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Development and Optimization of a Rapid Assay Kit for the Detection of Vibrio Cholerae in Bivalves

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Development and optimization of a rapid assay kit for the detection of *Vibrio cholerae* in bivalves

By

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A rapid assay kit for Vibrio cholerae (Vc) was developed to detect and quantify Vc cells in oyster samples within 24 h. The kit, formulated within a two -phase (liquid and solid) 96-well plate, can detect biomarker expression of Vc when the enrichment broth and incubation temperature are optimized. The kit showed 91 % selectivity and 92 % specificity when tested with 23 inclusive Vc and 106 exclusive non-Vc strains. The kit was further optimized using 47 samples of oysters, clams, and soil. There was no significant difference in most probable number between the kit, conventional PCR and BAX PCR regardless of agar heating method (autoclaved vs. boiled). The kit's limit of detection was below 5 cfu/g. The kit is a reliable method for the detection of V. cholerae in bivalve samples.

DEDICATION

This work is dedicated to my mother Ms. Yolanda Carter, brother Jaelon Carter, and all those who work to illuminate the darkness of mystery with the light of knowledge.

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TABLE OF CONTENTS

DEDICATION ii

ACKNOWLEDGEMENTS iii

LIST OF TABLES vi

LIST OF FIGURES vii

CHAPTER

I. INTRODUCTION 1

II. LITERATURE REVIEW 1

2.1 *Vibrio cholerae* 1
2.2 Pathway of Infection 3
2.3 Biochemical Properties 4
2.4 Isolation and Identification 5

III. MATERIALS AND METHODS 8

3.1 Vibrio strains 8
3.2 Sample preparation 9
3.3 pH measurement in oyster tissues incubated with different V. cholerae enrichment broths 11
3.4 Preparation of the solid phase of the *V. cholerae* kit 11
3.5 Preparation of the liquid phase of the *V. cholerae* kit 12
3.6 Serial dilution of samples for inoculation into V. cholerae assay kit and most probable number method 13
3.7 Most probable number determination by BAX PCR, conventional PCR, and V. cholerae assay kit 14
3.8 Conventional PCR detection of V. cholerae cultured on TCBS 17
3.9 BAX PCR detection of V. cholerae in enriched MPN tubes 19
3.10 Sensitivity analysis the V. cholerae assay kit 20
3.11 Statistical analysis 21

IV. RESULTS AND DISCUSSION 23

4.1 pH change of selected enrichment broths when incubated with oyster tissue....23
4.2 Determination of the selectivity and specificity of the V. cholerae assay kit with inclusive and exclusive V. cholerae strains....24
4.3 Optimal incubation temperature for the Vibrio cholerae kit....25
4.4 Determination of the sensitivity of the V. cholerae assay kit....26
4.5 Effect of autoclaving the assay kit solid phase....27
4.6 Influence of an antimicrobial compound in the assay kit solid phase at 42 °C....29

V. SUMMARY AND CONCLUSIONS....40

REFERENCES....41

LIST OF TABLES

3.1 Fresh oyster □, canned oyster ■, soil ■, and clam ■ samples used for optimization of the assay kit between 12/2013 and 9/2014 10

3.2 Conversion of 3-tube MPN numbers to cfu/unit of sample (Source: FDA) 16

4.1 Specificity and selectivityof the Vc assay kit. 34

LIST OF FIGURES

3.1 Vc assay kit post incubation for 24 h at 42 °C.....15

4.1 pH change of enrichment broths during 24 h incubation at 36 °C.....31

4.2 Determination of kit specificity with inclusive Vc strains and exclusive non-*cholerae Vibrios* after incubation at 36 °C for 24 h 32

4.3 Determination of kit specificity with inclusive Vc strains and exclusive non-*cholerae Vibrios* after incubation at 42 °C for 24 h 33

4.4 MPN cfu/g of Vc cells in oyster tissues response to incubation temperature 35

4.5 Determination of sensitivity of the Vc kit using two serially diluted V. cholerae isolates 36

4.6 Most probable number (MPN) cfu/g of Vc cells in oyster tissues after incubation for 24 h at 42 °C. 37

4.7 Most probable number (MPN) cfu/g of Vc cells in oyster tissues after incubation for 24 h at 42 °C. 38

4.8 Effect of an antimicrobial on MPN cfu/g of Vc 39

CHAPTER I

INTRODUCTION

Vibrio cholerae is a marine and estuarine pathogen and the causative agent of cholera. The species can survive and proliferate in environmental conditions that include pH from 6.0 – 9.6, temperatures from 10 to 43 °C, and salinities from 0 to 6 % (Davis et al., 1981; Pollitzer, 1955; Sakazaki and Donovan, 1984). *V. cholerae* typically enters the system through the gastrointestinal tract and cells colonize the small intestine after their exposure to bile triggers biofilm formation (Hung et al., 2006). Common vectors for *V. cholerae* include contaminated water sources, fish, and shellfish (Wong et al., 2012). Toxigenic serogroups *V. cholerae* O1 and O139 are typically more virulent due to the production of cholera toxin, but non-O1 and O139 serogroups can also cause disease. *Vibrio cholerae* typically blooms in the wild when water temperatures exceed 17 °C leading to 1.2–4.3 million infections and 28,000 – 142,000 deaths a year worldwide with most infections concentrated in third world regions with poor sanitation (Ali et al., 2012; Pfeffer et al., 2003). In the United States, *V. cholerae* is monitored by the Food and Drug Administration with a set allowable level of 0 detected cells/25 g of sample (FDA, 2014). Despite this monitoring, 737 infections and 37 deaths in the United States occurred from 1996 to 2010 (Newton et al., 2012). Infected patients are stricken with nausea, vomiting, fever, and diarrhea (WHO Scientific Working Group, 1980).

Undercooked or raw oysters are the primary route of *V. cholerae* infection in the United States. Oyster producers typically test for the foodborne pathogen *V. cholera*. However, detection according to the FDA method requires multiple days of enrichment and plating on selective media to confirm results (Kaysner and DePaola, 2004). Alternative methods, such as conventional polymerase chain reaction (PCR) and BAX PCR can be laborious and cost-restrictive, especially to seafood producers. Biochemical test based confirmation alternatives, such as API20NE test strips (bioMérieux, Durham, NC, USA), require multiple days of enrichment and incubation also. This leads to a situation where seafood producers may release product to market before laboratory results have been obtained. Thus, existing testing methods for *V. cholera* are not practical for industrial settings

The objective of this study was to validate a novel rapid assay kit formulated to be specific to *V. cholerae* using various samples of fresh and canned oysters, clams, and soil.

CHAPTER II

LITERATURE REVIEW

2.1 *Vibrio cholerae*

Vibrio cholerae is a 1.0 μm rod shaped, gram-negative, motile, facultative anaerobe that is commonly found in marine and estuarine waters around the world and the causative agent of the disease cholera (Maheshwari et al., 2011). A number of environmental factors affect the enrichment of V. cholerae. This microbe exhibits growth within a pH range of 6.0 – 9.6 with optimal growth occurring between 7.6 and 8.6 (Sakazaki and Donovan, 1984). V. cholerae can grow in salinities from 0 % to 6 % NaCl (Davis et al., 1981). The optimal multiplication temperature for this species is 37 °C with growth at temperatures between 10 °C and 43 °C (Pollitzer, 1955). There are more than 100 species within the Vibrio genus, however, only V. cholerae, V. parahaemolyticus, and V. vulnificus have been reported to be pathogenic to humans (Thompson et al., 2004). V. cholerae species are divided into at least 139 serogroups (O1 - O139) that is based upon somatic antigen profile (Kaper et al., 1995). Cholera toxin (CT) profiles further divide serogroup O1 into Classical and El Tor biotypes (Kim et al., 2014). Serogroup O1 was at one time the only group known to produce CT until isolates that are now known as V. cholerae O139 was the causative agent during a 1992 outbreak in India and Bangladesh (Shimada et al., 1993). Non-O1/O139 serogroups have also been

shown to cause both gastrointestinal and extraintestinal infection (Aguinaga et al., 2009; Khan et al., 2013) in certain cases.

V. cholerae can typically be found in temperate and tropical waters throughout the year with peak numbers occurring during warmer months, most notably once water temperatures are greater than 17 °C (Pfeffer et al., 2003). Ingestion of or exposure to contaminated water sources as well as consumption of raw or underdone known vectors such as fish and shellfish like oysters are typical methods of infection (Senderovich et al., 2010; Wong et al., 2012). Epidemiological studies reported that as few as 103 V. cholerae cells can cause symptomatic infection as characterized by diarrhea, nausea, vomiting, abdominal cramps, bloody or rice water stool, or fever for up to 3 days (WHO Scientific Working Group, 1980).

The ubiquitous nature of this organism combined with poor sanitation led to eight cholera pandemics that began with an 1817 O1 outbreak that originated in the Indian Ganges delta and continues in modern times with the 0139 outbreak from 1992 (Faruque et al., 1998). Poor sanitation was not the only factor that contributed to each of these pandemics causing tens to hundreds of thousands of casualties over short time frames, as lack of medical knowledge during the time period played a significant role until Robert Koch isolated V. cholerae during the fifth pandemic and developed the postulates that bear his name (Koch, 1884). As medical knowledge grew and sanitation practices proliferated, V. cholerae became less of a health threat in developed nations. Developing and undeveloped nations still face significant threat, especially to children 5 and under who are 50 % of reported cases and deaths, from outbreaks resulting in 1.2–4.3 million infections and 28,000 – 142,000 deaths a year with heavy concentration in endemic

regions such as southern Asia and Africa (Ali et al., 2012). This is a stark comparison to countries such as the United States where clean water and food sources are easily secured. Analysis of V. cholerae infections in the US reported to the COVIS and FoodNet outbreak monitoring networks from 1996 to 2010 found only 40 toxigenic serogroup O1 infections and 697 non-O1/O139 infections with a total of 37 deaths (Newton et al., 2012). Modern efforts to spread hygienic awareness are closing this gap and have reduced rates of infection in endemic regions such as Haiti (Jutla et al., 2013).

2.2 Pathway of Infection

The first major barrier for V. cholerae cells upon ingestion is the highly acidic host gastric juice. The cadA gene encodes for an inducible lysine decarboxylase that allows the cells to better tolerate the low pH environment (Merrel and Camilli, 1999). This adaptation does not make cells immune to stomach acid, however, as studies comparing infection rates between hypochlorhydric patients and normochlorhydric patients found hypochlorhydric patients were more likely to display symptomatic infection (Nalin et al., 1978). Once live cells reach the Duodenum, exposure to bile salts triggers expression of the vpsR genes which leads to biofilm formation (Hung et al., 2006). Resistance of V. cholerae to bile salts is a key factor in this mechanism (Goulart et al., 2010). This biofilm provides a stable matrix for the attachment and growth of cells to the intestinal epithelium. Studies have shown that the virulence of V. cholerae is determined by the presence of a gene cluster known as the toxR pathogenicity island that codes for accessory colonization factor (ACF) as well as toxin-coregulated pilus (TCP) by comparing intestinal colonization rates of normal cells against mutants (Attridge et al., 1996). The toxR cluster also contains the genes for the production of CT. TCP and ACF

work in tandem to anchor V. cholerae cells to intestinal epithelial cells (Kovach et al., 1996). CT and TCP expression reaches optimal levels at pH 6.5 indicating V. cholerae may have evolved these genes as a specific response to intestinal conditions in potential host species (Taylor et al., 1987). Production of CT begins as a response to pH, and anaerobiosis triggers a regulatory cascade during which activated AphB promotes the synthesis of TcpP. This progression leads to ToxT expression which then upregulates ctxAB which are the genes that directly encode for CT (Cobaxin et al., 2014). Outer membrane vesicles (OMV) collect CT on the cell surface. OMVs bind to GM1 gangliosides on the surface of the epithelial cells, which triggers endocytocis (Chatterjee and Chaudhuri, 2011). Once inside the cell, the toxin enters the Endoplasmic Reticulum (ER) via the Golgi. Inside the ER, the active A subunit of CT is enzymatically cleaved off. This liberated subunit then seeks out the adenyl cyclase regulating α subunit of the G protein. Interaction with Gα increases levels of the secondary messenger cAMP. The loss of the ability to regulate cAMP levels in the epithelial cells leads to apoptosis, rupturing, and dislodging into the intestinal lumen. This is the cause the trademark watery diarrhea of cholera (Fujinaga, 2006). The production of inflammatory cytokines is also exhibited by epithelial cells as a response to V. cholerae infection leading to physical discomfort and nausea (Bandyopadhaya et al., 2007).

2.3 Biochemical Properties

As previously indicated, *V. cholerae* exhibits growth in the presence of NaCl at a concentration of 0 % to 6 %. In addition, *V. cholerae* shows production of acid by fermentation of galactose, maltose, D-mannitol, D-mannose, sucrose, and trehalose. The species has also been shown to utilize Jordan tartrate, acetate, corn oil lipase,

deoxyribonuclease, Christensen citrate, Simmons cirate. Indole, methyl red (in the presence of NaCl), Voges-Proskauer, lysine decarboxylase, ornithine decarboxylase, D-glucose acid, oxidase, ONPG, and reduction of nitrite to nitrate have been identified as phenotypic properties as well. Growth in 0 % NaCl and utilization of sucrose are key biochemical properties for differentiation of *V. cholerae* serogroups from other *Vibrios* due to the low utilization rate of sucrose and the requirement of NaCl by non-*cholerae* spp. *(*Davis et al., 1981).

The antibiotic profile for toxigenic *V. cholerae* is characterized by resistance to ampicillin, nalidixic acid, tetracycline, erythromycin, kanamycin, streptomycin, polymyxin B, trimethoprimsulfamethoxazole, and colistin with intermediate resistance to chloramphenicol and azithromycin. Non-toxigenic serogroups only display resistance to erythromycin, streptomycin, polymyxin B, and colistin with intermediate resistance to ampicillin (Yu et al., 2012). Previous research indicated antibiotic resistance of *V. cholerae* fluctuates rapidly due to a combination of the inability to carry plasmids that confer resistance and natural environments free of antibiotic selective pressure. When utilized in concert, these phenotypic descriptors form a fingerprint of the bacterium that can be used for identification purposes (Klontz et al., 2014).

2.4 Isolation and Identification

According to the Food and Drug Administration (FDA) of the Unites States, V. cholerae transmission occurs directly through the fecal-oral or indirectly through contact with contaminated food or water sources. Due to the virulence of the microbe, toxigenic and non-toxigenic V. cholerae must be at non-detectable levels in a 25 g sample of ready-to-eat fishery products (FDA, 2014). Accurate detection of cells essential in tests for V.

cholerae. The FDA methodology for identification begins with 25 g of sample homogenized in 225 mL of alkaline peptone water (APW, pH 8.6). When analyzing raw oysters, a second flask with 25 g of sample and 2475 mL is prepared. Most probable number (MPN) enumeration may also be performed by serially diluting the sample into MPN-tubes. The inoculum is incubated for 8 to 24 h at 35 ± 2 °C. Fresh oyster samples are incubated for 18 to 21 h at 42 ± 0.2 °C as Depaola et al (1988) found this temperature improved the recovery of V. cholerae cells from fresh oyster tissues by 25 %. During incubation, Thiosulfate-Citrate-Bile-Sucrose (TCBS), modified Cellobiose Polymyxin Colistin (mCPC), or Cellobiose Colistin (CC) agar plates are prepared and dried. After enrichment, inoculum is removed from the incubator and samples are streaked for isolation onto the agar plates using a 3 mm sterile loop. Plates are then incubated for 18 to 24 h at 35 ± 2 °C for TCBS and 24 h at 39.5 ± 0.5 °C for mCPC/CC. Suspicious colonies (defined as 2 mm yellow smooth flattened colonies on TCBS or small smooth green to purple colonies on mCPC/CC) are streaked on non-selective 1 % Tryptone/1 % NaCl (T1N1), 1 % Tryptone/3 % NaCl (T1N3), or 2 % NaCl Trypticase Soy (2 % NaCl TSA) agars and incubated overnight at 35 ± 2 °C. Motility test stabs are also prepared and incubated overnight at 35 ± 2 °C (Kaysner and DePaola, 2004).

Confirmation of suspected colonies is attained via conventional PCR, BAX real-time PCR, API20NE biochemical test strips, or phenotype studies. The potential DNA primer targets for the identification of V. cholerae using conventional PCR are genes that code for the outer membrane protein (ompW, 588 bp), regulate expression of genes for virulence (toxR, 776), or produce cholera toxin (ctx, 777 bp or ctxA, 301 bp) (Nandi et al., 2000). An interlaboratory study which compared and contrasted a variety of Vibrio

species identification methods found PCR correctly identifies the target organism 96 % of the time and biochemical tests correctly identify the target 86 % of the time. They concluded biochemical tests were better utilized in concert with PCR (Croci et al, 2006).

CHAPTER III

MATERIALS AND METHODS

3.1 Vibrio strains

Stock cultures of 23 *Vibrio cholerae* (Vc), 48 *Vibrio vulnificus* (Vv), 48 *Vibrio parahaemolyticus* (VP) and 19 other *Vibrio* species (non-Vc) were kindly provided by the Food and Drug Administration Gulf Coast research laboratory located in Dauphin Island, Alabama. Following the methods of Janda et al (1988), all obtained strains were enriched to stationary growth phase at 37 °C for 24h in 150 µL of 1 % NaCl 1 % alkaline peptone water (APW, pH 8.6) held within a sterile 96 microwell plate (Costar, Fisher Scientific, PA, USA). One hundred µL of each strain was then transferred into a 2 mL round bottom internally threaded cryogenic vial (Corning Fisher Scientific, PA, USA) that held a solution of 600 µL of APW to 300 µL glycerol before being moved to -70 °C frozen storage. To make the working stock solutions, frozen samples were retrieved from a -70 °C frozen storage and allowed to thaw on the laboratory bench to room temperature prior to use. Purity of the strains was confirmed via polymerase chain reaction (PCR) (detailed in section 3.8). For experiments that required larger volumes, the stocks were stored in 18 mL capped test tubes containing 10 mL of 1% NaCl buffered alkaline peptone water (BAPW, pH 8.6).

3.2 Sample preparation

Selected Vc kit formulations were validated using 40 market obtained samples consisting of 35 fresh oyster samples, 2 canned oyster samples, and 2 fresh clam samples as well as 1 environmental soil sample. The samples were primarily sourced from markets in the Southeastern United States with emphasis on states directly bordering the Gulf of Mexico (Table 3.1). After purchase, all samples were held in refrigerated storage at 5 °C either under ice or inside a portable cooler for up to 7 days until further processed for inoculation into the Vc assay kits.

Fresh oyster and clam samples were hand shucked via a shucking knife that had been previously sterilized by spray with 70 % EtOH prior to exposure to a gas flame. The flesh of the shucked oysters or clams and any liquid contained within the shell was deposited into a labeled 1.538 L sterile round bottom Whirl-Pak stomacher bag (Nasco, WI, USA). This process was repeated until 6 – 12 oysters or clams had been shucked. The stomacher bag was sealed and the sample was homogenized by moderate stomaching for 60 s. Samples were immediately inoculated into the assay kit using methods detailed in section 3.6.

Canned samples were unsealed and oyster tissues were transferred into labeled sterile 1.538 L Whirl-Pak bag using a spoon that had been sterilized by 70 % EtOH and a gas flame. Soil samples were transferred into sterile 1.538 L Whirl-Pak using a spoon that was sterilized by 70 % EtOH and a gas flame. Liquid associated with the beach sand was also poured into the stomacher bag. All samples were stomached either by hand or in a stomacher for one minute. Stomached samples were stored at 5° C when not in use.

Table 3.1 Fresh oyster □, canned oyster ■, soil ■, and clam ■ samples used for optimization of the assay kit between 12/2013 and 9/2014

Sample	Purchase Location	Source	Type	Process
1	Louisiana		fresh	
2	Louisiana		fresh	pressure treated
3	Florida		fresh	
4	Texas		fresh	
5	Slidell, LA		plastic cup	pasteurized
6	Louisiana		fresh	
7	Louisiana		soil	
8	Louisiana		fresh	
9	Kenner, LA		fresh	
10	Pass Christian, MS	Louisiana	fresh	
11	Cherrystone Creek, VA		fresh	
12	Biloxi, MS		fresh	
13	Multiple		fresh	Sample 9 & 12, 24h at 25°C
14	Starkville, MS	Louisiana	fresh	
15	Starkville, MS		fresh	Sample 14, 24h at 25°C
16	Starkville, MS	Maryland	fresh	
17	Starkville, MS	Maryland	fresh	Sample 16, 24h at 25°C
18	Texas		fresh	
19	Texas		fresh	Sample 17, 3.5h at 25°C
20	Starkville, MS	Maryland	fresh	
21	Starkville, MS	Maryland	fresh	Sample 20, 2.5h at 25°C
22	Starkville, MS	Maryland	fresh	0~12h at 25°C
23	Memphis, TN	Louisiana	fresh	0~12h at 25°C
24	St. Bernard's Parish, LA		fresh	0~3h at 25°C, 1 week at 6°C
25	Hopedale, LA		fresh	
26	Port Sulfur, LA		fresh	
27	Bayou LeBatre, AL		plastic cup	pasteurized
28	N/A		fresh	
29	Louisiana		fresh	
30	Alabama	Louisiana	fresh	
31	Memphis, TN	Louisiana	fresh	
32	Apalachicola, FL		fresh	
33	Apalachicola, FL		fresh	
34	Apalachicola, FL		fresh	
35	Mississippi Gulf Coast	Louisiana	fresh	
36	Louisiana		fresh	
37	Virginia		fresh	
38	Mississippi Gulf Coast		fresh	
39	Apalachicola, FL		fresh	
40	Alabama		fresh	

3.3 pH measurement in oyster tissues incubated with different V. cholerae enrichment broths

Twenty-five g of sample was obtained by harvesting the flesh of fresh or canned oysters, as described in section 3.2, and placed into labeled 1.538 L sterile round bottom Whirl-Pak stomacher bags (Nasco, WI, USA) with either alkaline peptone water (225 mL APW, pH 8.6 ± 0.2), buffered alkaline peptone water with 1% NaCl sodium and potassium phosphate (225 mL BAPW, pH 8.6 ± 0.2), or 225 mL alkaline peptone water with 2x the concentration of buffering agents (2xBAPW, pH 8.6 ± 0.2) to obtain a 1:10 dilution ratio. Three labeled Whirl-Pak bags with no samples were additionally filled with 225 mL of either APW, BAPW, or 2xBAPW as control cases. The pH of each sample bag and control bag was measured and recorded as the 0 hour pH value. All bags were placed into a 36 °C incubator for a period of 4 hours after which the pH was recorded once again. This value became the 4 hour pH time point. The bags were returned to the 36 °C incubator for an additional 4 hours, and the pH was measured again after 8 hours. This process was repeated for 12, 16, 20, and 24 hour time points.

3.4 Preparation of the solid phase of the *V. cholerae* kit

The Vc assay kits were designed with two phases (solid and liquid). Due to the confidential nature of the product, the full formulation of the solid phase cannot be disclosed at this time. However, the process by which the assay kit was done is discussed, below. The chemical components for the solid phase were individually weighed into an Erlenmeyer flask. A volume of distilled water determined by the final volume of the solid phase was added to the flask using a graduated cylinder. The solution was mixed using a magnetic stir bar and a hot plate. Once all dry components were dissolved, the pH of the

solution was adjusted to 8.6 ± 0.2 with 10 N NaOH. Granulated agar was added to the solution based upon the volume of the solid phase that was produced. After boiling for 1 min, the solution with agar was autoclaved at 121 °C for 15 min at 15 psi.

The autoclaved solution was removed from the autoclave and returned to a plate stirrer, and an additional antimicrobial component was added to the solution. Furthermore, a pH sensitive chromogenic dye was added to the solution to allow for identification of positive reaction by their distinct yellow coloration as compared to the blue to green coloration from a negative reaction. The solid phase solution was allowed to cool to a temperature of 50 ± 5 °C. Once cooled, 800 µL was pipetted into each well of a sterile square V-bottom 2 mL prolypropylene 96 well plate (Costar, Fisher Scientific, PA, USA) and allowed to solidify by cooling to 25 °C.

3.5 Preparation of the liquid phase of the *V. cholerae* kit

The liquid phase of the Vc assay kit was formulated with 10 g/L peptone (Fisher Scientific, PA, USA), 10 g/L granulated NaCl (Fisher Scientific, PA, USA) and distilled deionized H2O that had been adjusted to a pH of 8.6 ± 2 by the addition of 10N NaOH (alkaline peptone water, APW). Di-sodium phosphate and potassium phosphate was optionally added to create a buffer system (BAPW, 2xBAPW). The pH of the liquid phase was adjusted to either 7.0 or 8.6 using 1 or 10 N NaOH and then autoclaved at 121 °C / 15 psi for 15 min. Broths were removed from the autoclave and allowed to cool to 25 °C. After the autoclaved liquid phase had cooled, 500 µL was pipetted into each well of a 96 well plate containing the solid phase that was prepared as described in section 3.4 of this paper, using a multichannel pipette. For the purposes of this paper, the liquid phase is assumed to be 2xBAPW unless otherwise noted.

3.6 Serial dilution of samples for inoculation into V. cholerae assay kit and most probable number method

Inoculation of the assay kit was performed via serial dilutions after the addition of the liquid phase. Dilutions ranged from 10^{0} to 10^{-5} in most probable number (MPN) tubes and 10^{-1} to 10^{-6} in the assay kit. Eighteen test tubes were filled with 10 mL of autoclaved APW or 2xBAPW. Additionally, 5 test tubes were filled with phosphate buffered saline (9 mL) (PBS, 1 L H_2O, 7.650 g NaCl, 0.724 g Na_2-Phosphate, 0.210 g K-Phosphate, pH 7.4) and then autoclaved. One PBS tube was placed with MPN triplets 2, 3, 4, 5, and 6 in the rack. Test tube triplets (APW or 2xBAPW) were labeled 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} for MPN triplets 1, 2, 3, 4, 5, and 6 respectively. The PBS tubes were labeled 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} to correspond to MPN triplets 2, 3, 4, 5, and 6.

One g homogenized sample was transferred into each of the 3 test tubes of APW or 2xBAPW labeled 10^{0}. Tubes were vortexed and returned to test tube rack. One gram of stomached sample was pipetted into the 10^{-1} labeled PBS tube. The PBS tube was vortexed to homogenize the solution. One hundred mg of sample was pipetted directly into each well of row A in a Vc assay kit prepared as detailed in section 3.5. One g of dilute sample was transferred from the 10^{-1} PBS tube to each of the tubes in MPN triplet 2 and the 10^{-2} PBS tube. Additionally, 100 mg was pipetted from the 10^{-1} PBS tube into each well of row B in the assay kit. This process was repeated until MPN triplets 3, 4, 5, and 6 and assay kit rows C, D, E, and F were inoculated. The inoculated assay kit was sealed using a 10.2 by 15.2 cm sheet of parafilm and labeled such that samples were divided into 4 groups of MPN triplets, then photographed before being placed into an incubator at 36 to 42 °C for 18 to 24 h. Capped and labeled MPN tubes were then placed in an incubator at a temperature of 36 °C for approximately 24 hours.

Post-incubation photographs of the assay kit were taken. Wells that exhibited a change in color from blue to yellow were considered positive for the presence of *V. cholerae* for all methods in this study. Blue and dark green wells were considered negative. Light green wells were considered suspected positives. Results of these wells were confirmed using conventional PCR identification. Wells broken by gas fermentation or other means were considered inconclusive.

3.7 Most probable number determination by BAX PCR, conventional PCR, and V. cholerae assay kit

Thiosulfate-Citrate-Bile salts-Sucrose agar (TCBS, Difco, Fisher Scientific, PA, USA) plates were prepared as directed then labeled to correspond to MPN tubes and any positive wells of a post-incubation assay kit. Approximately 10 µL of culture was streaked onto the TCBS plates from each MPN tubes and positive well using a sterile disposable loop. Confirmation via the BAX PCR method was performed during this time as described in section 3.9. TCBS plates were incubated at 36 °C for 24 hours after which photographs were taken. Suspected positive colonies were then confirmed via the conventional PCR method (section 3.8).

After completion of both confirmation methods, the results from the assay kit, conventional PCR, and BAX PCR were converted to a 3 tube MPN number. To begin, the 12 columns of wells in the assay kit were divided into 4 groups of 3 and each group was examined for light green and yellow (positive) wells or dark green and blue (negative) wells (Figure 3.1). The point at which *V. cholerae* had been diluted to extinction was considered the extinction triplet. Dilution to extinction was defined as three negative results in the same dilution triplet. If dilution to extinction was not

obtained, the final dilution triplet became the extinction triplet. Tubes or wells in the extinction triplet confirmed positive for *V. cholerae* were tallied and recorded as a 1, 2 or 3 dependent upon the number of positive results. This became the last digit of a 3 digit number. Subsequently, originating from the extinction triplet, the number of tubes positive for *V. cholerae* in the next highest dilution MPN triplet was recorded as the second digit. Tubes positive for *V. cholerae* growth in the third highest dilution MPN triplet were recorded as the first digit. This resulted in a three digit code where the first digit represents the number of positive results from the lowest dilution ratio triplet, the second represents the number of positive results from the intermediate dilution ratio triplet, and the last represents the number of positive results from the highest dilution ratio triplet. Table 3.2 was referenced for a match to this three digit MPN code and converted to a MPN value (V_{MPN}). The equation $CFU/g = V_{MPN} \times 10^{D}$, where CFU/g was the approximated number of *V. cholerae* colony forming units per gram of oyster tissue in the original sample and D was the dilution factor for the intermediate dilution triplet.

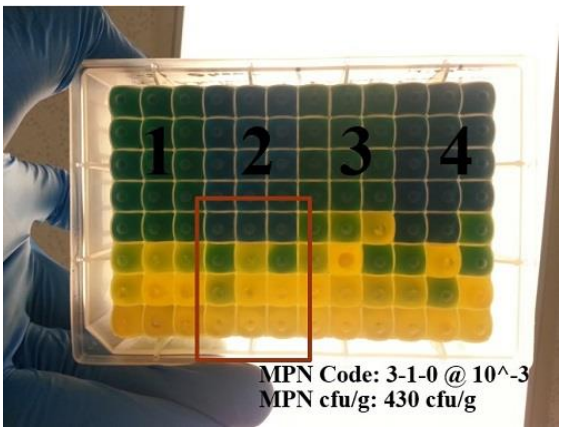


Figure 3.1 Vc assay kit post incubation for 24 h at 42 °C

Note: Row A (10^{-1} dilution) is the bottom row and row H (10^{-8}) is the top row in photographs of Vc kits utilized in this paper.

Table 3.2 Conversion of 3-tube MPN numbers to cfu/unit of sample (Source: FDA)

1st Triplet	2nd Triplet	3rd Triplet	MPN value
0	0	0	< 0.03
0	0	1	0.03
0	1	0	0.03
0	1	1	0.061
0	2	0	0.062
0	3	0	0.094
1	0	0	0.036
1	0	1	0.072
1	0	2	0.11
1	1	0	0.074
1	1	1	0.11
1	2	0	0.11
1	2	1	0.15
1	3	0	0.16
2	0	0	0.092
2	0	1	0.14
2	0	2	0.2
2	1	0	0.15
2	1	1	0.2
2	1	2	0.27
2	2	0	0.21
2	2	1	0.28
2	2	2	0.35
2	3	0	0.29
2	3	1	0.36
3	0	0	0.23
3	0	1	0.38
3	0	2	0.64
3	1	0	0.43
3	1	1	0.75
3	1	2	1.2
3	1	3	1.6
3	2	0	0.93
3	2	1	1.5
3	2	2	2.1
3	2	3	2.9
3	3	0	2.4
3	3	1	4.6
3	3	2	11
3	3	3	> 11

3.8 Conventional PCR detection of V. cholerae cultured on TCBS

Utilizing the methodology of Nandi et al (2000), Vc stock cultures and suspected positives on TCBS were confirmed using polymerase chain reaction (PCR) with the genes encoding the outer membrane protein of *V. cholerae* as the primer target. Suspected colonies on TCBS were mixed utilizing a sterile loop into a 2 mL microcentrifuge tube containing 200 µL of autoclaved DI-H_2O. A known *V. cholerae* sample and a blank sample containing only DI-H_2O were processed as positive and negative controls. The PCR tubes were numbered and arranged in a floating rack to be suspended in 100 °C water for 5 min to lyse cellular membranes, thus liberating DNA from the nucleus. Tubes were vortexed, followed by centrifugation at 14,000 RPM for a minimum of 5 min to separate DNA from cellular material. Nuclease free water (7.5 µL) (Promega, WI, USA) was pipetted into 200 µL PCR tubes in the proceeding step. One µL of the forward primer (omp w64F 5'– CACCAAGAAGGTGACTTTATTGTG, Sigma-Aldrich, MO, USA), 1.0 µL of the reverse primer (omp w367R 5'– GGTTTGTCGAATTAGCTTCACC, Sigma-Aldrich, MO, USA), 12.5 µL of Gotaq Green 2x Master Mix (Promega, WI, USA) were also pipetted into each PCR tube. Lastly, 3.0 µL of the supernatant from the centrifuged tubes was transferred into the PCR tube filled with the primers, Master Mix and water. The PCR tubes were numbered corresponding to 2 mL microcentrifuge tubes and placed in a thermocycler. Initial denaturation was at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s, and extension at 72 °C for 30 s. Final extension was performed at 72 °C for 5 min. The thermocycler lid was kept at 105° C during this process. Thermocycled samples were held at 8 °C until the gel was ready to be loaded.

Electrophoresis was performed using a 1.5% agarose gel. The gel was prepared by mixing 60 mL 5x TAE (24.2 g Tris Base Powder, 5.71 mL Acetic Acid, 10 mL 0.5 M Ethylenediaminetetraacetic acid, 1 L DI-H_2O) that was diluted to 0.5x and 0.90 g of electrophoresis grade agarose powder in a 125 mL Erlenmeyer flask. The gel was corked using a paper towel and microwaved on high power to a temperature of 100 °C and held for 5 s. The flask was removed from the microwave and swirled gently to suspend powder agarose before being returned to the microwave and heated to 100 °C once more. This process was repeated until all agarose was thoroughly dissolved, as defined by a lack of refractive crystalline structures in the solution when held up to a light source. The agarose gel was allowed to cool to a temperature of 50 °C before 5 µL of ethidium bromide was added. The gel was poured into a mold with two 13 prong well combs then allowed to cool and solidify.

The solidified gel was slowly placed at an angle into an electrophoresis system filled with 0.5x TAE to prevent air bubbles. Additional 0.5x TAE was added to the electrophoresis system until the liquid level rose above the surface of the gel. At this time, 5 µL of a 0.1 to 1 kbp DNA Ladder was transferred into the first well of each row. 5 µL of each thermocycled PCR product was pipetted into a well. Samples and associated well numbers were recorded for reference. After all samples were loaded into wells, the gel was run for 20 min at 135 volts. After the electrophoresis step, the gel was photographed under 258 nm ultraviolet light. Bands at 588 base pairs were considered a positive result for the presence of *Vibrio cholerae*. PCR products were stored for up to 2 weeks at -20 °C.

3.9 BAX PCR detection of V. cholerae in enriched MPN tubes

BAX PCR was also performed to detect Vc cells cultured in MPN tubes as an additional detection method. Incubated MPN tubes (sec. 3.6) were set aside. Twelve mL of lysis buffer (DuPont Nutrition and Health, DE, USA) was combined with 150 µL of protease (DuPont Nutrition and Health, DE, USA) in a capped container and mixed by hand shaking. Two hundred µL of this solution was pipetted into lysis tubes. Once all tubes were filled with lysis reagent, a 5 µL aliquot was transferred from each enriched MPN tube to a lysis tube. Lysis tubes were capped and heated at 37 ± 2°C for 20 min in a dry block heater. This was followed by 10 min of heating at 95 ± 2°C. After heating, the lysis tubes were placed into a cooling block and cooled for 5 min. BAX PCR tubes (BAX System Real-Time PCR Assay Vibrio cholerae/parahaemolyticus/vulnificus, DuPont Nutrition and Health, DE, USA) were arranged into a separate cooling block and associated round top caps were removed and discarded. Lysis tubes were uncapped and 30 µL of lysate was transferred from the lysis tubes to the BAX tubes using an 8 channel pipette. Lysis tubes were recapped and stored at 5 °C in a labeled rack. The PCR tubes were capped with flat optical real time PCR caps (DuPont Nutrition and Health, DE, USA) then placed into a 7500 Fast Real Time PCR (Applied Biosystems, Fisher Scientific, PA, USA). BAX software was initialized on a laboratory Windows 7 PC. A rack file was created by highlighting tubes to be tested and selecting a species dependent software protocol for PCR. Tube representations were labeled appropriately and the rack file was saved. After saving, PCR was run according to the BAX Vibrio software protocol for approximately 75 min. The results of the process were saved to a flash drive.

Estimated cfu/g of V. cholerae cells in the sample was determined via the MPN method (sec. 3.7).

3.10 Sensitivity analysis the V. cholerae assay kit

To determine the sensitivity of the *V. cholerae* kit, a known *V. cholerae* stock culture was enriched to the stationary growth phase in a capped test tube containing 10 mL of APW for 24 h at 36 °C. The enriched stock culture was diluted 1:10 from 10^{0} to 10^{-7} by serial dilutions. One mL of enriched sample was pipetted to an autoclaved test tube containing 9 mL of PBS which was labeled the 10^{-1} dilution. One mL was then pipetted from the 10^{-1} tube into a second 9 mL PBS tube labeled 10^{-2}. This was repeated until a final dilution of 10^{-7} was obtained. An assay kit was prepared as previously described and each well was filled with 900 µL of APW. Row A in the assay kit was defined as the 10^{-1} and was prepared by pipetting 100 µL of the 10^{0} dilution (enriched stock culture) into each well. Afterwards, row B was defined as the 10^{-2} dilution by pipetting 100 µL from the 10^{--1} dilution PBS tube into each well. Next, 100 µL of the 10^{--2} PBS dilution was added to each well of row C in the assay kit which was defined as the 10^{-3}. This process was repeated until all dilutions were inoculated into a row with assay kit rows D, E, F, G, and H being defined as dilutions 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} respectively. One hundred µL aliquots of each well from assay kit rows F, G, and H (dilutions 10^{-6}, 10^{-7}, and 10^{-8}) were transferred directly onto aerobic plate count Petrifilms (3M, MN, USA) labeled to match the row and column position of the well utilized to for inoculation. Assay kits were sealed with a 10.2 by 15.2 cm sheet of parafilm prior to photographing and placed into an incubator for 24 hours at 42 °C. Petrifilms were incubated for 24 hours at 36 °C.

The assay kit was removed from the incubator and photographs were taken. Wells that had changed in color from blue to yellow were recorded as a positive result while blue wells were recorded as negative. The total number of positive results was tallied for assay kit rows F, G, and H. Petrifilms were retrieved from the incubator and examined for growth. Total number of Petrifilms with growth for each dilution level were tallied and colonies were enumerated by hand counting.

3.11 Statistical analysis

The pH determination study (sec. 4.1) utilized samples 1 to 5 for a total of 5 replicates. The results were reported as means ± standard error. Significance was determined through two tailed t-test analysis contrasting experimental versus control values. The experimental results were also contrasted against each other ($\alpha = 0.05$, Microsoft Excel 2010, Microsoft, WA, USA).

The selectivity and specificity determination study (sec. 4.2) considered individual isolates repeats for tests done at 36 °C, 42 °C, and 42 °C with antibiotics. Positive and negative results were tabulated and then selectivity and specificity were determined Selectivity was defined as the percentage of true positives correctly identified. Specificity was defined as the percentage of true negatives correctly identified.

Optimal incubation temperature determination (sec. 4.3) utilized sample 3, sample 6 twice, and samples 8 to 14 for an n of 10. Differences in mean colony forming unit (cfu)/g ± standard error of V. cholerae obtained from MPN analysis of the assay kit incubated at 36 or 42 °C and a conventional PCR control were determined via two tailed t-test analysis ($\alpha = 0.05$, Microsoft Excel 2010, Microsoft, WA, USA).

Sensitivity determination (sec. 4.4) utilized a two tailed t-test ($\alpha = 0.05$, Microsoft Excel 2010, Microsoft) for analysis of MPN obtained mean ± standard error cfu/g V. cholerae of the assay kit and a conventional PCR control.

Autoclaving (sec 4.5) and the addition of antimicrobial compound A (sec. 4.6) studies both utilized two tailed t-test analysis of MPN obtained mean ± standard error cfu/g V. cholerae between assay kits and controls unless otherwise noted. ($\alpha = 0.05$, Microsoft Excel 2010, Microsoft). Market samples 16 – 27 were used for the autoclaving study with samples 22 and 23 repeated 5 times and sample 24 repeated twice for a n of 21. Antimicrobial A concentration study utilized market samples 28 to 40. Samples 29, 30, 35, and 37 to 40 were analyzed twice. The results for samples 29 and 30 were excluded from statistical analysis due to missing control data.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 pH change of selected enrichment broths when incubated with oyster tissue

This experiment tracked the pH of a mixture of homogenized sample and enrichment broths for different incubation times (t = 0, 4, 8, 12, 16, and 24 h) at 36 °C (Figure 4.1). The pH (t = 24 h) of the controls without oyster tissues was 8.28 ± 0.09 for APW, 8.41 ± 0.05 for BAPW, and 8.37 ± 0.03 for 2xBAPW (n = 5). This was not significantly higher or lower than the initial pH values (t = 0) of 8.41 ± 0.14 for APW, 8.44 ± 0.04 for BAPW, and 8.49 ± 0.02 (n = 5). The pH (t = 24 h) of all oyster-containing experimental broths decreased to 5.20 ± 0.13, 5.33 ± 0.13, and 6.52 ± 0.17 from initial pH (t=0) values of 8.31 ± 0.05, 8.29 ± 0.04, and 8.27 ±0.05 in APW, BAPW, and 2xBAPW respectively (n = 5). Figures 4.1a, 4.1b, and 4.1c show a single experimental/control pair for each of the 3 broths. At t = 4 h the pH of each experimental broth was lower than the associated control ($P < 0.05$). A reduction in pH ($P < 0.05$) was observed at 0 h immediately following addition of oyster samples to 2xBAPW. This result is unexpected and may be an artifact attributed to the addition of the oyster tissues to the broth, however, further investigation is required for a full determination.

The pH of the 2xBAPW experimental sample (t = 24 h) was greater ($P < 0.05$) than APW and BAPW experimental samples (Fig. 4.1d). Experimental APW and BAPW pH values were not different ($P > 0.05$) at 24 h. The experimental pH of BAPW became

lower than that of 2xBAPW ($P < 0.05$) at 12 h. While there was no difference in pH values at 0 and 24 H for APW when compared to BAPW experimental broths, the pH at 8 h was lower for APW ($P < 0.05$). This was hypothesized to be due to buffering action slowing the rate at which the pH of the broth decreased, but having no effect the endpoint pH due to low buffering capacity. This is supported by BAPW reaching the final observed pH between 8 to 12 h of incubation whereas reduction to the final observed pH for APW and 2xBAPW occurred from 4 to 8 h.

The pH of APW and BAPW broths were noted to drop below 6.0 at 12 h. Decrease of pH in oyster tissue can be explained by microbial fermentation of natural sugar (glycogen) in oyster tissue. While not sufficient for *V. cholerae* lethality alone, the low pH of the enrichment broth in concert with naturally occurring antimicrobial compounds in oyster tissue has been shown to inhibit *V.* cholerae growth (DePaola et al, 1998).Prior studies also reported that pH reduced Vc biofilm production by up to 55 % and had an effect on flagellum functionality (Halang et al., 2013; Hostacká, 2010; Sakazaki and Donovan, 1984). As these factors affect the selectivity of the assay kit, 2xBAPW was selected as the liquid phase for the following studies to optimize the enrichment environment.

4.2 Determination of the selectivity and specificity of the V. cholerae assay kit with inclusive and exclusive V. cholerae strains

Twenty-three *V. cholerae,* 48 *V. parahaemolyticus,* 48 *V. vulnificus*, and 10 non *cholera/parahaemolyticus/vulnificus Vibrio spp.* isolates were transferred from working stocks on a 96 microwell plate into wells of the prepared assay kits using a sterilized 48 pin multi-blot inoculator (Fisher Scientific, PA, USA). After 24 h of incubation at 36 or

42 °C, the assay kits were read for changes in color (Fig. 4.2, Fig. 4.3). At 36 °C, 21/23 Vc, 0/48 Vp, 0/48 Vv, and 0/10 non-Vc wells exhibited the desired yellow color development of a positive result in the Vc assay kit. At 42 °C, 21/23 Vc, 1/48 Vp, 0/48 Vv, and 0/10 non-Vc wells showed a positive reaction. Since none of the Vibrio strains with antibiotics showed a positive result, these compounds were eliminated from further formulations.

The results at both 36 and 42 °C showed excellent selection of Vc isolates with little to no cross reaction from non-Vc spp in 24 h of incubation (Table 4.1). When incubated at 36 or 42 °C, the kit was found to be 91 % selective for positive identification of Vc. At 42 °C with antibiotics, Vc selectivity dropped to 0 %. Specificity for *V. cholerae* amongst other Vibrios was 100 % for the assay kit when incubated at 36 °C or 42 °C with antibiotics. At 42 °C incubation, specificity dropped to 98 %. As the assay kit achieved more than 90 % selectivity and specificity for Vc against non-Vc spp, real world market sample analysis was performed.

4.3 Optimal incubation temperature for the Vibrio cholerae kit

Two *V. cholerae* kits were prepared as previously described and inoculated with serial dilutions of the same sample alongside 18 MPN tubes containing 10 mL of APW. The first assay kit was labeled S-N_{36} (S-N = sample number), photographed, and incubated for 24 h at 36 °C. The second assay kit was labeled S-N_{42}, photographed, and incubated for 24 h at 42 °C. After 24 h, the assay kits were removed from incubators and photographed. The assay kits were sectioned into 4 triplets which were the basis for MPN analysis. MPN tubes were processed as previously described and confirmed using conventional PCR (sec 3.8) to be used as a control series for statistical analysis. MPN

cfu/g was 1700 ± 1400 cfu/g for 36 °C, 1.4 ± 0.98 for 42 °C, and 0.69 ± 0.59 for PCR (Fig. 4.4). There was no difference ($P > 0.05$) between the MPN cfu/g of each assay kit and the control. While the results were not significant, false positive reactions due to *Aeromonas hydrophila* growth was observed in multiple assay kits at 36 °C.

Cross reactions from *A. hydrophila* were an issue that effected the accuracy and specificity of the kit at 36 °C. Like *V. cholerae, A. hydrophila* has been shown to grow in low to moderate salinity, reduce NO_3 to NO_2, ferment sucrose, be Voges-Proskauer and indole positive, and exhibit oxidase positive reactions (Janda et al., 1988). The growth range of *A. hydrophila* is 4 to 42 °C with optimal growth at 28 °C (Uddin et al., 1997) and the growth range of Vc is 10 to 42 °C with optimal growth at 37 °C (Pollitzer, 1955). This is corroborated by DePaola and Hwang (1995) who show incubation at 42 °C with alkaline peptone water improves recovery of *V. cholerae*. Thus, a higher incubation temperature should be more selective for Vc. Incubation at 42 °C reflected this hypothesis and showed less contamination with counts that were closer to the true value set by the conventional PCR control (Fig. 4.4).

4.4 Determination of the sensitivity of the V. cholerae assay kit

The Vc assay kits were validated for sensitivity by two-tailed *t*-test of MPN cfu/mL values that were obtained from the assay kit and aerobic plate count Petrifilms (3M, MN, USA). Wells were inoculated by serial dilutions of *V. cholerae* isolates that were selected from stock sources at random from 10^{-1} to 10^{-8} and enriched for 24 h at 36°C. One hundred µL was transferred from for each well of rows F, G, and H (10^{-6}, 10^{-7}, and 10^{-8} dilutions respectively) onto labeled Petrifilms to be used for MPN analysis. After 24 h of incubation at 42°C, the assay kits were examined and photographed, Petrifilms

were examined for growth and colonies were counted. MPN analysis performed using Petrifilms and assay kit rows F, G, and H approximated 8 log *V. cholerae* cfu/mL (8.8 x $10^8 \pm 1.5$ x 10^8 cfu/mL) for the assay kit as well as the Petrifilms (4.2 x $10^8 \pm 2.3$ x 10^8 cfu/mL). These results were not different ($P > 0.05$) therefore there limit of detection for the *V. cholerae* assay kit was considered to be < 5 cfu/mL (Fig. 4.5).

Vogel et al (2014) determined the limit of detection (LoD) of a SystemSure Plus with isolates of *Escherichia coli* (LoD = 5.88 cfu/mL) and *Staphylococcus aureus* (LoD = 4.37 cfu/mL).They enriched the cells to create growth curves and then compared against a growth curve generated by the SystemSure. In this study, counts obtained from Petrifims which contained the same inoculum that was in the wells of the kit were compared. Since FDA regulations required no *V. cholerae* cells be detectable in 25 g of sample for food products (FDA, 2014), this was the benchmark utilized in development of the Vc assay kit.

4.5 Effect of autoclaving the assay kit solid phase

For this study, assay kits for which the autoclaving step at 121 °C, 15 psi for 15 min was omitted were compared against assay kits that had been autoclaved. Additionally, the liquid phase of the kit was 2x phosphate buffered 1 % NaCl peptone water adjusted to a pH of either 7.0 or 8.6 (2xBPW, 2xBAPW). All assay kits were incubated for 24 h at 42 °C. The conventional PCR control series for this study had a MPN *V. cholerae* count of 0 ± 0 cfu/g. As the assay kit results could only equal or exceed this value, one-tailed *t*-tests were used for comparing results to the control series throughout this section.

2xBPW replicate *V. cholerae* MPN approximations were 46 ± 44 cfu/g for assay kits that were not autoclaved and 25 ± 21 cfu/g for assay kits that were autoclaved (Fig. 4.6a). The estimated cfu/g *V. cholerae* for the autoclaved and non-autoclaved kits were not found to be higher than the control or each other ($P > 0.05$, n=21).

The 2xBAPW *V. cholerae* MPN approximations were 22 ± 20 cfu/g in non-autoclaved assay kits and 22 ± 17 cfu/g for the autoclaved assay kits (Fig. 4.7b). The results for the 2xBAPW autoclaved and non-autoclaved kits were also found to not be higher than the control or each other.

The results for 2xBPW and 2xBAPW were combined and analyzed disregarding the broth pH (Fig. 4.8). MPN Vc cfu/g was 34 ± 24 for the non-autoclaved assay kits and 15 ± 8.8 for the autoclaved kits. The non-autoclaved assay kit results was not different from the autoclaved assay kit results or the control ($P > 0.05$), however, significance was observed for autoclaved assay kits (23 ± 13) compared against the control (0 cfu/g, $P < 0.05$).

Research has long shown the effect of autoclaving on the sanitation of laboratory equipment and media (del Campo et al., 2014; Palenik and Curnberlander, 1993; Sheldrake et al., 1995). The liquid phase of the *V. cholerae* assay kit was autoclaved to minimize contamination due to being a non-selective enrichment broth, however the necessity of the autoclave process for the solid phase was not as clear due to the selective nature of the formulation. This study sought to determine if there was a difference in MPN cfu/g Vc due to the autoclaving versus not autoclaving the solid phase of the assay kit. We determined that there was no difference in MPN cfu/g with a liquid phase at pH 7.0 or pH 8.6 ($P >0.05$) (Fig. 4.6, Fig 4.7). This result has high impact on the versatility

of the Vc assay kit since the autoclave process is not necessary which makes the kit a better option for smaller companies that may not have autoclaves or for companies or researchers who perform field work with portable incubators.

4.6 Influence of an antimicrobial compound in the assay kit solid phase at 42 °C

MPN cfu/g of Vc cells were obtained from the sample inoculated Vc assay kits that were formulated with 0, 0.10, 0.25, 0.50, 0.75, or 1.0 % of an antimicrobial compound in the solid phase and incubated for 24 h at 42 °C. MPN tubes were filled with either 10 mL of 1 % APW or 1 % 2xBAPW that were inoculated with homogenized oyster tissues as previously described and incubated for 24 h at 36 °C. These MPN tubes were utilized to generate each of conventional PCR, BAX_{APW} PCR, and BAX_{2xBAPW} PCR MPN cfu/g controls.

MPN *V. cholerae* results were 1500 ± 1400 cfu/g, 2600 ± 2500 cfu/g, and 0.14 ± 0.14 for BAX_{APW} PCR, BAX_{2xBAPW} PCR, and conventional PCR controls respectively. Approximate cfu/g for varied concentrations of the antimicrobial compound were 3100 ± 1700 at 0 %, 7500 ± 4100 at 0.10 %, 4600 ± 2800 at 0.25%, 4900 ± 2700 at 0.50 %, 7700 ± 5500 at 0.75 %, and 3800 ± 2000 at 1.0 % (Fig. 4.9). The large error in these data is due to variation in the MPN counts among samples as some samples were found to have very large cfu/g of *V. cholerae* while others contained little to none.

Results indicate that there was no difference between BAX_{APW} and BAX_{2xBAPW}, BAX_{APW} and conventional PCR, or BAX_{2xBAPW} and conventional PCR ($P > 0.05$, $n = 17$) MPN *V. cholerae* cfu/g. The results of assay kits with 0, 0.10, 0.25, 0.50, 0.75, and 1.0 % concentrations of the antimicrobial compound exhibited no effect ($P > 0.05$) on the MPN cfu/g Vc when compared to any control. Prior studies reported *V. cholerae* resistance to

the surfactant properties of the antimicrobial compound via a putative phosphoporin that was encoded by vca1008 was key to pathogenesis (Goulart et al., 2010). This study sought to utilize resistance to the antimicrobial compound as a method of selection for *V. cholerae.* However, the results showed that the antimicrobial compound had neither a beneficial, nor harmful effect on MPN cfu/g (Fig 4.8). As a result, the antimicrobial compound was removed from the formulation as a cost cutting measure. Also, the removal of the compound crucially simplified production of assay kits as the post-autoclave addition could lead to contamination of the solid phase and impact results.

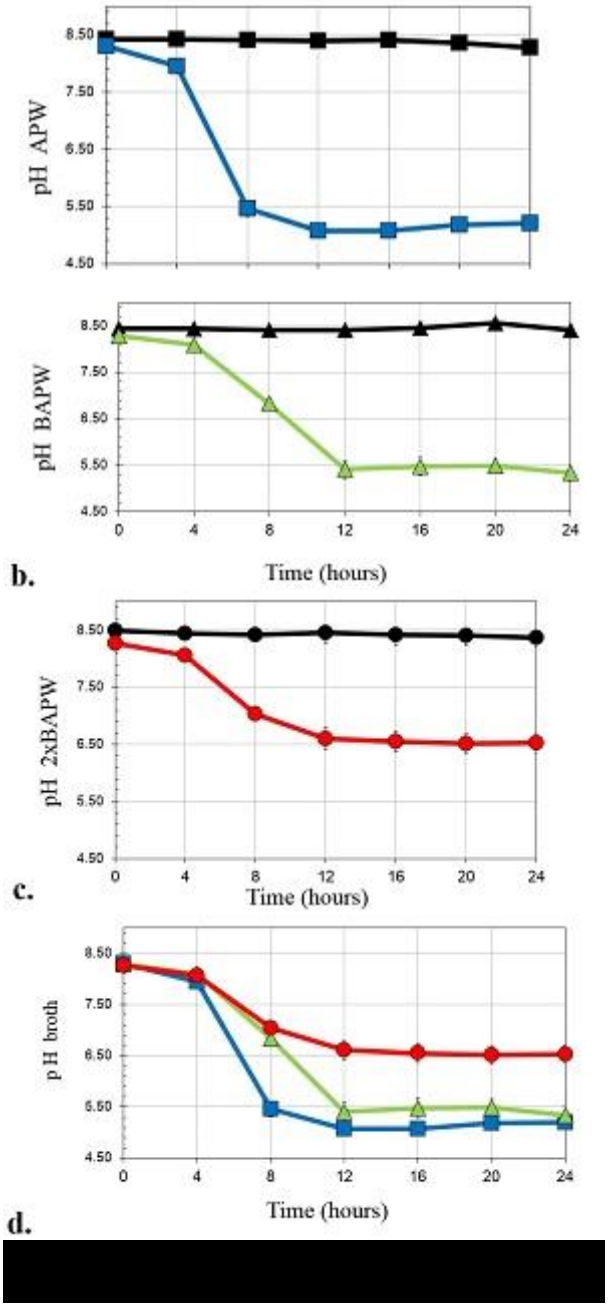


Figure 4.1 pH change of enrichment broths during 24 h incubation at 36 °C

Note: Samples were tested with or without oyster tissues for a.) Alkaline peptone water APW (■control, ■experimental), b.) Buffered APW, BAPW (▲control, ▲experimental), and c.) 2x BAPW (●control, ●experimental). Figure 3.2d) shows experimental results for all 3 enrichment broths on a single graph.

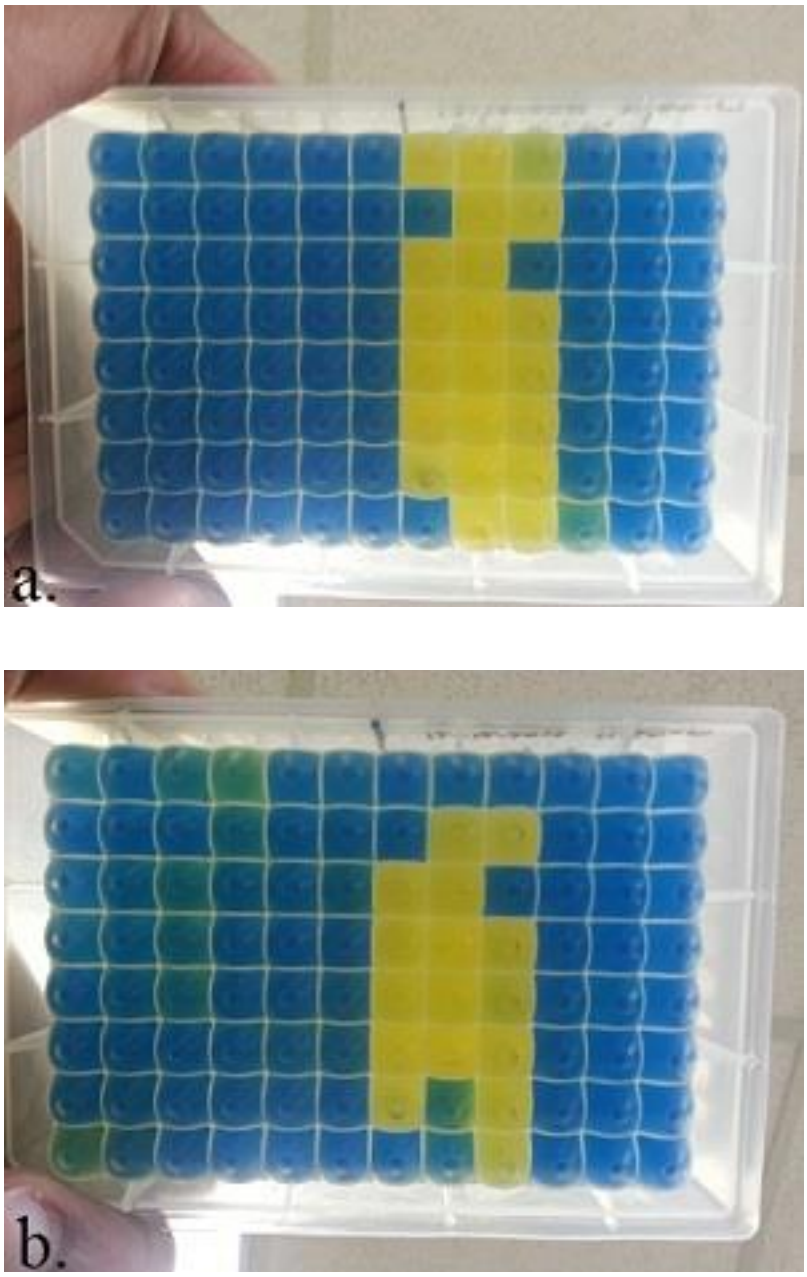


Figure 4.2 Determination of kit specificity with inclusive Vc strains and exclusive non-*cholerae Vibrios* after incubation at 36 °C for 24 h

Note: a.) The first 6 colums of wells in assay kit were inoculated with *Vibrio parahaemolyticus* and the last 6 were half *V cholerae* and half non-Vc, Vv, Vp *Vibrios*. b.) The first 6 colums of wells in assay kit were inoculated with *Vibrio vulnificus* and the last 6 were half *V cholerae* and half non-Vc, Vv, Vp *Vibrios*.

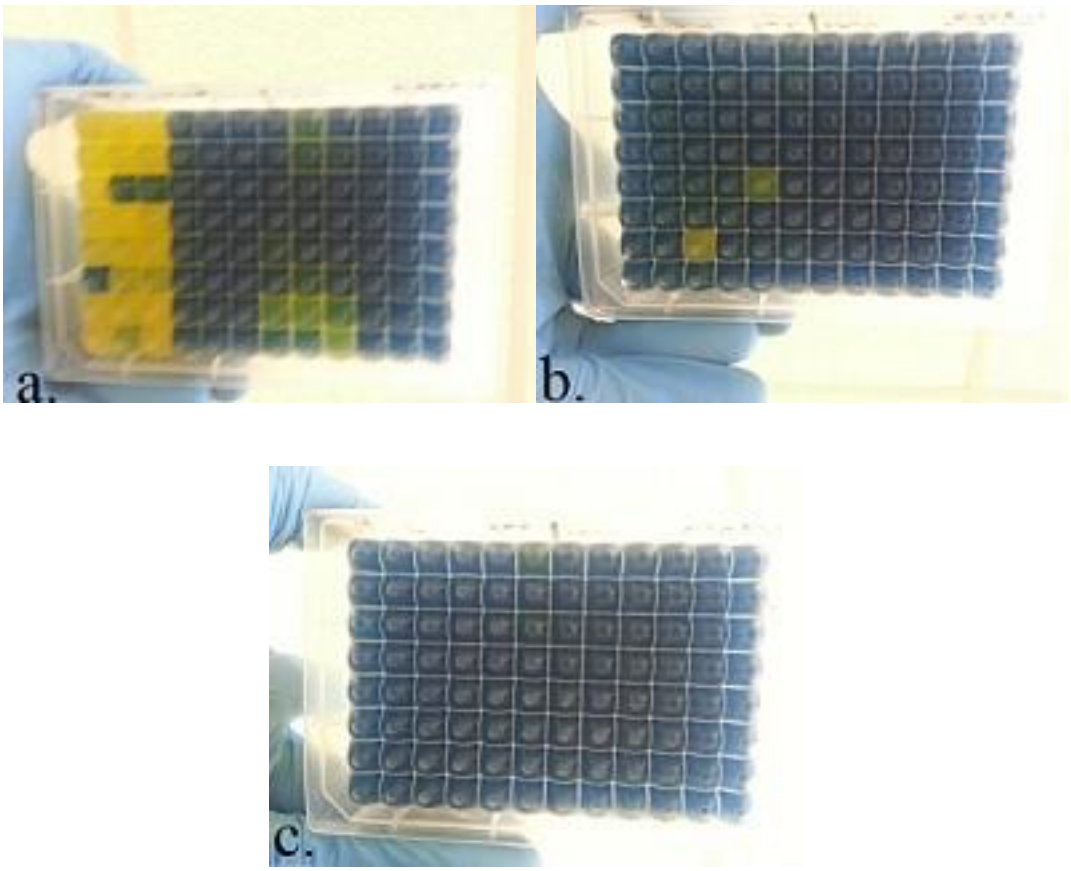


Figure 4.3 Determination of kit specificity with inclusive Vc strains and exclusive non-*cholerae Vibrios* after incubation at 42 °C for 24 h

Note: Assay kit solid phases were formulated with or without antibiotics. a.) Vc, b.) Vp, and c.) Vv isolates were utilized. Antibiotics were added to the solid phase in the right 6 columns of the assay kits

Table 4.1 Specificity and selectivityof the Vc assay kit.

without added compounds, 24 h at 36 °C							
	Tested	True+	True-	(+)	(-)	Selectivity	Specificity
V. cholerae	23	23	25	21	2	91%	92%
V. parahaemolyticus	48	0	48	0	48	0%	0%
V. vulnificus	48	0	48	0	48	0%	0%
Other *Vibrio spp*	10	0	10	0	10	0%	0%

without added compounds, 24 h at 42 °C							
	Tested	True +	True -	Yellow (+)	Blue (-)	Selectivity	Specificity
V. cholerae	23	23	25	21	2	91%	92%
V. parahaemolyticus	48	0	48	1	47	0%	2%
V. vulnificus	48	0	48	0	48	0%	0%
Other *Vibrio spp*	10	0	10	0	10	0%	0%

with added compounds, 24 h at 42 °C							
	Tested	True +	True -	Yellow (+)	Blue (-)	Selectivity	Specificity
V. cholerae	23	23	25	0	23	0%	8%
V. parahaemolyticus	48	0	48	0	48	0%	0%
V. vulnificus	48	0	48	0	48	0%	0%
Other *Vibrio spp*	10	0	10	0	10	0%	0%

Note: Selectivity was defined the percentage of positive wells that were correctly identified and specificity was defined as the difference between 100 % and percentage of negative wells that were correctly identified. Assay kits with added antibiotic compounds were observed to be unable to detect known *V. cholerae* (0 % specificity).

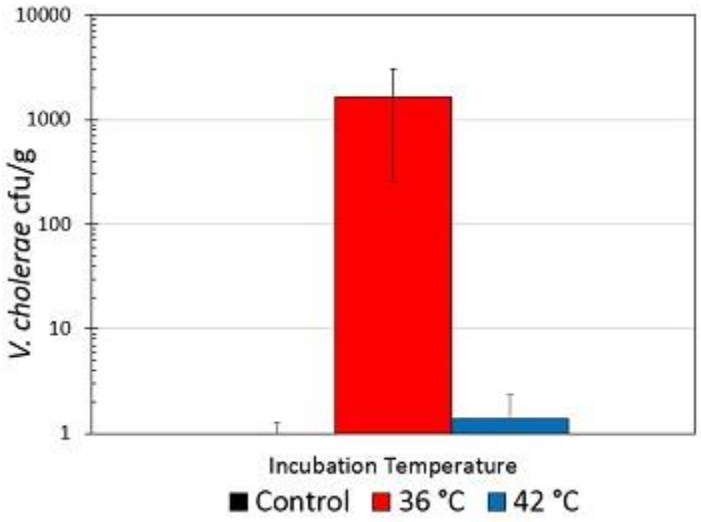


Figure 4.4 MPN cfu/g of Vc cells in oyster tissues response to incubation temperature

Note: MPN tube conventional PCR (control, ■) and V. cholerae kits incubated at 36 °C (■) or 42 °C (■) for 24 h. Control (■) not shown as V. cholerae cfu/g value was below the scale of this figure.

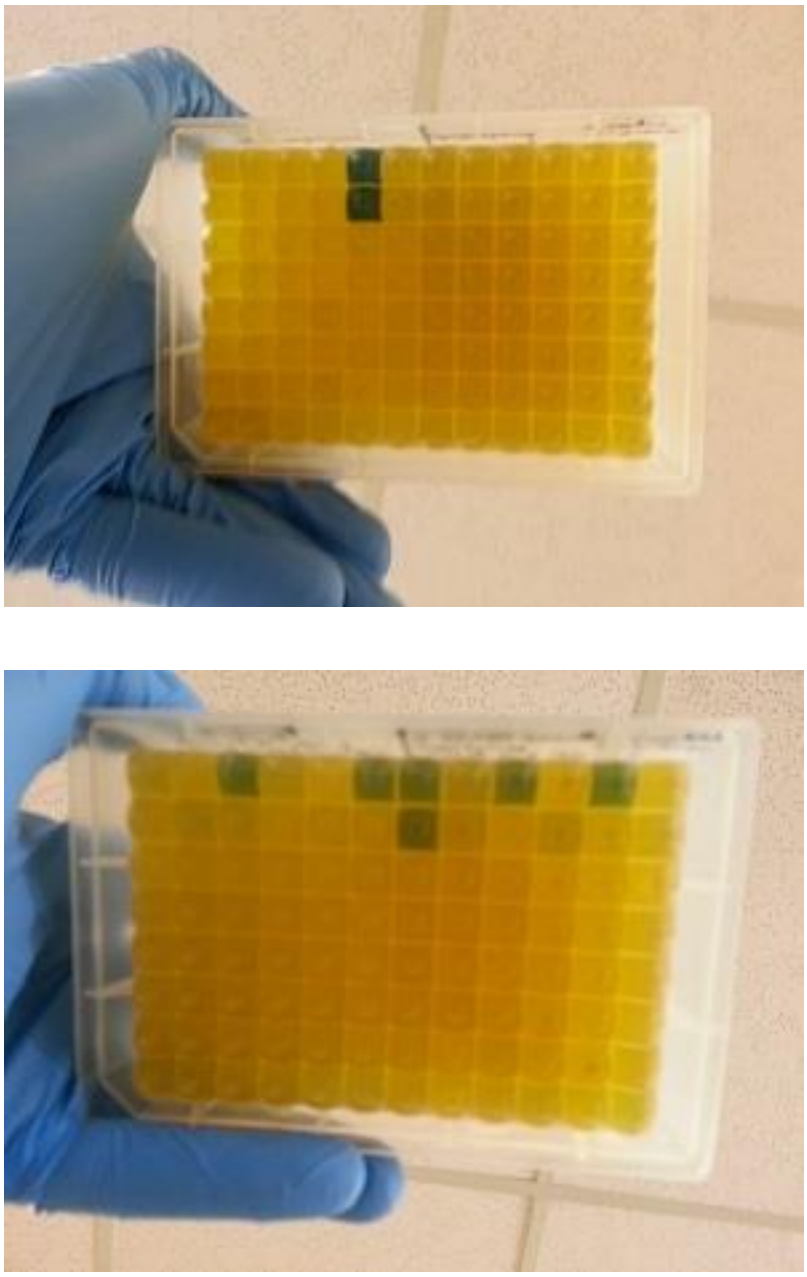
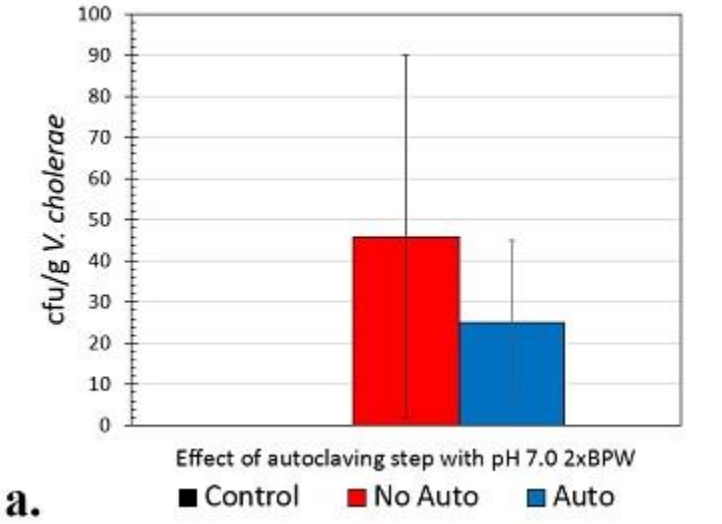


Figure 4.5 Determination of sensitivity of the Vc kit using two serially diluted V. cholerae isolates

Note: Isolates were incubated in Vc assay kit at 42 °C for 24 h. The 108 dilution (top row in photograph) showed a mean count < 1 cfu/g



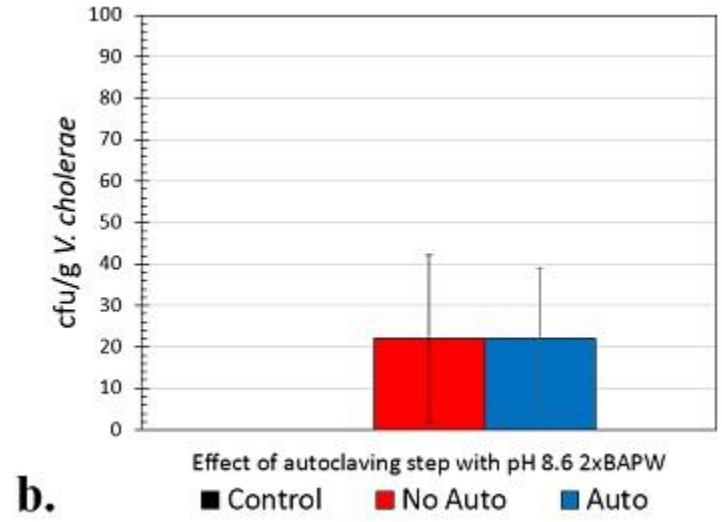


Figure 4.6 Most probable number (MPN) cfu/g of Vc cells in oyster tissues after incubation for 24 h at 42 °C.

Note: Series were a MPN tube conventional PCR (control, ■) and V. cholerae assay kits with solid phases that were (Auto, ■) or were not autoclaved (No Auto, ■). The liquid phases were: a.) 2x buffered peptone water (BPW, pH 7.0) or b.) 2x buffered alkaline

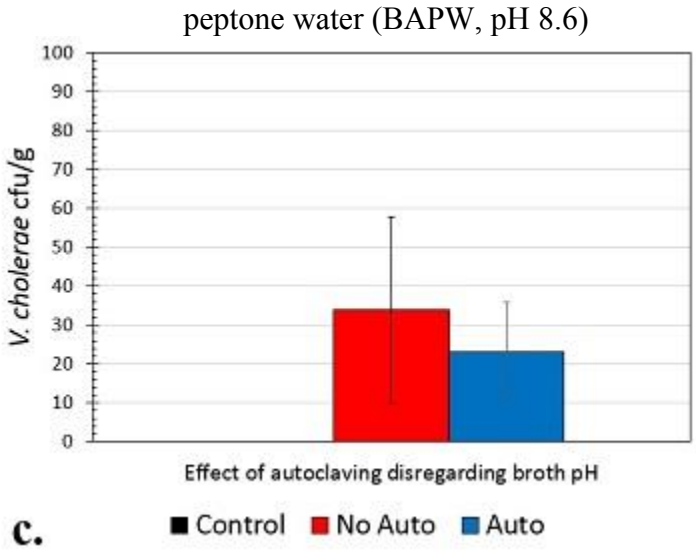


Figure 4.7 Most probable number (MPN) cfu/g of Vc cells in oyster tissues after incubation for 24 h at 42 °C.

Note: Liquid phases were 2xBPW and 2xBAPW, however, the pH of liquid phase was not considered in this analysis. Series were MPN tube conventional PCR (control, ■) and V. cholerae kits with solid phases that were (Auto, ■) or were not autoclaved (No-Auto, ■).

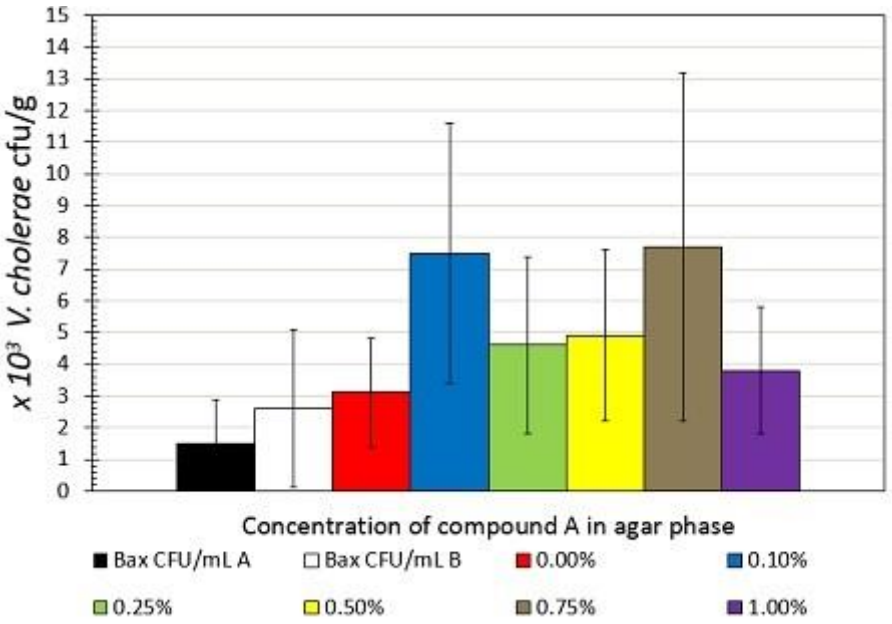


Figure 4.8 Effect of an antimicrobial on MPN cfu/g of Vc

Note: The compound was added to the solid phase of *V. cholerae* kit on most probable number (MPN) cfu/g after incubation for 24 h at 42 °C. PCR control V. cholerae cfu/g is not displayed here as it does not meet the scale for the figure.

CHAPTER V

SUMMARY AND CONCLUSIONS

The novel *V. cholerae* rapid assay kit was developed and optimized with oyster tissues to detect viable *V. cholerae* cells within a 24 h time period against other Vibrios and background microorganisms with a limit of detection below 5 cfu/mL. This kit maintained high levels of confidence in results despite indication that buffering capability of the liquid phase would significantly affect the pH of the kit and potentially confound results. The Vc assay kit also retained selection capability without autoclaving the solid phase portion. Incubation at 36 °C showed increased cross reaction from *Aeromonas hydrophila* leading to false positive results. Studies of the assay kit performed with inclusive and exclusive Vc strains reported 91 % selectivity and 92 % specificity. In almost every experiment ran in for this paper, the Vc assay kit performed at near equal footing with methods that are either more expensive (BAX PCR) or take longer to obtain a confirmed result (conventional PCR). Therefore, it was determined the *V. cholerae* rapid test kit can be considered an alternative method for 24 h determination of *V. cholerae* presence in a sample of oyster tissue.

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