensitive, rapid, and quantitative method for the detection of *V. vulnificus* in oysters. Comparable sensitivity was obtained using the two real-time platforms, with the assay limits of detection to be approximately 1-10 CFU/reaction of *V. vulnificus* for pure culture, up to 100-fold more sensitive than PCR. When applied to spiked oyster samples, the real-time LAMP assays were able to detect 6.4×10^4 CFU/g of oyster without enrichment, 1,000 fold more sensitive than PCR. Standard curves generated for detecting *V. vulnificus* in both pure culture and spiked oyster samples showed good linear regression between cell counts and the fluorescence *Ct* or turbidity *Tt* values. Future testing with natural or commercial oyster samples is desired to further evaluate the efficacy of the assay.

References

Aoi, Y., Hosogai, M. & Tsuneda, S. (2006). Real-time quantitative LAMP (loop-mediated isothermal amplification of DNA) as a simple method for monitoring ammonia-oxidizing bacteria. *J Biotechnol* **125**, 484-491.

Campbell, M. S. & Wright, A. C. (2003). Real-time PCR analysis of *Vibrio vulnificus* from oysters. *Appl Environ Microbiol* **69**, 7137-7144.

Centers for Disease Control and Prevention (2010). Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food--10 states, 2009. *MMWR Morb Mortal Wkly Rep* **58**, 418-422.

Chen, S. & Ge, B. (2010). Development of a toxR-based loop-mediated isothermal amplification assay for detecting *Vibrio parahaemolyticus*. *BMC Microbiol* **10**, 41.

Cholera and Other Vibrio Illness Surveillance (2009). Summary of human *Vibrio* isolates reported to CDC 2007. Available at <http://www.cdc.gov/national-surveillance/PDFs/CSTEVibrio2007.pdf>. Accessed on April 28, 2010.

Coleman, S. S., Melanson, D. M., Biosca, E. G. & Oliver, J. D. (1996). Detection of *Vibrio vulnificus* biotypes 1 and 2 in eels and oysters by PCR amplification. *Appl Environ Microbiol* **62**, 1378-1382.

DePaola, A., Jones, J. L., Woods, J. & other authors (2010). Bacterial and viral pathogens in live oysters: 2007 United States market survey. *Appl Environ Microbiol* **76**, 2754-2768.

Fall, J., Chakraborty, G., Kono, T., Maeda, M., Itami, T. & Sakai, M. (2008). Establishment of loop-mediated isothermal amplification method (LAMP) for the detection of *Vibrio nigripulchritudo* in shrimp. *FEMS Microbiol Lett* **288**, 171-177.

Goto, M., Hayashidani, H., Takatori, K. & Hara-Kudo, Y. (2007). Rapid detection of enterotoxigenic *Staphylococcus aureus* harbouring genes for four classical enterotoxins, SEA, SEB, SEC and SED, by loop-mediated isothermal amplification assay. *Lett Appl Microbiol* **45**, 100-107.

Han, F., Walker, R. D., Janes, M. E., Prinyawiwatkul, W. & Ge, B. (2007). Antimicrobial susceptibilities of *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates from Louisiana Gulf and retail raw oysters. *Appl Environ Microbiol* **73**, 7096-7098.

Han, F. & Ge, B. (2008). Evaluation of a loop-mediated isothermal amplification assay for detecting *Vibrio vulnificus* in raw oysters. *Foodborne Pathog Dis* **5**, 311-320.

Hara-Kudo, Y., Yoshino, M., Kojima, T. & Ikedo, M. (2005). Loop-mediated isothermal amplification for the rapid detection of *Salmonella*. *FEMS microbiology letters* **253**, 155-161.

Hara-Kudo, Y., Nemoto, J., Ohtsuka, K., Segawa, Y., Takatori, K., Kojima, T. & Ikedo, M. (2007). Sensitive and rapid detection of Vero toxin-producing *Escherichia coli* using loop-mediated isothermal amplification. *Journal of medical microbiology* **56**, 398-406.

Hill, W. E., Keasler, S. P., Trucksess, M. W., Feng, P., Kaysner, C. A. & Lampel, K. A. (1991). Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. *Appl Environ Microbiol* **57**, 707-711.

- Kaysner, C. A. & DePaola, A. (2001).** *Vibrio*. In *Bacteriological Analytical Manual online*, Chapter 9. Edited by U.S Food and Drug Administration. Rockville, MD.
- Martin, S. J. & Siebeling, R. J. (1991).** Identification of *Vibrio vulnificus* O serovars with antilipopolysaccharide monoclonal antibody. *J Clin Microbiol* **29**, 1684-1688.
- Monis, P. T., Giglio, S. & Saint, C. P. (2005).** Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. *Analytical biochemistry* **340**, 24-34.
- Mori, Y., Nagamine, K., Tomita, N. & Notomi, T. (2001).** Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochemical and biophysical research communications* **289**, 150-154.
- Mori, Y., Kitao, M., Tomita, N. & Notomi, T. (2004).** Real-time turbidimetry of LAMP reaction for quantifying template DNA. *Journal of biochemical and biophysical methods* **59**, 145-157.
- Mori, Y., Hirano, T. & Notomi, T. (2006).** Sequence specific visual detection of LAMP reactions by addition of cationic polymers. *BMC Biotechnol* **6**, 3.
- Nagamine, K., Hase, T. & Notomi, T. (2002).** Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and cellular probes* **16**, 223-229.
- Nemoto, J., Kojima, T., Kusaba, K., Nagasawa, Z., Oishi, H. & Nakashima, M. (2008).** Rapid detection of *Vibrio vulnificus* using a loop-mediated isothermal amplification method. *Kansenshogaku Zasshi* **82**, 407-413.
- Nemoto, J., Sugawara, C., Akahane, K., Hashimoto, K., Kojima, T., Ikedo, M., Konuma, H. & Hara-Kudo, Y. (2009).** Rapid and specific detection of the thermostable direct hemolysin gene in *Vibrio parahaemolyticus* by loop-mediated isothermal amplification. *J Food Prot* **72**, 748-754.
- Njiru, Z. K., Mikosza, A. S., Armstrong, T., Enyaru, J. C., Ndung'u, J. M. & Thompson, A. R. (2008).** Loop-Mediated Isothermal Amplification (LAMP) Method for Rapid Detection of *Trypanosoma brucei rhodesiense*. *PLoS Negl Trop Dis* **2**, e147.
- Nordstrom, J. L., Vickery, M. C., Blackstone, G. M., Murray, S. L. & DePaola, A. (2007).** Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. *Appl Environ Microbiol* **73**, 5840-5847.
- Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. (2000).** Loop-mediated isothermal amplification of DNA. *Nucleic acids research* **28**, E63.

- Oliver, J. D., Warner, R. A. & Cleland, D. R. (1982).** Distribution and ecology of *Vibrio vulnificus* and other lactose-fermenting marine vibrios in coastal waters of the southeastern United States. *Appl Environ Microbiol* **44**, 1404-1414.
- Oliver, J. D. (2006).** *Vibrio vulnificus*, p. 349-366. Edited by F. L. Thompson, B. Austin, and J. Swings (ed.), *The biology of vibrios*. Washitonton, DC: ASM press.
- Panicker, G., Myers, M. L. & Bej, A. K. (2004).** Rapid detection of *Vibrio vulnificus* in shellfish and Gulf of Mexico water by real-time PCR. *Appl Environ Microbiol* **70**, 498-507.
- Panicker, G. & Bej, A. K. (2005).** Real-time PCR detection of *Vibrio vulnificus* in oysters: comparison of oligonucleotide primers and probes targeting *vhA*. *Appl Environ Microbiol* **71**, 5702-5709.
- Parida, M. M., Santhosh, S. R., Dash, P. K. & other authors (2007).** Rapid and real-time detection of Chikungunya virus by reverse transcription loop-mediated isothermal amplification assay. *J Clin Microbiol* **45**, 351-357.
- Ren, C. H., Hu, C. Q., Luo, P. & Wang, Q. B. (2009).** Sensitive and rapid identification of *Vibrio vulnificus* by loop-mediated isothermal amplification. *Microbiol Res* **164**, 514-521.
- Strom, M. S. & Paranjpye, R. N. (2000).** Epidemiology and pathogenesis of *Vibrio vulnificus*. *Microbes and infection / Institut Pasteur* **2**, 177-188.
- Takahashi, H., Hara-Kudo, Y., Miyasaka, J., Kumagai, S. & Konuma, H. (2005).** Development of a quantitative real-time polymerase chain reaction targeted to the *toxR* for detection of *Vibrio vulnificus*. *J Microbiol Methods* **61**, 77-85.
- U. S. Food and Drug Administration (2007).** National shellfish sanitation program guide for the control of molluscan shellfish 2007.
- U.S. Food and Drug Administration (2009).** *Vibrio vulnificus*. Available at <http://www.fda.gov/Food/FoodSafety/FoodborneIllness/FoodborneIllnessFoodbornePathogensNaturalToxins/BadBugBook/ucm070473.htm>. Accessed: May 13, 2010.
- Wright, A. C., Miceli, G. A., Landry, W. L., Christy, J. B., Watkins, W. D. & Morris, J. G., Jr. (1993).** Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. *Appl Environ Microbiol* **59**, 541-546.
- Wright, A. C., Garrido, V., Debuex, G., Farrell-Evans, M., Mudbidri, A. A. & Otwell, W. S. (2007).** Evaluation of postharvest-processed oysters by using PCR-based most-probable-number enumeration of *Vibrio vulnificus* bacteria. *Appl Environ Microbiol* **73**, 7477-7481.
- Yamazaki, W., Ishibashi, M., Kawahara, R. & Inoue, K. (2008a).** Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of *Vibrio parahaemolyticus*. *BMC Microbiol* **8**, 163.

Yamazaki, W., Seto, K., Taguchi, M., Ishibashi, M. & Inoue, K. (2008b). Sensitive and rapid detection of cholera toxin-producing *Vibrio cholerae* using a loop-mediated isothermal amplification. *BMC Microbiol* **8**, 94.

Yamazaki, W., Taguchi, M., Ishibashi, M., Kitazato, M., Nukina, M., Misawa, N. & Inoue, K. (2008c). Development and evaluation of a loop-mediated isothermal amplification assay for rapid and simple detection of *Campylobacter jejuni* and *Campylobacter coli*. *Journal of medical microbiology* **57**, 444-451.

Yano, A., Ishimaru, R. & Hujikata, R. (2007). Rapid and sensitive detection of heat-labile I and heat-stable I enterotoxin genes of enterotoxigenic *Escherichia coli* by Loop-Mediated Isothermal Amplification. *Journal of microbiological methods* **68**, 414-420.

Yoda, T., Suzuki, Y., Yamazaki, K., Sakon, N., Kanki, M., Aoyama, I. & Tsukamoto, T. (2007). Evaluation and application of reverse transcription loop-mediated isothermal amplification for detection of noroviruses. *Journal of medical virology* **79**, 326-334.

Part III: Quantitative Detection of Virulent-Type *Vibrio vulnificus* in Raw Oysters by Real-Time Loop-Mediated Isothermal Amplification

Introduction

Vibrio vulnificus is a Gram-negative, halophilic bacterium that inhabits warm coastal and estuarine waters worldwide. The organism is capable of causing fatal diseases such as primary septicemia and wound infections, with reported mortality rates over 50% and 25%, respectively (Oliver, 2006). At-risk groups for fatal *V. vulnificus* infections include people with immunocompromising conditions, diabetes, and elevated serum iron concentrations due to chronic liver disease or alcohol abuse (Strom & Paranjpye, 2000). The primary source of *V. vulnificus* infection has been reported to be the consumption of raw or undercooked seafood, particularly oysters (Rippey, 1994). Therefore, rapid and sensitive detection assays are needed to facilitate better control of potential *V. vulnificus* infections from oyster consumption.

Besides being an opportunistic human pathogen, epidemiological data suggested that only a small percentage of *V. vulnificus* strains in oysters are virulent (Cholera and Other *Vibrio* Illness Surveillance, 2008; Jackson *et al.*, 1997). However, none of the virulence factors identified to date (Jones & Oliver, 2009), have been shown to be unique for virulent *V. vulnificus*. Therefore, alternative strategies are sought. Recently, several biomarkers have been explored to differentiate virulent- (i.e. clinical-) from non-virulent- (i.e. environmental-) type *V. vulnificus* strains, although with varied degree of success. Using a randomly amplified polymorphic DNA method, a virulence-correlated gene (*vcg*) was identified in *V. vulnificus* (Warner & Oliver, 1999). Testing 55 randomly selected *V. vulnificus* strains using this biomarker showed that 90% of clinical isolates possessed the *vcgC* sequence variant and 93% of environmental isolates had the *vcgE* variant (Rosche *et al.*, 2005). One study characterizing *vcg* genotypes among *V. vulnificus* oyster and seawater isolates recovered from the eastern coast of North Carolina found that 84.4% of the 880 oyster isolates had the *vcgE* type, while a similar

distribution of the two *vcg* genotypes (46.9% *vcgE* versus 53.1% *vcgC*) was found among 292 seawater isolates (Warner & Oliver, 2008). Secondly, polymorphism in 17 bp of the *V. vulnificus* 16S rRNA gene has been explored to differentiate between clinical and environmental strains. A study showed that the majority (31 out of 33) of nonclinical *V. vulnificus* isolates had 16S rRNA type A, whereas a significant percentage (24 out of 34) of clinical isolates belonged to 16S rRNA type B (Nilsson *et al.*, 2003). Real-time PCR assays based on 16S rRNA polymorphism have been developed to differentiate virulent from non-virulent ones (Gordon *et al.*, 2008; Vickery *et al.*, 2007). Lastly, the capsular polysaccharide operon (CPS) has been identified, which showed a significant association between clinical isolates and CPS allele 1 whereas environmental isolates were associated with CPS allele 2 (Chatzidaki-Livanis *et al.*, 2006a; Chatzidaki-Livanis *et al.*, 2006b). In a previous study, we characterized 349 *V. vulnificus* isolates from the Gulf Coast and retail oysters in Louisiana using these biomarkers (Han *et al.*, 2009). CPS tended to give a low estimate of potentially clinical important *V. vulnificus* strains compared to other biomarkers and the agreement between CPS and other biomarkers was poor, whereas *vcg* and 16S rRNA demonstrated good correlations (Han *et al.*, 2009).

The application of biomarkers for the detection of virulent-type *V. vulnificus* has been mostly used in PCR or real-time PCR assays. However, for both PCR and real-time PCR, a dedicated thermal cycler is needed, which is rather expensive and hinders the wide application of such assays. Loop-mediated isothermal amplification (LAMP), is a novel nucleic acid amplification assay developed in 2000 that utilizes four to six primers to specifically recognize six to eight regions of the target DNA sequence and amplify millions of DNA copies under isothermal conditions (60-65°C) within an hour (Notomi *et al.*, 2000). Additionally, LAMP can be quantitative (i.e., real-time LAMP) by measuring the formation of a by-product (magnesium

pyrophosphate) during LAMP amplification, which correlates with the amount of amplified DNA and could be monitored by a real-time turbidimeter (Mori *et al.*, 2001; Mori *et al.*, 2004). Since its invention, LAMP has emerged as a powerful tool by various investigators for the rapid detection of multiple bacterial and viral agents (Goto *et al.*, 2007; Hara-Kudo *et al.*, 2005; Hara-Kudo *et al.*, 2007; Yamazaki *et al.*, 2008c; Yoda *et al.*, 2007). Very recently, our research group and two others independently developed LAMP assays for *V. vulnificus* detection, of which two targeted the *V. vulnificus* hemolysin (*vvhA*) gene (Han & Ge, 2008; Ren *et al.*, 2009) and one targeted the *V. vulnificus* *toxR* gene (Nemoto *et al.*, 2008). However, no LAMP assay has been developed for the detection of virulent-type *V. vulnificus*.

The present study aimed to develop a real-time-LAMP assay suitable for the quantitative detection of virulent-type *V. vulnificus* in raw oysters by targeting *vcgC* and 16S rRNA type B.

Materials and Methods

Target and LAMP Primer Design. The *V. vulnificus* *vcgC* (GenBank accession number AY626575) and 16S rRNA B type (GenBank accession number X76334) were selected as the target for LAMP primer design. A set of six primers, two outer and two inner, as well as two loop primers, that recognize eight distinct regions of the target sequence (Figure 5-7) was designed using the PrimerExplorer software (V4, Fujitsu Limited, Japan; <http://primerexplorer.jp/e>). Forward inner primer (FIP) consisted of a complementary sequence of F1 and a sense sequence of F2. Similarly, backward inner primer (BIP) was a combination of a complementary sequence of B1 and a sense sequence of B2. The two outer primers, forward outer primer and backward outer primer, were F3 and B3, respectively. LF and LB were two Loop primers designed to accelerate the amplification reaction. For 16S rRNA, specific primers with the potential to differentiate between A and B type sequences were designed using a special

feature of the software. All primers were synthesized by the Integrated DNA Technologies (Coralville, IA).

Bacterial Strains and DNA Templates Preparation. *V. vulnificus* clinical strain ATCC 33815 (*vcgC* +, 16S rRNA B +) was used for the sensitivity testing in pure culture and spiked oysters. Another 33 *vcgC* + and 50 *vcgC* - (i.e., *vcgE* type) *V. vulnificus*, as well as 30 other *Vibrio* and 12 non-*Vibrio* strains were used to evaluate *vcgC* LAMP assay specificity (Table 5-4). Similarly, 33 16S rRNA B + and 50 16S rRNA B - (i.e., A type) *V. vulnificus*, as well as 30 other *Vibrio* and 12 non-*Vibrio* strains were used to evaluate 16S rRNA LAMP assay specificity. All *Vibrio* strains were grown overnight at 35°C on trypticase soy agar or in broth (TSA or TSB; BD Diagnostic Systems, Sparks, MD) supplemented with 2% NaCl. Non-*Vibrio* strains were grown on Luria-Bertani agar or blood agar (BD Diagnostic Systems).

Table 5 - 4 Bacterial strains used in LAMP to detect virulent-type *Vibrio vulnificus*

Strain group	Strain ID and serotype ^a	Source and reference
<i>V. vulnificus</i> , <i>vcgC</i> -type (n = 33)	V199, V214, V241, V246, V332, V358, V360, V363, V371, V377, V385, V388, V389, V414, V419, V443, V444, V447, V457, V465, V469, V499, V501, V504, V513, V534, V544, V545, V552, V560, V576, V601, V616	Gulf and Retail oyster, Louisiana (Han <i>et al.</i> , 2007)
<i>V. vulnificus</i> , <i>vcgE</i> -type (n = 50)	V209, V213, V217, V223, V225, V238, V239, V240, V242, V248, V252, V260, V261, V274, V280, V292, V297, V299, V302, V304, V308, V324, V327, V328, V333, V337, V350, V354, V364, V372, V381, V383, V387, V392, V416, V432, V433, V438, V470, V471, V474, V477, V484, V490, V546, V574, V584, V598, V606, V631	Gulf and Retail oyster, Louisiana (Han <i>et al.</i> , 2007)
Other <i>Vibrio</i> spp. (n = 30)		
<i>Vibrio parahaemolyticus</i>	ATCC 17802; O1:K1	Shirasu food poisoning, Japan
	ATCC 27969	Blue crab, Maryland
	ATCC 33847	Gastroenteritis, Maryland
	ATCC 49529; O4:K12	Feces, California
	CT-6636; O3:K6	Clinical, Connecticut

Table continued

	M350A; O5 NY477; O4:K8 TX-2103; O3:K6 8332924; O1:K56 83AO8757 83AO9148 83AO9756; O4:K12 84AO1516; O4:K12 84AO4226 916i, 541-0-44c, V68, V69, V 86, V155	Oyster, Washington Oyster, New York Clinical, Texas Oyster, Gulf of Mexico Clinical, feces Clinical, feces Clinical, feces Clinical, feces Clinical, feces Gulf and Retail oyster, Louisiana (Han <i>et al.</i> , 2007) Spoiled horse mackerel, Japan
<i>Vibrio alginolyticus</i>	ATCC 17749	Seawater, Hawaii
<i>Vibrio cholerae</i>	ATCC 33787	NCTC, United Kingdom
<i>Vibrio cincinnatiensis</i>	ATCC 14035; O:1 ATCC 35912	Blood/cerebrospinal fluid, Ohio
<i>Vibrio fluvialis</i>	ATCC 33809	Human feces, Bangladesh
<i>Vibrio harveyi</i>	ATCC 14126	Dead amphipod, Massachusetts
<i>Vibrio mimicus</i>	ATCC 35084 ATCC 33653 ATCC 33655 ATCC 14048	Brown shark, Maryland Human ear, North Carolina Feces, Tennessee Salt marsh mud, Georgia
Non- <i>Vibrio</i> spp. (<i>n</i> = 12)		
<i>Campylobacter jejuni</i>	81-176	Human
<i>Enterobacter aerogenes</i>	ATCC 13048	Sputum, South Carolina
<i>Enterococcus faecalis</i>	ATCC 29212	Urine
<i>Escherichia coli</i>	ATCC 25922	Human
<i>Listeria monocytogenes</i>	ATCC 13932; 4b	Spinal fluid, Germany
<i>Litonella anguillarum</i>	ATCC 19264	Ulcerous lesion in cod, UK
<i>Pseudomonas aeruginosa</i>	ATCC 27853	Human blood
<i>Salmonella enterica</i>	LT2; Typhimurium	Unknown
<i>Shigella flexneri</i>	ATCC 12022; 2b	Unknown
<i>Shigella sonnei</i>	ATCC 25931	Human feces, Panama
<i>Staphylococcus aureus</i>	ATCC 29213	Wound
<i>Streptococcus pneumoniae</i>	ATCC 49619; type 59	Sputum, Arizona

^a ATCC, American Type Culture Collection, Manassas, VA; NCTC, the National Collection of Type Cultures, London, United Kingdom.

DNA templates were prepared by suspending a single bacterial colony grown on appropriate agar plates in 500 µl of TE (10 mM Tris, pH 8.0; 1 mM EDTA; Sigma-Aldrich, St.

Louis, MO) and heated at 95°C for 10 min in a dry heating block. After centrifuge at 12,000 *g* for 2 min, the supernatants were stored at -30°C until use.

To prepare templates for sensitivity testing, an overnight *V. vulnificus* ATCC 33815 culture was diluted 50 fold in TSB and incubated at 35°C for 5 h with shaking at 100 rpm. Serial 10-fold dilutions of the mid-log phase culture were made in phosphate-buffered saline (PBS; Sigma-Aldrich) and aliquots of each dilution were used to prepare DNA templates similarly by the boiling method. The exact cell counts in the templates were obtained by standard plate counting on TSA with 2% NaCl.

Real-time LAMP Reaction. The real-time LAMP reaction mix (25 μ l) consisted of the following: 1 \times Thermo buffer (New England Biolabs, Ipswich, MA), 6 mM of MgSO₄, 0.8 M of betaine (Sigma-Aldrich), 1.4 mM of deoxynucleotide triphosphate (dNTP), 0.2 μ M of each outer primer (F3 and B3), 1.6 μ M of each inner primer (FIP and BIP), 0.8 μ M of each loop primer (LF and LB), 8 U of *Bst* DNA polymerase (New England Biolabs), and 2 μ l of DNA template. A positive control and a negative control were included in each LAMP run. The reaction was carried out at 63°C for 1 h and terminated at 80°C for 5 min in a real-time turbidimeter (LA-320C; Teramecs, Kyoto, Japan), which acquired turbidity readings of the LAMP reaction mix at 650 nm every 6 seconds. The cutoff values for positive samples were determined when turbidity increased above the threshold value, which was fixed at 0.1.

PCR. As a comparison, two sets of PCR targeting the *vcgC* gene were performed: one using the *vcgC* LAMP outer primers (F3/B3) and the other one using P1/P3 (Rosche *et al.*, 2005). Similarly, two sets of PCR targeting the 16S rRNA B type were performed: one using the 16S rRNA B LAMP outer primers (F3/B3) and the other one using B F1/ B R1 (Warner & Oliver, 2008). The 25 μ l PCR mixture contained 1 \times PCR buffer, 0.2 mM of each dNTP, 1.5 mM of

MgCl₂, 0.5 unit of GoTaq Hot Start Polymerase (Promega, Madison, WI), 0.2 μM of forward and reverse primer, and 2 μl of DNA template. The PCR reaction was conducted using 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min in a Bio-Rad C1000 Thermal Cycler (Hercules, CA). PCR products were analyzed by electrophoresis on 2.0% agarose gel containing ethidium bromide, and visualized under UV light, and documented by a Gel Doc XR system (Bio-Rad).

Real-time LAMP Optimization. The assay optimization was performed using *V. vulnificus* ATCC 33815 by varying assay parameters including the concentrations of MgCl₂ (2 to 10 mM in 2 mM increments), betaine (0 to 1 M in 0.2 M increments), dNTP (0.4 to 2 mM in 0.4 mM increments), enzyme (2 to 10 U in 2 U increments), inner primer (1.2 to 2.0 μM in 0.2 μM increments), outer primer (0.05 to 0.4 μM in 0.05 μM increments) and loop primer (0.2 to 1.0 μM in 0.2 μM increments), assay temperature (60 to 65°C in 2.5 °C increments) and duration (30 to 60 min in 15 min increments). Each of the optimization experiments were repeated three times.

Real-time LAMP Specificity and Sensitivity. A total of 125 bacterial strains (Table 5-4) were used to determine the real-time LAMP specificity. Aliquots (2 μl) of each DNA template were subjected to both real-time LAMP and PCR amplifications. Specificity tests were repeated twice.

To determine real-time LAMP sensitivity, aliquots (2 μl) of the 10-fold serial dilutions of sensitivity templates prepared above were subjected to both real-time LAMP and PCR amplifications. Sensitivity tests were repeated five times.

Quantification of Virulent-type *V. vulnificus* Cells in Spiked Oysters. Oyster samples were obtained from local seafood restaurants and determined to be *V. vulnificus*-free as described previously (Han *et al.*, 2007). Oyster samples were processed following a previous study (Yamazaki *et al.*, 2008a) with slight modifications. Briefly, 25 g of oyster sample was mixed with 225 ml of alkaline peptone water (APW; BD Diagnostic Systems) and homogenized in a food stomacher (Model 400; Tekmar Company, Cincinnati, OH) for 90 s to generate 1:10 oyster in APW homogenate. Aliquots (100 μ l) of serial 10-fold dilutions of a mid-log phase *V. vulnificus* ATCC 33815 were inoculated into 900 μ l of the 1:10 oyster in APW homogenate. The spiked oyster samples were mixed well and centrifuged at 900 *g* for 1 min to remove oyster tissues. The supernatants were transferred to a fresh tube and centrifuged at 10,000 *g* for 5 min to pellet bacterial cells. After removing the supernatants, pellets were resuspended in 100 μ l of TE and boiled for templates as described above. Aliquots (2 μ l) of the supernatant were used for both real-time LAMP and PCR amplifications. Three sets of independent spiking experiments were performed, and the real-time LAMP and PCR reactions were repeated three times for each set of inoculation.

Data Analysis. For specificity data, means and standard deviations of *Tt* (time threshold) for turbidimeter were calculated by using the Microsoft Excel software (Microsoft, Seattle, WA). For sensitivity data, means and standard deviations of *Tt* values for detecting 10-fold serial dilutions of *V. vulnificus* ATCC 33815 in pure culture and spiked oyster homogenates were calculated similarly using Microsoft Excel. The limits of detection (CFU/reaction in pure culture or CFU/g in spiked oysters) were determined. In spiked oyster samples, CFU/reaction was calculated by using $\text{CFU/g} \times 0.09 \text{ g/ml} \times 10 \times 2 \times 10^{-3}$, i.e., $\text{CFU/g} \times 1.8 \times 10^{-3}$.

Standard curves to quantify *V. vulnificus* in pure culture and spiked oysters were generated by plotting *Tt* values against log CFU/reaction for pure culture or log CFU/g for spiked oyster and linear regression was calculated using Microsoft Excel. Amplification efficiency (E; %) was obtained by using the formula $[10^{(-1/\text{slope})} - 1] \times 100$.

Results

LAMP Primer Design. We designed a LAMP assay targeting *vcgC*, which indicates that six specific locations on the *vcgC* were recognized by the LAMP primers, along with detailed information on each LAMP primer (Figure 5-7).

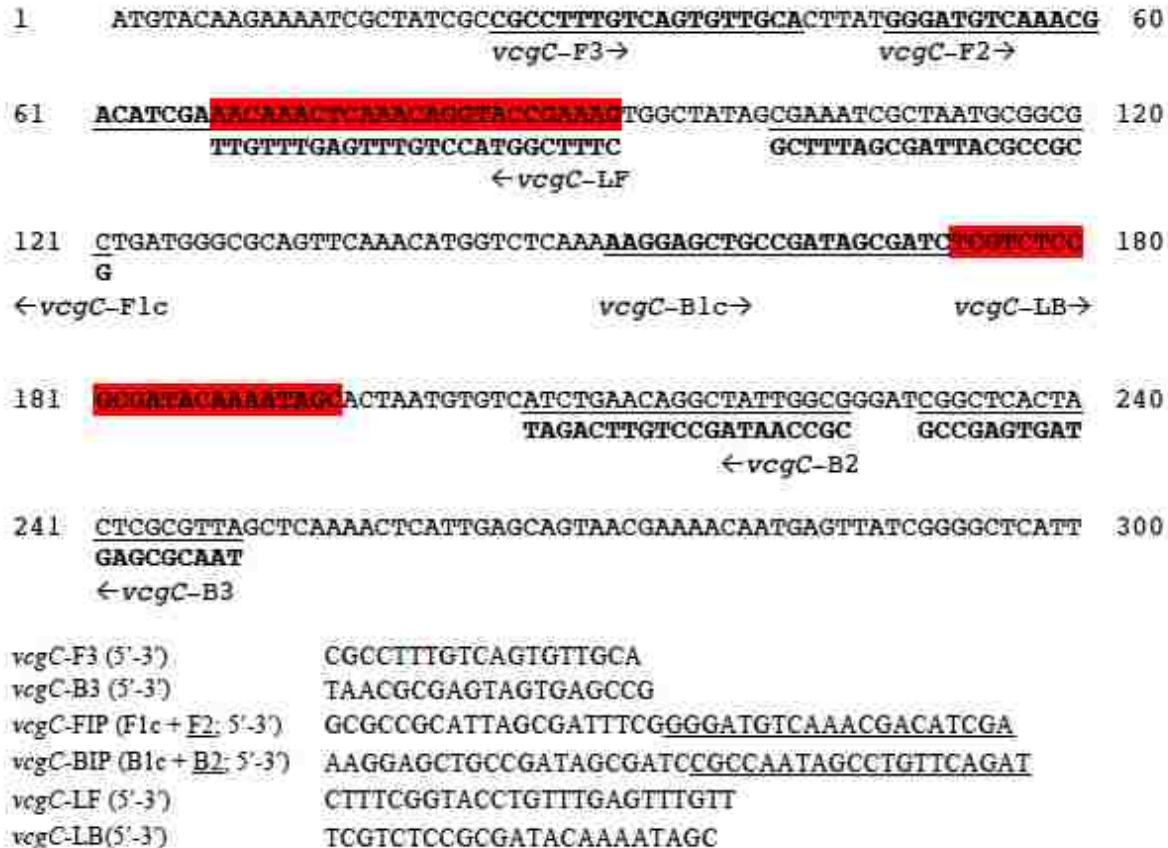


Figure 5 - 7 Partial nucleotide sequence of the *vcgC* type of *Vibrio vulnificus* (accession number AY626575).

F3 and B3 are the forward outer primer and backward outer primer, respectively. FIP and BIP are the forward inner primer and the backward inner primer, respectively. LF and LB are the Loop F and Loop B primer, respectively.

For 16S rRNA, we designed a total of 13 pairs of primers and tested their specificity, which indicated none of those primers could differentiate virulent-type *V. vulnificus* from non-virulent-type strains. The only difference is the virulent-type strains would amplify faster than non-virulent-type ones (~10 min earlier); however, within one hour, all *V. vulnificus* strains could be detected in the turbidimeter regardless of virulent-type and non-virulent-type strains. Figure 5-8 showed the amplification graph by one set of primer in the turbidimeter using *V. vulnificus* ATCC 33815 (16S rRNA B +, black line, $T_t = 19.8$ min) and *V. vulnificus* WR1 (16S rRNA B -, green line, $T_t = 26.2$ min), suggesting non-specific amplification. Therefore, after testing 13 pairs of primers, we withdrew 16S rRNA B type as a target for the remaining sections of this study.

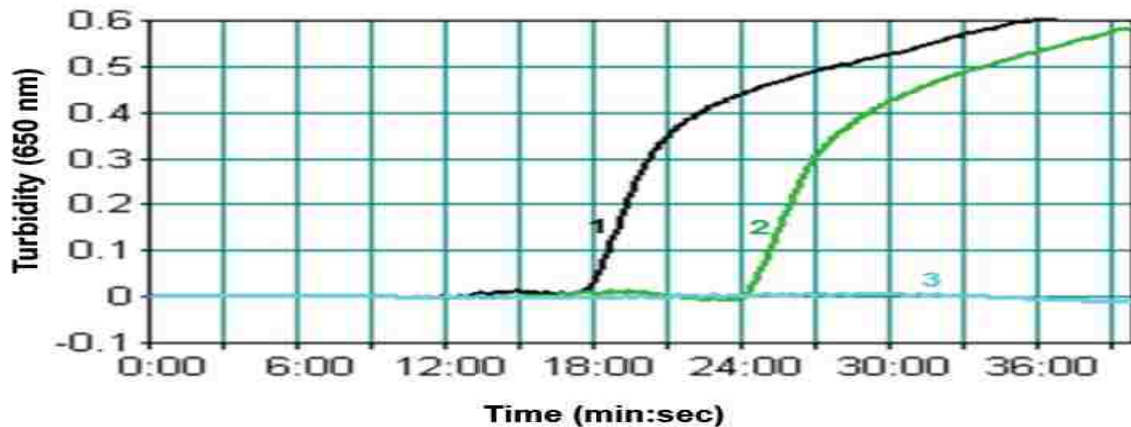


Figure 5 - 8 16S rRNA-LAMP using *Vibrio vulnificus* ATCC 33815 and WR1. Sample 1 is *V. vulnificus* ATCC 33815. Sample 2 is *V. vulnificus* WR1. Sample 3 is water.

Real-time LAMP Optimization. The optimized LAMP assay reagent mix and reaction condition for *vcgC* were: 6 mM MgCl₂, 0 M betaine, 1.2 mM dNTP, 10 U *Bst* DNA polymerase, 2.0 μM each inner primer, 0.05 μM each outer primer, and 1.0 μM each loop primer at 65°C for 40 min. Figure 5-9 showed the amplification graph in the turbidimeter using *V. vulnificus* ATCC 33815 under optimized condition (red line, $T_t = 18.27$ min) compared to the prototype condition

(black line, $Tt = 26.33$ min). Besides decreasing Tt values, the optimized LAMP conditions also achieved higher signal intensity.

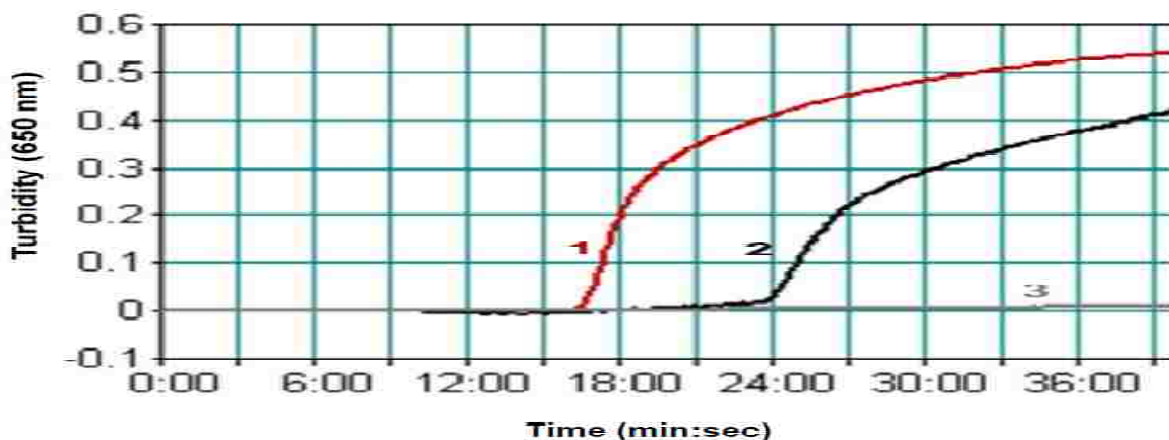


Figure 5 - 9 Comparison between optimized LAMP and prototype LAMP using *Vibrio vulnificus* ATCC 33815.

Sample 1 is under optimized condition. Sample 2 is under prototype condition. Sample 3 is water.

Specificity of the Real-time LAMP Assay. Among 125 bacterial strains (Table 5-4) used to determine LAMP specificity, no false positive or false negative results were observed. The Tt values for the 33 *vcgC*-type *V. vulnificus* ranged from 16.1 to 22.3 min with an average of 18.17 ± 1.45 min. For the other 92 strains, no Tt value was obtained, indicating negative results for real-time LAMP.

For PCR results, 33 *vcgC*-type *V. vulnificus* could amplify using both F3/B3 and P1/P3 primers while no PCR amplification was observed for the other 92 bacterial strains, indicating good specificity (data not shown).

Sensitivity and Quantitative Capability of Real-time LAMP Assay. Figure 5-10 presents sensitivity of the *vcgC* real-time LAMP assay in turbidimeter and two sets of PCR amplification when testing 10-fold serial dilutions of *V. vulnificus* ATCC 33815 DNA templates. A representative turbidity graph and a standard curve are shown in Figure 5-10A and 5-10B,

respectively. For templates ranging from 5.4×10^4 to 5.4 CFU/reaction, based on five repeats, the average T_t values fell between 17.5 and 31.0 min. In two out of five repeats, amplification of the 0.5 CFU template occurred. Therefore, the limit of detection for the real-time LAMP assay was 0.5-5.4 CFU/reaction. Based on the standard curve (Figure 5-10B); the real-time LAMP assay had an r^2 value of 0.97 and an E value of 105%.

When testing the same set of *V. vulnificus* ATCC 33815 templates by PCR using F3/B3 and P1/P3, F3/B3 PCR detected 5.4×10^2 CFU/reaction (Figure 5-10C) while P1/P3 PCR detected 5.4×10^3 CFU/reaction (Figure 5-10D), which were up to 1,000-fold less sensitive than real-time LAMP assay.

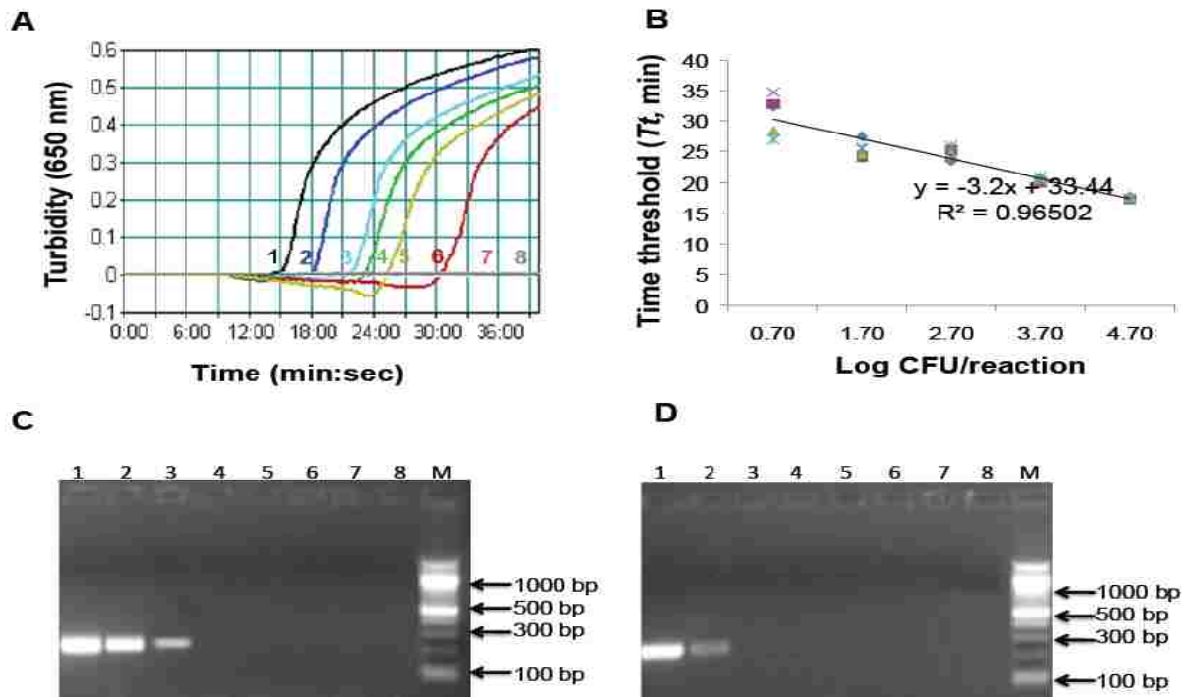


Figure 5 - 10 Sensitivity of *vcgC* real-time LAMP and PCR when testing serial-diluted *Vibrio vulnificus* ATCC 33815 DNA templates. (A) A representative real-time LAMP turbidity graph; (B) A standard curve of real-time LAMP generated based on five independent repeats. (C) PCR using F3/B3 primer (226 bp). (D) PCR using P1/P3 primer (278 bp). Samples (1-7) correspond to 10-fold serial dilutions of *V. vulnificus* ATCC 33815 cells ranging from 5.4×10^4 to 0.05 CFU/reaction; sample 8 is water. M is molecular DNA marker.

Real-time LAMP in Spiked Oysters. A representative turbidity graph and a standard curve of *vcgC* LAMP in spiked oyster are shown in Figure 5-11A and 5-11B, in comparison with PCR using F3/B3 PCR (Figure 5-11C) and P1/P3 PCR (Figure 5-11D). In three independent spiking experiments, the average T_t values ranged from 17.47 to 25.7 min. The limit of detection by real-time LAMP assay was 4.52 CFU/reaction (i.e., 2.5×10^3 CFU/g) of *V. vulnificus* ATCC 33815 in spiked oyster samples without enrichment. However, for PCR assays using F3/B3 primer, the limit of detection in spiked oysters was 2.5×10^6 CFU/g; and PCR using P1/P3 could detect 2.5×10^7 CFU/g, 1,000-fold less sensitive than that of real-time LAMP assay. Standard curves generated for the quantitative detection of *V. vulnificus* cells in spiked oyster samples had an r^2 value of 0.99.

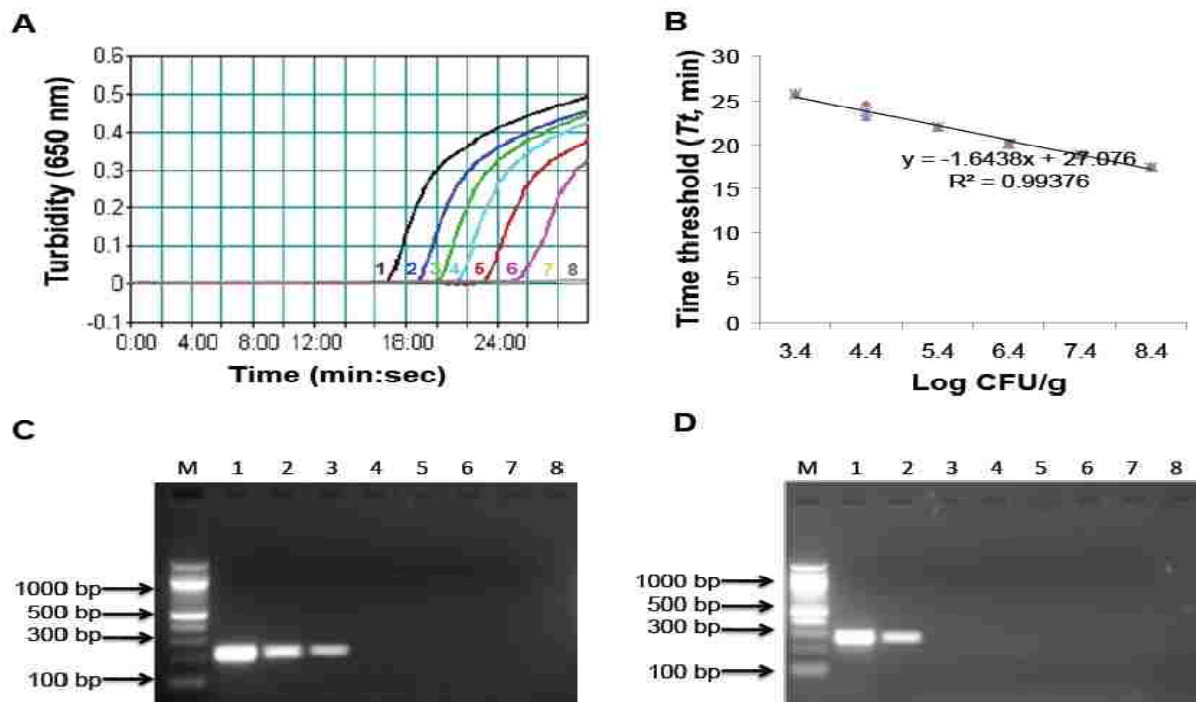


Figure 5 - 11 Quantitative detection of *vcgC* real-time LAMP and PCR using *Vibrio vulnificus* ATCC 33815 in spiked oysters.

(A) A representative real-time LAMP turbidity graph; (B) A standard curve of real-time LAMP generated based on three independent repeats. (C) PCR using F3/B3 primer (226 bp). (D) PCR using P1/P3 primer (278 bp). Samples (1-7) correspond to 10-fold spiked oyster sample ranging from 2.5×10^8 to 250 CFU/g, sample 8 is water. M is molecular DNA marker.

Discussion

We previously designed a LAMP assay targeting the *V. vulnificus* *vvhA* gene, which is a species-specific gene (Han & Ge, 2008), followed by developing a real-time LAMP assay to quantify total *V. vulnificus* (Chapter 4, Part II). Besides our studies, two other LAMP assays for *V. vulnificus* detection were reported, one targeting the *vvhA* gene (Ren *et al.*, 2009) and the other the *toxR* gene (Nemoto *et al.*, 2008), both species-specific genes. To our knowledge, this is the first report examining the quantitative capability of real-time LAMP for the detection of virulent-type *V. vulnificus* in oysters by targeting virulence-type biomarkers.

We chose *vcgC* and 16S rRNA B type as target genes based on our previous study (Han *et al.*, 2009). The mutual exclusivity of *vcgC* and *vcgE* sequences variants in *V. vulnificus* made *vcgC* a good candidate for virulent-type *V. vulnificus* characterization/detection, which was confirmed by several studies (Han *et al.*, 2009; Rosche *et al.*, 2005; Warner & Oliver, 2008). Practically, if a strain shows positive for *vcgC* by using *vcgC* LAMP, it would show negative for *vcgE*. When applying the *vcgC* LAMP in microbial ecology or epidemiological studies, it is acceptable to run the *vcgC* LAMP only, no need to test *vcgE*.

For the other target, 16S rRNA B, we failed to generate satisfactory results with LAMP design. Among a total of 13 pairs of primers designed, none could effectively differentiate virulent-type *V. vulnificus* from non-virulent strains. Alignment between 16S rRNA A (non-virulent-type, GenBank X76333) and B type (virulent-type, GenBank X76334) showed a difference of 17 bases out of 1,535 bases, with most of the polymorphism centered near helix 10 of the secondary structure for bacterial 16S rRNA (Van de Peer *et al.*, 1996). The alignment between 16S rRNA A and B type identified three variable regions, positions 177 to 186 (contain 5 bases), 437 to 483 (contain 8 bases), 999 to 1016 (contain 4 bases), which could be used as

targets to design primers for differentiation of B type from A type. Our LAMP primer design, although tried on different positions of the three variable regions in our 13 pairs of primer sets, could not succeed in sufficient differentiation of 16S rRNA B type from A type. In the previously studies, the 16S rRNA was used in a real-time format which applied primer and probe to obtain specific amplification (Vickery *et al.*, 2007).

Using the optimized conditions of *vcgC* LAMP, the amplification time to a positive result was greatly accelerated compared to the prototype, as shown in Figure 5-9, which requires less than 20 min to be positive in a turbidimeter. Surprisingly, 0 M betaine was found to be most effective during assay optimization, which suggested that in a LAMP assay, betaine should be deleted to make the assay more efficient and cost-effective. Also, using the optimized conditions, the *T_t* values for the 33 *vcgC*-type *V. vulnificus* in the specificity test had an average of 18.17 min, while the prototype condition has an average amplification time 27.17 min (data not shown). Currently, most reported LAMP studies did not conduct the optimization; instead using the amplification kit originally developed by the Eiken company (Tokyo, Japan). Our study, however, emphasized the importance of optimization studies for each specific primer set designed.

Among a total of 33 *vcgC*-type and 50 *vcgE*-type *V. vulnificus*, as well as 30 other *Vibrio* and 12 non-*Vibrio* strains tested, the real-time LAMP assay achieved 100% inclusivity and 100% exclusivity, indicating high specificity. Similarly, the two sets of PCR, F3/B3 and P1/P3, were highly specific for virulent-type, non-virulent-type *V. vulnificus* and other strains.

In addition to high specificity, the *vcgC* real-time LAMP assay was able to detect 0.5-5 CFU/reaction of *V. vulnificus*, in contrast to 500-5 × 10³ CFU/reaction by the two PCR assays. We previously reported a *vvhA*-based LAMP sensitivity of 20 CFU/reaction and a PCR

sensitivity of 202 CFU/reaction (Han & Ge, 2008) and further a real-time LAMP that could detect 1-10 CFU/reaction and a PCR comparison of 100 CFU/reaction (chapter 5, part II), which are comparable to findings of this study. A *toxR*-based LAMP assay for *V. vulnificus* reported a minimum detection level of 1 CFU per test (Nemoto *et al.*, 2008), whereas another *vvhA*-based LAMP assay found it to be 10-fold more sensitive than conventional PCR, although the exact cell number was not reported (Ren *et al.*, 2009). Additionally, increased sensitivity (at least 10-fold) of LAMP compared to PCR was reported in studies on the detection of other *Vibrio* spp. (Fall *et al.*, 2008; Yamazaki *et al.*, 2008a; Yamazaki *et al.*, 2008b; Yamazaki *et al.*, 2010) or other foodborne pathogens (Hara-Kudo *et al.*, 2005; Hara-Kudo *et al.*, 2007; Yano *et al.*, 2007).

The strong linear correlation ($r^2 = 0.97-0.99$) between the number of *V. vulnificus* cells in the LAMP reaction and the associated *Tt* values over a dynamic range of template concentrations (10^4 to 10^0 CFU/reaction in pure culture and 10^5 to 10^0 CFU/reaction in spiked oyster sample) illustrates the quantitative capability of the real-time LAMP assays when detecting virulent-type *V. vulnificus* in both pure culture and spiked oysters. Very few reports have examined the quantitative ability of LAMP, most on viruses (Mekata *et al.*, 2009; Parida *et al.*, 2006). Recently, a real-time LAMP assay was developed to target *toxR* in *V. parahaemolyticus*, and the linear correlation (r^2) between the number of *V. parahaemolyticus* cells in the LAMP reaction and the associated *Tt* values was 0.94 (Chen & Ge, 2010).

In spiked oyster samples, a detection limit of 4.5 *V. vulnificus* CFU/reaction was found for real-time LAMP assay, which translates to 2.5×10^3 CFU/g of oyster sample. In contrast, the detection limit of two PCR assays was $2.5 \times 10^6 / 2.5 \times 10^7$ CFU/g, indicating real-time LAMP was much less prone to inhibitor effects in oyster samples compared to PCR. Nonetheless, no extensive sample preparation other than homogenization and two simple centrifugation steps

were required. This significantly reduced the total assay time. Combined with less than 1 h for the real-time LAMP assay, the complete LAMP detection system was markedly faster than either PCR or conventional methods. On the other side, LAMP can be combined with overnight enrichment or pre-enrichment for 6 h to achieve higher sensitivity, although in that situation, the quantitative capability of real-time LAMP would not be possible.

Conclusion

The real-time LAMP assays targeting *vcgC* developed in this study was a highly specific, sensitive, rapid, and quantitative method for the detection of virulent-type *V. vulnificus* in oysters. The detection limit of the real-time LAMP assay was 0.5-5 CFU in pure cultures, 1,000-fold more sensitive than conventional PCR. In spiked oyster samples, the real-time LAMP assay was able to detect 2.5×10^3 CFU/g of *V. vulnificus* without enrichment. Standard curves generated in both pure culture and spiked oyster samples showed good linear relationship between virulent-type *V. vulnificus* cell counts and the turbidity signals. This assay may facilitate regulatory and oyster industry personnel to better control potential *V. vulnificus* risks associated with oyster consumption. Future testing with natural oyster samples is desired to further evaluate the LAMP efficacy in a setting more close to application.

References

Chatzidaki-Livanis, M., Hubbard, M. A., Gordon, K., Harwood, V. J. & Wright, A. C. (2006a). Genetic distinctions among clinical and environmental strains of *Vibrio vulnificus*. *Appl Environ Microbiol* **72**, 6136-6141.

Chatzidaki-Livanis, M., Jones, M. K. & Wright, A. C. (2006b). Genetic variation in the *Vibrio vulnificus* group 1 capsular polysaccharide operon. *J Bacteriol* **188**, 1987-1998.

Chen, S. & Ge, B. (2010). Development of a *toxR*-based loop-mediated isothermal amplification assay for detecting *Vibrio parahaemolyticus*. *BMC Microbiol* **10**, 41.

Cholera and Other *Vibrio* Illness Surveillance (2008). Summary of human *Vibrio* isolates reported to CDC 2007. Available at

<http://www.cdc.gov/national-surveillance/PDFs/CSTE-Vibrio2007.pdf>. Accessed on March 1, 2010.

Fall, J., Chakraborty, G., Kono, T., Maeda, M., Itami, T. & Sakai, M. (2008). Establishment of loop-mediated isothermal amplification method (LAMP) for the detection of *Vibrio nigripulchritudo* in shrimp. *FEMS Microbiol Lett* **288**, 171-177.

Gordon, K. V., Vickery, M. C., DePaola, A., Staley, C. & Harwood, V. J. (2008). Real-time PCR assays for quantification and differentiation of *Vibrio vulnificus* strains in oysters and water. *Appl Environ Microbiol* **74**, 1704-1709.

Goto, M., Hayashidani, H., Takatori, K. & Hara-Kudo, Y. (2007). Rapid detection of enterotoxigenic *Staphylococcus aureus* harbouring genes for four classical enterotoxins, SEA, SEB, SEC and SED, by loop-mediated isothermal amplification assay. *Lett Appl Microbiol* **45**, 100-107.

Han, F., Walker, R. D., Janes, M. E., Prinyawiwatkul, W. & Ge, B. (2007). Antimicrobial susceptibilities of *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates from Louisiana Gulf and retail raw oysters. *Appl Environ Microbiol* **73**, 7096-7098.

Han, F. & Ge, B. (2008). Evaluation of a loop-mediated isothermal amplification assay for detecting *Vibrio vulnificus* in raw oysters. *Foodborne Pathog Dis* **5**, 311-320.

Han, F., Pu, S., Hou, A. & Ge, B. (2009). Characterization of Clinical and Environmental Types of *Vibrio vulnificus* Isolates from Louisiana Oysters. *Foodborne Pathog Dis* **6**, 1251-1258.

Hara-Kudo, Y., Yoshino, M., Kojima, T. & Ikedo, M. (2005). Loop-mediated isothermal amplification for the rapid detection of *Salmonella*. *FEMS microbiology letters* **253**, 155-161.

Hara-Kudo, Y., Nemoto, J., Ohtsuka, K., Segawa, Y., Takatori, K., Kojima, T. & Ikedo, M. (2007). Sensitive and rapid detection of Vero toxin-producing *Escherichia coli* using loop-mediated isothermal amplification. *Journal of medical microbiology* **56**, 398-406.

Jackson, J. K., Murphree, R. L. & Tamplin, M. L. (1997). Evidence that mortality from *Vibrio vulnificus* infection results from single strains among heterogeneous populations in shellfish. *J Clin Microbiol* **35**, 2098-2101.

Jones, M. K. & Oliver, J. D. (2009). *Vibrio vulnificus*: disease and pathogenesis. *Infect Immun* **77**, 1723-1733.

Mekata, T., Sudhakaran, R., Kono, T., Supamattaya, K., Linh, N. T., Sakai, M. & Itami, T. (2009). Real-time quantitative loop-mediated isothermal amplification as a simple method for detecting white spot syndrome virus. *Lett Appl Microbiol* **48**, 25-32.

- Mori, Y., Nagamine, K., Tomita, N. & Notomi, T. (2001).** Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochemical and biophysical research communications* **289**, 150-154.
- Mori, Y., Kitao, M., Tomita, N. & Notomi, T. (2004).** Real-time turbidimetry of LAMP reaction for quantifying template DNA. *Journal of biochemical and biophysical methods* **59**, 145-157.
- Nemoto, J., Kojima, T., Kusaba, K., Nagasawa, Z., Oishi, H. & Nakashima, M. (2008).** Rapid detection of *Vibrio vulnificus* using a loop-mediated isothermal amplification method. *Kansenshogaku Zasshi* **82**, 407-413.
- Nilsson, W. B., Paranjpye, R. N., DePaola, A. & Strom, M. S. (2003).** Sequence polymorphism of the 16S rRNA gene of *Vibrio vulnificus* is a possible indicator of strain virulence. *J Clin Microbiol* **41**, 442-446.
- Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. (2000).** Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* **28**, E63.
- Oliver, J. D. (2006).** *Vibrio vulnificus*, p. 349-366. Edited by F. L. Thompson, B. Austin, and J. Swings (ed.), *The biology of vibrios*. Washitonton, DC: ASM press.
- Parida, M. M., Santhosh, S. R., Dash, P. K., Tripathi, N. K., Saxena, P., Ambuj, S., Sahni, A. K., Lakshmana Rao, P. V. & Morita, K. (2006).** Development and evaluation of reverse transcription-loop-mediated isothermal amplification assay for rapid and real-time detection of Japanese encephalitis virus. *J Clin Microbiol* **44**, 4172-4178.
- Ren, C. H., Hu, C. Q., Luo, P. & Wang, Q. B. (2009).** Sensitive and rapid identification of *Vibrio vulnificus* by loop-mediated isothermal amplification. *Microbiol Res* **164**, 514-521.
- Rippey, S. R. (1994).** Infectious diseases associated with molluscan shellfish consumption. *Clin Microbiol Rev* **7**, 419-425.
- Rosche, T. M., Yano, Y. & Oliver, J. D. (2005).** A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical or environmental isolation. *Microbiol Immunol* **49**, 381-389.
- Strom, M. S. & Paranjpye, R. N. (2000).** Epidemiology and pathogenesis of *Vibrio vulnificus*. *Microbes and infection / Institut Pasteur* **2**, 177-188.
- Van de Peer, Y., Nicolai, S., De Rijk, P. & De Wachter, R. (1996).** Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Res* **24**, 86-91.
- Vickery, M. C., Nilsson, W. B., Strom, M. S., Nordstrom, J. L. & DePaola, A. (2007).** A real-time PCR assay for the rapid determination of 16S rRNA genotype in *Vibrio vulnificus*. *J Microbiol Methods* **68**, 376-384.

Warner, E. & Oliver, J. D. (2008). Population structures of two genotypes of *Vibrio vulnificus* in oysters (*Crassostrea virginica*) and seawater. *Appl Environ Microbiol* **74**, 80-85.

Warner, J. M. & Oliver, J. D. (1999). Randomly amplified polymorphic DNA analysis of clinical and environmental isolates of *Vibrio vulnificus* and other *vibrio* species. *Appl Environ Microbiol* **65**, 1141-1144.

Yamazaki, W., Ishibashi, M., Kawahara, R. & Inoue, K. (2008a). Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of *Vibrio parahaemolyticus*. *BMC Microbiol* **8**, 163.

Yamazaki, W., Seto, K., Taguchi, M., Ishibashi, M. & Inoue, K. (2008b). Sensitive and rapid detection of cholera toxin-producing *Vibrio cholerae* using a loop-mediated isothermal amplification. *BMC Microbiol* **8**, 94.

Yamazaki, W., Taguchi, M., Ishibashi, M., Kitazato, M., Nukina, M., Misawa, N. & Inoue, K. (2008c). Development and evaluation of a loop-mediated isothermal amplification assay for rapid and simple detection of *Campylobacter jejuni* and *Campylobacter coli*. *Journal of medical microbiology* **57**, 444-451.

Yamazaki, W., Kumeda, Y., Misawa, N., Nakaguchi, Y. & Nishibuchi, M. (2010). Development of a Loop-Mediated Isothermal Amplification Assay for Sensitive and Rapid Detection of the *tdh* and *trh* Genes of *Vibrio parahaemolyticus* and Related *Vibrio* Species. *Appl Environ Microbiol* **76**, 820-828.

Yano, A., Ishimaru, R. & Hujikata, R. (2007). Rapid and sensitive detection of heat-labile I and heat-stable I enterotoxin genes of enterotoxigenic *Escherichia coli* by Loop-Mediated Isothermal Amplification. *Journal of microbiological methods* **68**, 414-420.

Yoda, T., Suzuki, Y., Yamazaki, K., Sakon, N., Kanki, M., Aoyama, I. & Tsukamoto, T. (2007). Evaluation and application of reverse transcription loop-mediated isothermal amplification for detection of noroviruses. *Journal of medical virology* **79**, 326-334.

CHAPTER 6: CONCLUSIONS

The incidence of infections of *Vibrio parahaemolyticus* and *V. vulnificus* due to the consumption of oysters has shown a sustained increase since 2001, indicating further measures are needed to prevent human *Vibrio* illness. Given the lack of information on the prevalence, antimicrobial susceptibility profiles, and genotypic pattern of those two vibrios in Louisiana oysters, as well as the lack of sensitive and cost-effective methods that could be applied in the field sites, we conducted this research project.

We isolated a total of 622 *Vibrio* isolates, consisting of 252 *V. parahaemolyticus* and 370 *V. vulnificus*, from a total of 94 Louisiana oyster samples collected quarterly over a 15-month period from the Gulf Coast and obtained weekly from four retail outlets for 7 months. Overall, the prevalence of one or both species fell between 58.3% and 100%. The antimicrobial susceptibility testing of a randomly selected subset of 319 isolates indicated that the only resistance detected was for ampicillin, with 136 (42.6%) of the isolates showing either intermediate or resistant phenotype, all being *V. parahaemolyticus*. This study represents the first report on both prevalence and antimicrobial susceptibility of *V. parahaemolyticus* and *V. vulnificus* from Louisiana Gulf and retail oysters since 1998.

Following the isolation, we characterized 349 *V. vulnificus* oyster isolates by the presence/absence of a *viuB*-associated fragment and genotypes of three biomarkers: *vcg*, 16S rRNA, and CPS. Genotyping data indicated that environmental-type *V. vulnificus* strains accounted for the majority of oyster isolates. Additionally, the presence of the *viuB* fragment (41%) was significantly associated with clinical genotypes of *V. vulnificus*. An interesting seasonal pattern was observed, with clinical-type *V. vulnificus* isolates more frequently associated with warmer months. This is the first study that a combination of these four biomarkers was used to independently evaluate their usefulness in predicting clinically important

V. vulnificus isolates from oysters. This study was followed up with the development of multiplex PCR assays and demonstrated the detection and characterization of general and virulent-type *V. vulnificus* in a single reaction.

Finally, we adopted a novel DNA amplification technique, LAMP to detect assays for total or virulent-type *V. vulnificus*. No false positive or false negative results were observed. The real-time LAMP assay, in both fluorescence- and turbidity-based real-time platforms could detect approximately 1-10 CFU per reaction of total *V. vulnificus* for pure culture, and 6.4×10^4 CFU/g for spiked oyster without enrichment. When real-time LAMP was applied for the quantitative detection of virulent-type *V. vulnificus* by targeting the *vcgC* gene, the detection limit of the real-time LAMP assay was 0.5-5 CFU in pure culture, and 2.5×10^3 CFU/g of *V. vulnificus* in spiked oyster without enrichment, up to 1,000-fold more sensitive than conventional PCR. Standard curves generated in both pure culture and spiked oyster sample testing showed good linear relationship between total or virulent-type *V. vulnificus* cell counts and the turbidity signals.

This dissertation research provided comprehensive information on the genotypes, population dynamics, and antimicrobial resistance profiles of the two important vibrios. The development of LAMP assays provided invaluable tools for the regulatory agencies and seafood industry to facilitate better control of *Vibrio* in seafood, thereby reducing the incidence of foodborne illnesses and deaths resulting from the consumption of raw oysters due to the presence of these important *Vibrio* spp.

The future study would include the continued surveillance of *Vibrio* in oysters to ensure seafood safety. Further evaluation of these assays using natural oysters will move these assays a step closer to field applications.

APPENDIX: COPYRIGHT PERMISSION LETTERS

Rightslink Printable License

Page 1 of 4

AMERICAN SOCIETY FOR MICROBIOLOGY LICENSE TERMS AND CONDITIONS

Mar 22, 2010

This is a License Agreement between Feifei Han ("You") and American Society for Microbiology ("American Society for Microbiology") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by American Society for Microbiology, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2394330608193
License date	Mar 22, 2010
Licensed content publisher	American Society for Microbiology
Licensed content publication	Applied and Environmental Microbiology
Licensed content title	Antimicrobial Susceptibilities of <i>Vibrio parahaemolyticus</i> and <i>Vibrio vulnificus</i> Isolates from Louisiana Gulf and Retail Raw Oysters
Licensed content author	Feifei Han, Beilei Ge
Licensed content date	Nov 1, 2007
Volume	73
Issue	21
Start page	7096
End page	7098
Type of Use	Dissertation/Thesis
Format	Electronic
Portion	Full article
Order reference number	
Title of your thesis / dissertation	Antimicrobial Susceptibility, Genotypic Characterization, and Molecular Detection of <i>Vibrio parahaemolyticus</i> and <i>Vibrio vulnificus</i> from Louisiana Oysters
Expected completion date	Aug 2010
Estimated size(pages)	150
Total	0.00 USD

Terms and Conditions

Publisher Terms and Conditions

The publisher for this copyrighted material is the American Society for Microbiology (ASM). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at <http://myaccount.copyright.com>).

1. ASM hereby grants to you a non-exclusive license to use this material. Licenses are for one-

<https://s100.copyright.com/App/PrintableLicenseFrame.jsp?publisherID=100&licenseID=...> 3/22/2010

payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10754976.

Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

**Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006**

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2394330608193 and noting the reason for cancellation.

Questions? customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

March 30, 2010

Feifei Han
Graduate Assistant
111 Food Science Building
Louisiana State University
Baton Rouge, LA 70803

Dear Feifei Han:

It is WITH PLEASURE that PERMISSION IS GRANTED for you to reprint the 2 articles, of which you are the first author: **Evaluation of a loop-mediated isothermal amplification assay for detecting *Vibrio vulnificus* in raw oysters**, Han, F. and B.Ge. 2008, *FOODBORNE PATHOGENS AND DISEASE*, 5:311-20, and **Characterization of clinical and environmental types of *Vibrio vulnificus* isolates from Louisiana oysters**, HAN, F. Pu, A. Hou, and B.Ge. 2009- *FOODBORNE PATHOGENS AND DISEASE*, 6:1251-8. The request for the aforementioned, is for your dissertation.

May I take the liberty of wishing you luck with your dissertation and continued success with all future endeavors.

Sincerely,


Esther Bicovery

**JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS**

Jun 17, 2010

This is a License Agreement between Feifei Han ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2451690015012
License date	Jun 17, 2010
Licensed content publisher	John Wiley and Sons
Licensed content publication	Letters in Applied Microbiology
Licensed content title	Multiplex PCR Assays for Simultaneous Detection and Characterization of <i>Vibrio vulnificus</i> Strains
Licensed content author	Han Feifei, Ge Beilei
Licensed content date	Jun 10, 2010
Start page	0
End page	0
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Electronic
Portion	Full article
Will you be translating?	No
Order reference number	
Total	0.00 USD

Terms and Conditions

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or a society for whom a Wiley Company has exclusive publishing rights in relation to a particular journal (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your Rightslink account (these are available at any time at <http://myaccount.copyright.com>).

Terms and Conditions

1. The materials you have requested permission to reproduce (the "Materials") are protected by copyright.
2. You are hereby granted a personal, non-exclusive, non-sublicensable, non-transferable, worldwide, limited license to reproduce the Materials for the purpose specified in the licensing process. This license is for a one-time use only with a maximum distribution equal to the number that you identified in the licensing process. Any form of republication granted by this license must be completed within two years of the date of the grant of this license (although copies prepared before may be distributed thereafter). Any electronic posting of the Materials is limited to one year from the date permission is granted and is on the condition that a link is placed to the journal homepage on Wiley's online journals publication platform at www.interscience.wiley.com. The Materials shall not be used in any other manner or for any other purpose. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher and on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Material. Any third party material is expressly excluded from this permission.
3. With respect to the Materials, all rights are reserved. No part of the Materials may be copied, modified, adapted, translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Materials without the prior permission of the respective copyright owner. You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Materials, or any of the rights granted to you hereunder to any other person.
4. The Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc or one of its related companies (WILEY) or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto.
5. WILEY DOES NOT MAKE ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND WAIVED BY YOU.
6. WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.
7. You shall indemnify, defend and hold harmless WILEY, its directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.
8. IN NO EVENT SHALL WILEY BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT,

NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

9. Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

10. The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

11. This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

12. These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in a writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

13. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.

14. WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

15. This Agreement shall be governed by and construed in accordance with the laws of England and you agree to submit to the exclusive jurisdiction of the English courts.

16. Other Terms and Conditions:

BY CLICKING ON THE "I ACCEPT" BUTTON, YOU ACKNOWLEDGE THAT YOU HAVE READ AND FULLY UNDERSTAND EACH OF THE SECTIONS OF AND PROVISIONS SET FORTH IN THIS AGREEMENT AND THAT YOU ARE IN AGREEMENT WITH AND ARE WILLING TO ACCEPT ALL OF YOUR OBLIGATIONS AS SET FORTH IN THIS AGREEMENT.

V1.2

Gratis licenses (referencing \$0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number

RLNK10801959.

Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

**Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006**

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2451690015012 and noting the reason for cancellation.

Questions? customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

**ELSEVIER LICENSE
TERMS AND CONDITIONS**

Jun 17, 2010

This is a License Agreement between Feifei Han ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard, Langford Lane Kidlington, Oxford, OX5 1GB, UK
Registered Company Number	1982084
Customer name	Feifei Han
Customer address	111 Food Science Building Baton Rouge, LA 70803
License Number	2451370116039
License date	Jun 17, 2010
Licensed content publisher	Elsevier
Licensed content publication	International Journal of Food Microbiology
Licensed content title	Quantitative Detection of <i>Vibrio vulnificus</i> in Raw Oysters by Real-Time Loop- Mediated Isothermal Amplification
Licensed content author	Feifei Han, Beilei Ge
Licensed content date	11 June 2010
Volume number	n/a
Issue number	n/a
Pages	1
Type of Use	Thesis / Dissertation
Portion	Full article
Format	Electronic
You are an author of the Elsevier article	Yes
Are you translating?	No
Order Reference Number	
Expected publication date	

Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
Value added tax 0.0%	0.00 USD
Total	0.00 USD
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at <http://myaccount.copyright.com>).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this

licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance

Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. Translation: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. Website: The following terms and conditions apply to electronic reserve and author websites:

Electronic reserve: If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:

This license was made in connection with a course,

This permission is granted for 1 year only. You may obtain a license for future website posting,

All content posted to the web site must maintain the copyright information line on the bottom of each image,

A hyper-text must be included to the Homepage of the journal from which you are licensing at <http://www.sciencedirect.com/science/journal/xxxxx> or the Elsevier homepage for books at <http://www.elsevier.com> , and

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

17. Author website for journals with the following additional clauses:

All content posted to the web site must maintain the copyright information line on the bottom of each image, and

the permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version.

A hyper-text must be included to the Homepage of the journal from which you are licensing at <http://www.sciencedirect.com/science/journal/xxxxx> , As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

18. Author website for books with the following additional clauses:

Authors are permitted to place a brief summary of their work online only.
A hyper-text must be included to the Elsevier homepage at <http://www.elsevier.com>

All content posted to the web site must maintain the copyright information line on the bottom of each image

You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

19. **Website** (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing at <http://www.sciencedirect.com/science/journal/xxxxx>. or for books to the Elsevier homepage at <http://www.elsevier.com>

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. **Other Conditions**: None

v1.6

Gratis licenses (referencing \$0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10801403.

Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

**Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006**

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2451370116039 and noting the reason for cancellation.

Questions? customer@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

VITA

Feifei Han was born in Qingdao, Shandong Province, People's Republic of China. After completing high school in her hometown, she attended China Agricultural University, Beijing, China, and graduated with a Bachelor of Science in food science and engineering in June 2004. She came to Louisiana State University for her doctoral program in food science in August 2005. Currently, she is a candidate for the degree of Doctor of Philosophy in food science in the College of Agriculture. She will receive her doctoral degree in summer 2010.

During her graduate career, she has been actively involved in multiple research projects and an active member in the Food Science Club. She received first place in the Louisiana Gulf Coast Section IFT 2010 Suppliers' Night Student Poster Competition. She was awarded Gamma Sigma Delta Outstanding Ph.D. Student in 2009, Gamma Sigma Delta Graduate Student Merit Honor Roll in 2008, LSU Food Science Department Barkate Scholarship in 2008, LSU Food Science Department Grodner Scholarship in 2007, and McCleskey Award by South Central Branch of the American Society for Microbiology in 2006.