EXAMINATION OF SELECTIVE BROTH CULTURE MEDIA FOR THE DETECTION AND QUANTIFICATION OF VIBRIO CHOLERAE IN DRINKING WATER USING AN ADAPTED COMPARTMENT BAG TEST

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ABSTRACT

Megan Lott: Examination of selective broth culture media for the detection and quantification of *Vibrio cholerae* in drinking water using an adapted Compartment Bag Test
(Under the direction of Mark Sobsey)

Cholera outbreaks may be mitigated by monitoring drinking waters for the bacterial disease agent, *Vibrio cholerae*. Present microbial methods for *V. cholerae* require advanced training and specialized equipment; these methods may not be appropriate for low-resource settings most vulnerable to cholera outbreaks. Our team hypothesized that the Aquagenx Compartment Bag Test (CBT) kit could be adapted for the direct detection and quantification of *V. cholerae* in drinking water in field settings. The present study evaluates selective culture media for adapted field use. The results of this study suggest that previously-described and commercially-available media are limited in specificity, exclusivity, and plating efficiency. We propose new selective broth culture media for use with the CBT. Preliminary results suggest that newly-proposed media demonstrate improved exclusivity over other existing culture media. These results support the further examination of broth media for direct quantification of *V. cholerae* with an adapted Cholera CBT.

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LIST OF ABBREVIATIONS

APW	Alkaline Peptone Water
BE	Beef Extract
BHI	Brain Heart Infusion
CBT	Compartment Bag Test
CFU	Colony Forming Units
CPC	Cellobiose, Polymyxin, Colistin Agar
CV	CHROMagar Vibrio Agar
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GSLS	Gelatin Salt Lauryl Sulfate
GTFCC	Global Task Force on Cholera Control
LB	Luria Broth
LBA	Luria Broth Agar
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry
MHB	Mueller-Hinton Broth
MIC	Minimum Inhibitory Concentration
MPN	Most Probable Number
MTT	Multiple Tube Test
SDS	Sodium Dodecyl Sulfate
SLS	Sodium Lauryl Sulfate
STT	Sucrose Teepol Tellurite Agar
TCBS	Thiosulfate-Citrate-Bile Salts-Sucrose Agar

TCI	Thiosulphate Chloride Iodide Agar
TTGA	Taurocholate Tellurite Gelatin Agar
TTGA	Taurocholate Tellurite Gelatin Broth
US EPA	United States Environmental Protection Agency
VBNC	Viable but Non-Culturable
VP	Vibrio parahaemolyticus Agar
WASH	Water, Sanitation and Hygiene
WHO	World Health Organization
YE	Yeast Extract

CHAPTER 1: INTRODUCTION

Vibiro cholerae is the etiologic agent of the water-borne illness which bears its name, cholera. Cholera is an acute diarrheal disease that causes watery stools, severe dehydration, and can lead to death if not treated promptly and properly. Since the 19th century, seven cholera pandemics have plagued global populations (Thompson, Austin, & Swings, 2006). The seventh pandemic began in Asia in 1961 and continues today (World Health Organization, 2018). Researchers estimate that there are as many as 2.9 million cases of cholera annually, which account for 95,000 deaths across 69 endemic countries (Ali, Nelson, Lopez, & Sack, 2015). The country of Yemen is currently facing the world's largest outbreak; between April and December of 2017, 1 million cases of cholera were suspected in this region alone (World Health Organization, 2018). With the threat of such outbreaks, cholera remains a pressing concern for global public health.

Cholera is transmitted through food and water contaminated with *V. cholerae*. Cholera outbreaks are often reported in low-income areas with inadequate or failing water and wastewater infrastructure (World Health Organization, 2018). Long-term solutions for cholera require investments for the provision of safe drinking water and proper sanitation. To strategize global efforts for such provisions, The World Health Organization (WHO) revitalized the Global Task Force on Cholera Control (GTFCC) in 2011. The GTFCC responded with a declaration to reduce cholera death by 90 percent by the year 2030 (Global Task Force on Cholera Control, 2017). As part of this commitment, the GFTCC calls for improved water, sanitation and hygiene

(WASH) systems. The Global Roadmap to 2030 calls specifically for provisions of rapid microbial test kits to prevent the transmission of *V. cholerae* and ensure safe water quality.

Field monitoring for *V. cholerae* can prevent disease transmission by identifying contaminated sources of water. However, many of the widely-accepted methods for detection of *V. cholerae* may not be appropriate for source monitoring in those low-resource settings that are vulnerable to cholera outbreaks. Current methods require advanced equipment and trained technicians to implement culture, molecular, or immunochemical assays for identification and enumeration of *V. cholerae* (Huq, 2013; Thompson et al., 2006). There is a need for improved, low-cost and portable microbial test kits for *V. cholerae*.

The Compartment Bag Test (CBT) is a field-friendly method for microbial monitoring of drinking water. The CBT is a low-cost, portable, and field-ready microbial test kit that was originally developed for the quantification of indicator bacteria in drinking and surface waters (Stauber, Miller, Cantrell, & Kroell, 2014). The test kit may be adapted for the direct quantification of *V. cholerae* if paired with an appropriate selective broth culture medium. The purpose of this current study was to evaluate selective culture media for direct quantification of *V. cholerae* for further application in the Compartment Bag Test.

Objectives

1. To examine the exclusivity of previously-described culture media for *V. cholerae.*
2. To compare the plating efficiency between culture media for *V. cholerae*.
3. To examine the exclusivity and efficiency of broth adaptations of solid agar media previously-described for selective culture of *V. cholerae.*
4. To examine the effect of incubation temperature at 37°C and 42°C on the exclusivity and plating efficiency of selective culture media for *V. cholerae.*
5. To examine the exclusivity of newly-formulated broth media intended for use with the Compartment Bag Test for quantification *V. cholerae*.

Experimental Design

The purpose of this study was to evaluate selective culture media for direct quantification of *V. cholerae* and to identify a culture medium appropriate for use in the CBT. An appropriate culture medium for an adapted Cholera CBT would be a broth medium that is selective against non-target microorganisms occurring in stored household drinking waters and natural surface water sources. The selectivity of the medium is imperative to limit false-positive results or inaccurate estimations of bacterial concentration of *V. cholerae*. An appropriate broth medium should enrich *V. cholerae* and be utilized for direct, efficient quantification of *V. cholerae* in drinking water samples. Selective growth of *V. cholerae* should be clearly visualized by chromogenic or fluorogenic change.

Many plating agars and enrichment broths have been described for the selective culture of *Vibrio spp.* and *V. cholerae*. The exclusivity of widely-used plating agars was examined by enumerating the extent of growth of non-target microorganisms from a simulated fecally-

contaminated drinking water sample. In this study, we defined exclusivity as a feature of a selective agar medium; it describes the medium's extent of inhibition of naturally-occurring non-target, non-vibrio organisms. The exclusivity of these plating agars was compared by incubation at both 37°C and 42°C.

The most selective and exclusive plating agars were adapted into broth media by centrifugation to eliminate the agar component. The exclusivity of these broth adaptations was compared to the exclusivity of previously-described selective broth enrichment media for *Vibrio spp.* and *V. cholerae.* Exclusivity was examined by quantifying the extent of growth of non-target organisms from a simulated fecally-contaminated drinking water sample. Exclusivity was compared for incubation temperatures of 37°C and 42°C.

Three selective media were compared based on their plating efficiency as both plating agars and broth media. Pure cultures of *V. cholerae* were plated onto modified taurocholate tellurite gelatin agar (TTGA), Thiosulfate-citrate-bile salts-sucrose agar (TCBS), CHROMagar Vibrio agar (CV), as well as non-selective Luria Broth (LB). The efficiency of these media was compared at incubation temperatures of 37°C and 42°C.

Recognizing the limitations of previously-described selective culture media, we sought to compose new selective media appropriate for use with the CBT for the exclusive quantification of *V. cholerae*. The exclusivity of these new culture media was examined by observing the extent of growth of spiked cultures of known gram-positive and gram-negative organisms, as well as *Vibrio mimicus*. The exclusivity of these new media was compared to the exclusivity of TTGB, TCBS, and CV and compared for incubation temperatures of 37°C and 42°C. The experimental design is outlined in **Figure 1**.

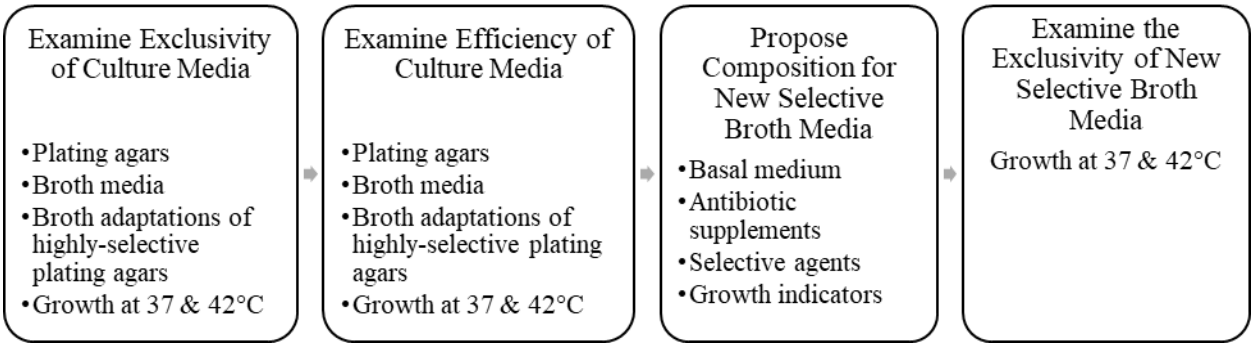

Figure 1. Experimental design for the identification and evaluation of culture media appropriate for quantification of *V. cholerae* with the Compartment Bag Test.

CHAPTER 2: REVIEW OF LITERATURE

The Genus *Vibrio*

The genus *Vibrio* belongs to the family of Vibrionaceae, which consists of gram negative organisms that are oxidase-positive and have polar flagella (Drasar, B.S and Forrest, 1996). Historically, this family has also included the genera: *Aeromonas*, *Photobacterium*, and *Plesiomonas* (Sneath, Mair, & Sharpe, 1986). Recently, molecular analyses have distinguished two new genera (*Listonella* and *Shewanella)*, as well as a new family, Aeromonadaceae (Koneman, Allen, & Janda, W.M. Schreckenberger, 1997).

Species belonging to the genus *Vibrio* are often classified as either *V. cholerae* or non-cholera *Vibrios* (Drasar, B.S and Forrest, 1996; Koneman et al., 1997). The primary habitat for most of these organisms is sea, brackish or freshwater (Forbes, Betty, Sahm, Daniel, Weissfeld, 2017; Thompson et al., 2006). A few vibrios can be isolated from freshwaters. These include *V. cholerae, V. mimicus, V. fluvialis, V. navarrensis, and V. andguillarum* (Thompson et al., 2006) Human infection by non-cholera *Vibrios* is typically associated with the consumption of contaminated seafood or experiencing wounds that become infected by contaminated waters (Kaysner, Charles and DePaola, 2004).

All *Vibrios* are gram-negative, fermentative and facultative anaerobes that grow best in alkaline conditions with NaCl between 1% and 3% (Drasar, B.S and Forrest, 1996). *Vibrio cholerae* and *V. mimcus*, however, do not strictly require NaCl for growth (Thompson et al., 2006). *V. cholerae* and *V. mimicus* share many of the same physiological and biochemical characteristics; they are lysine decarboxylase and ornithine decarboxylase-positive, but arginine

dihydrolase-negative. *Vibrio mimicus* was once considered a subspecies of *V. cholerae*, but molecular analyses have distinguished the two species (Koneman et al., 1997). *Vibrio mimicus* may be differentiated from *V. cholerae* by the inability to ferment sucrose and a negative reaction on the Voges-Proskauer Test that is used to determine if an organism produces acetylmethyl carbinol from glucose fermentation (Kaysner, Charles and DePaola, 2004).

Vibrio cholerae

There are more than 140 strains of *V. cholerae* that are described and differentiated based on serogroup (Drasar, B.S and Forrest, 1996). Most of the strains associated with the cholera disease belong to the O1 serogroup (Drasar, B.S and Forrest, 1996). These strains produce the cholera enterotoxin, the main virulence factor associated with cholera (Thompson et al., 2006).

Strains of the O1 serogroup are classified by biovar, either as Classical or El Tor. During this seventh pandemic of cholera, the El Tor biotype has replaced the Classical biotype as the predominant etiologic agent of epidemic cholera (Drasar, B.S and Forrest, 1996). The O1 serogroup is divided further into serotypes of Inaba, Ogawa and Hijokima based on antigenic factors (Dhiman Barua and William B. Greenough III, 1992).

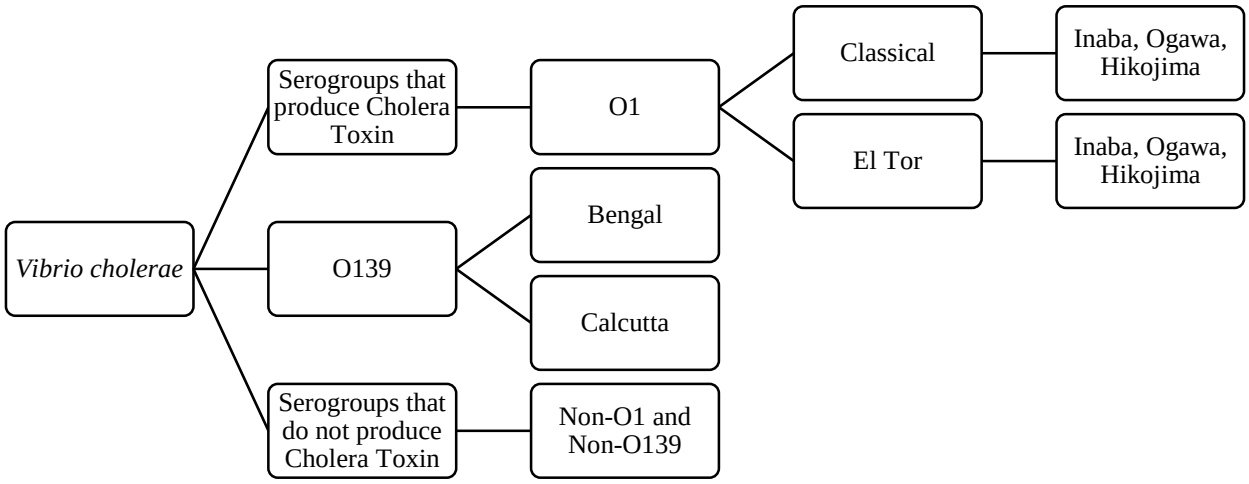

Figure 2. Classifications of *V. cholerae* by toxin-production, biovar, and serogroup. Adapted in part from Banerjee et al. 2014.

Strains of *V. cholerae* Non-O1 have been known to cause diarrheal illness similar to cholera (Drasar, B.S and Forrest, 1996). Some Non-O1 serotypes produce enterotoxins indistinguishable from the cholera toxin (Dhiman Barua and William B. Greenough III, 1992). The O139 strain of *V. cholerae* emerged in 1993 during a large cholera outbreak across India and Bangladesh (Kaysner, Charles and DePaola, 2004). The O139 Bengal strain produces the same cholera toxin produced by those relevant strains of *V. cholerae* O1. Researchers suspect that the *V. cholerae* O139 strain may be responsible for an eighth cholera pandemic (Dhiman Barua and William B. Greenough III, 1992; Drasar, B.S and Forrest, 1996; Kaysner, Charles and DePaola, 2004). The relationship between these classifications of *V. cholerae* strains is described in **Figure 2**. Evidence suggests that mobile genetic elements can confer virulence to other Non-O1 and Non-O139 serogroups, and that these organisms may lead to potential disease outbreaks (Li, Shimada, Morris, Sulakvelidze, & Sozhamannan, 2002).

Vibrio cholerae in the Aquatic Environment

Vibrio cholerae is a natural inhabitant of aquatic environments and may be isolated from fresh, brackish, and coastal waters (Dhiman Barua and William B. Greenough III, 1992; Huq, 2013; Thompson et al., 2006). *V. cholerae* can be isolated from environmental samples, even in regions where cholera is not endemic (Huq, 2013). The bacterium can be free-swimming, or found in association with sediment, zooplankton and shellfish. Sediment can harbor *V. cholerae*, and stressed bacteria can enter a dormant state to ensure long-term survival in natural waters (Dhiman Barua and William B. Greenough III, 1992; Huq, 2013). *Vibrio* species associate well with copepods and shellfish, as these zooplankton and shellfish facilitate increases in bacterial density (Huq, 2013; Thompson et al., 2006). The complex ecology of *V. cholerae* requires that sources of drinking water be monitored routinely for microbial quality and detection of the cholera bacterium, especially in areas where cholera occurs in humans and *V. cholerae* are present in environmental waters.

The Epidemiology of Cholera

Cholera is a waterborne and foodborne disease transmitted by the etiologic agent, *V. cholerae*. This bacterium is transmitted through the fecal-oral route upon consumption of food or drinking water contaminated with these organisms (Dhiman Barua and William B. Greenough III, 1992). An infectious dose of up to 10^{11} viable organisms of *V. cholerae* may be required to induce illness, but as few as 10^{3} viable vibrios have been reported to induce a diarrheal illness (Thompson et al., 2006). Once ingested, the cholera bacterium colonizes the small intestine and produces the cholera toxin (Thompson et al., 2006). This toxin induces the excretion of water and electrolytes from the host's intestinal cells. The resulting diarrhea and vomiting can lead to

severe dehydration and even death. Patients with acute cholera may excrete up to 20 liters of stool per day, containing viable organisms of *V. cholerae* (Dhiman Barua and William B. Greenough III, 1992). Without proper sanitation, these organisms can contaminate household and environmental waters.

Disease outbreaks are typically associated with poor or failing water and wastewater infrastructure (World Health Organization, 2018). Cholera can persist as an endemic disease, spreading through local populations in the same region over time. Today, cholera is endemic to Southern Asia, parts of Africa, the Middle East and Latin America (Thompson et al., 2006). Epidemic cholera emerges in new regions where local transmission is not often recognized or reported. The risk of disease transmission can increase during disaster events in the face of flooding, failing infrastructure, or displacement of large populations (Watson, Gayer, & Connolly, 2007). Humanitarian crises may similarly displace groups to overcrowded camps without provisions for clean water and sanitation (World Health Organization, 2018).

Disease control requires improvements in sanitation and provisions for clean drinking water in low-resource and disaster settings. Outbreaks from the seventh cholera pandemic have been associated with waters from rivers and other sources that are used without treatment (Dhiman Barua and William B. Greenough III, 1992). Drinking water wells can be contaminated by fecal waste from cases of cholera within households and communities. Waters that are collected, treated and stored can be re-contaminated in households with limited sanitation and hygiene. Routine microbial surveillance of reservoirs and source waters can help ensure the safety of drinking water and reduce the risk of diarrheal illness by identifying contaminated drinking waters so that actions can be taken to eliminate the contamination by treatment processes (Bain et al., 2012).

Methods for Cultivation and Enumeration *V. cholerae*

Routine water quality monitoring can help reduce the risk of transmission of waterborne cholera. Microbial surveillance for *V. cholerae* can identify contamination in surface water sources, wells, or stored water supplies. Field-based monitoring can evaluate the safety of drinking waters before use, examine the efficacy of point-of-use disinfection, or identify hotspots for *V. cholerae* contamination. By identifying water contaminated with the organisms, remedial actions such as water treatment can be taken to eliminate the contamination.

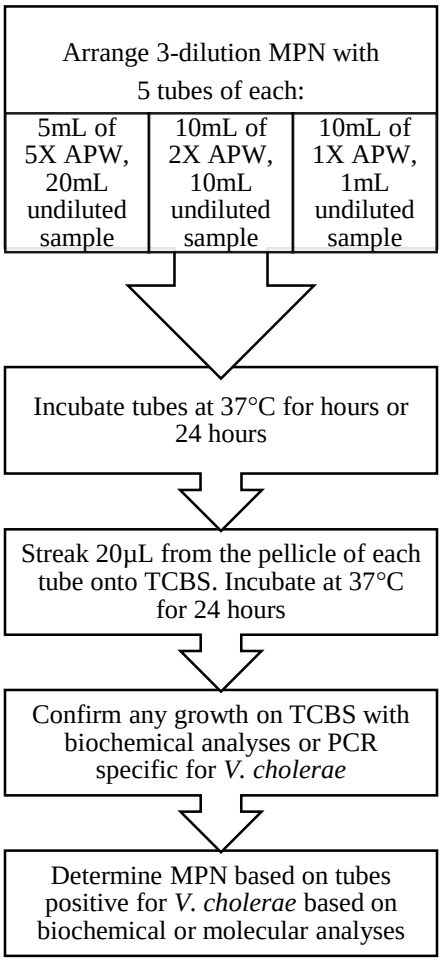

Figure 3. EPA Standard Analytical Protocol for *V. cholerae* O1 and O139 in Drinking and Surface Water

Current methods for the detection and quantification of *V. cholerae* include culture, molecular, immunochemical, or biochemical assays (Huq, 2013; Thompson et al. 2006). Many of these methods require specialized media and reagents, laboratory equipment and trained personnel that limit the applicability of these methods for routine field-based water quality monitoring. Recent advancements in field-based technologies may improve microbial surveillance in low-resource settings where prevention measures are often most needed.

To detect and isolate *Vibrio cholerae* from environmental samples by culture-based methods, the samples are often pre-enriched in non-selective and/or selective broth culture media and the cultures are then streaked onto selective agar plating media (Thompson et al., 2006).

Biochemical, immunological and/or molecular assays are often required to confirm that these presumptive isolates are *V. cholerae* and to determine their specific strain or type.

Vibrio cholerae can be quantified in environmental samples by traditional culture-based methods. Standard methods require multiple procedures, including pre-selective enrichment and biochemical or molecular confirmation of presumptive *V. cholerae* organisms. The methods described by the International Standards Organization (ISO) for the isolation of Vibrio spp. suggest that organisms from environmental media should be enriched in selective broth and then plated onto TCBS and an additional selective medium (Hartnell et al., 2018; ISO, 2017). Any presumptive colonies should then be confirmed using biochemical assays with an oxidase test, string test, or Voges-Proskaur Test. Molecular assays, such as the polymerase chain reaction (PCR) can confirm presumptive colonies as well.

The EPA *Standard Analytical Protocol for V. cholerae O1 and O139 in Drinking Water and Surface Water* describes a similar multi-step, culture-based method for quantification of *V. cholerae*. Using these methods, *V. cholerae* can be quantified by a multiple-tube assay coupled with isolation on selective agar media. In this assay, sample volumes are inoculated into 15 tubes containing selective enrichment media at varying sample concentrations and volumes (USEPA, 2010). These tubes are incubated at 37°C for either 6-8 hours, or 24 hours. An inoculum from each tube is streaked onto selective plating media and incubated for another 24 hours. The bacterial concentration, or Most Probable Number (MPN), is estimated based on the number of positive results from these tubes and plates. **Figure 3** outlines these procedures. Alternatively, *V. cholerae* can be enumerated by direct plating or by membrane filtration, assuming all organisms in the sample are viable (Thompson et al., 2006; Thomson & Gunsch, 2015). Culture-based methods may be limited because *V. cholerae* organisms can enter into a

viable but nonculturable state (VBNC), during which they do not grow or form colonies on traditional culture media (Huq, 2013). Alternatively, *V. cholerae* can be enumerated by flow cytometry, colony hybridization, or culture-independent methods (Huq, 2013; Thompson et al., 2006).

Culture-independent methods may be used to enumerate *V. cholerae* from environmental samples. Fluorescence in situ hybridization (FISH) has been used to quantify *Vibrios* based on extracted nucleic acids (Huq, 2013; Thompson et al., 2006). Real-time PCR may be used to quantify *V. cholerae* (Thompson et al., 2006). The direct fluorescent antibody – direct viable count method can be used to distinguish viable from VBNC organisms of *V. cholerae* while estimating the count of organisms (Huq, 2013; Kahler et al., 2015).

These methods require laboratory equipment and trained personnel that limit the applicability of these methods for routine field-based water quality monitoring. Some of these methods may be adapted for field-use by implementing portable devices that employ the same culture-based or immunological assays. Recent advancements in such field-based technologies may improve microbial surveillance in low-resource settings where prevention measures are often most needed.

Field-Based Methods for Detection and Enumeration of *V. cholerae*

Field-based methods for detection of *V. cholerae* may improve microbial surveillance in low-resource settings. Emerging field-based methods implement both culture-based and immunological assays for surveillance for *V. cholerae*. A novel paper-based analytical device (PAD) was developed to examine the presence/absence of *V. cholerae* from a 3mL sample of pre-enriched drinking water (Briquaire et al., 2017). Similarly, the CV™ Dipstick is a novel test

validated for surveillance of *V. cholerae* in household and stored drinking water (Rashid et al., 2017). The dipstick test utilizes antibodies that are specific to O1 and O139 serotypes to confirm the presence or absence of *V. cholerae* in a 1mL pre-enriched sample. Field-based electrochemical immunosensors (biosensors) may be used for environmental surveillance of *V. cholerae*, again confirming the presence of the organism in a pre-enriched sample (Cecchini, Fajs, Cosnier, & Marks, 2016; Sharma, Goel, Singh, & Rao, 2006).

These alternative and novel methods may be useful tools for field-based monitoring of waters vulnerable to contamination. However, the methods above only analyze small volumes of sample (5 µL – 3mL) for the presence or absence of *V. cholerae* and they cannot be used readily to quantify the bacterial concentration in test waters unless modified to a multiple volume quantal assay format. A portable flow-cytometer may be used for rapid assays to enumerate *V. cholerae* from drinking water reservoirs, but the equipment may not be cost-efficient for low-resource settings (Righetto et al., 2015). Furthermore, many of these non-culture-based methods are based on detecting *V. cholerae* cells as physical objects or are based on detection of specific nucleic acids or antigens. They do not provide evidence that the detected cells or cell constituents are culturable or infectious unless applied after an initial culture procedure.

An alternative field-based method for quantifying *V. cholerae* may be the application of the Aquagenx Compartment Bag Test (CBT). The CBT is a portable, low-cost water quality test used currently for the quantification of *Escherichia coli* and H_2S -producing bacteria in drinking water and environmental samples.

The Compartment Bag Test

The Aquagenx CBT is ideal for water quality monitoring in low-resource settings. The test kit is a low-cost, portable, and easy-to-use quantal method for quantification of culturable bacteria of *E. coli* or H_2S -producing bacteria (Aquagenx, 2018). The CBT is a clear plastic bag designed for 100mL samples. Water samples are mixed with a selective culture medium and dispensed into the CBT. The bag is divided into multiple compartments of different volumes (1mL, 3mL, 10mL, 30mL, and 56mL). Samples are incubated overnight and these individual compartments are scored for growth based on chromogenic change of the culture medium. The results are reported as an most probable number, MPN/100mL, with corresponding confidence intervals as described in the user "look-up" tables described by Gronewold, Sobsey, & McMahan (2017). The CBT Kit instructions are shown in **Figure 4**.

Figure 4. CBT Kit Instructions. Accessed from Aquagenx LLC at https://www.aquagenx.com/how-to-use-the-cbt/.

The CBT was originally designed for quantification of fecal indicator organisms. The CBT has been validated as a reliable method for quantifying *E. coli* and H_2S -producing bacteria in water, with results comparable to traditional methods for quantification and enumeration of bacteria (McMahan et al., 2011; 2012; 2017; Stauber et al., 2014). The CBT kit has been further validated recently for the quantification of H_2S-producing bacteria (Tipton, Stewart, Sobsey, &

Whalen, 2017). The Compartment Bag Test may be adapted for the direct quantification of *V. cholerae* if paired with an appropriate selective and differential culture medium.

Selective Culture Media for *Vibrio* and *V. cholerae*

Many broth media and plating agars have been described for the selective enrichment and culture of *Vibrio spp.* (Dhiman Barua and William B. Greenough III, 1992; Donovan & van Netten, 1995; Koneman et al., 1997; Thompson et al., 2006). Several additional commercially-available media may be utilized for selective isolation of *Vibrio spp.* These media are described in **Table 1**. Almost none of these media are capable of selecting for a single species of *Vibrio* (Thompson et al., 2006).

The most commonly used medium for *V. cholerae* is TCBS. The major disadvantage of this medium is its limited selectivity and specificity against non-vibrios (Donovan & van Netten, 1995). Species of *Acinetobacter, Aeromonas, Alcaligenes, Enterobacter, Escherichia coli, Pasteurella, Pseudomonas, Salmonella,* and *Proteus* are all able to grow on TCBS (Donovan & van Netten, 1995). Another limitation of TCBS is that this medium cannot distinguish specifically between *V. cholerae* and other sucrose fermenting *Vibrios* such as *V. alginolyticus, V. fluvialis, V. furnissii*, and *V. metschnikovii*, (Thompson et al., 2006).

For the purposes of this study, we assume that of the *Vibrios* species, *V. cholerae, V. mimicus, V. fluvialis, V. navarrensis, and V. andguillarum* may be present in the drinking water samples examined by an adapted Cholera Compartment Bag Test (Thompson et al., 2006). The primary habitats for other *Vibrio spp.* are predominately brackish, coastal, or sea waters that may not be suitable as drinking water sources as they are too high in salinity or dissolved solids (Koneman et al., 1997). An appropriate medium for an adapted Cholera CBT should inhibit non-

target non-Vibrio organisms, and distinguish between *V. cholerae* and *V. mimicus*. Such a medium would employ nutrients, specific substrates, selective agents, and growth indicators for the selective broth culture of *V. cholerae.*

Basal Media

Of the Vibrio media previously described in literature, basal media are composed of peptone alone or in combination with beef extract, yeast extract, or cellobiose. Columbia Blood Agar Base or Brain Heart Infusion Broth may be appropriate for culturing *V. cholerae* as well (Beazley, 1992; Dhiman Barua and William B. Greenough III, 1992). Gelatin may be incorporated into differential media, as most vibrios hydrolyze gelatin (Kaysner, Charles and DePaola, 2004). However, other non-target organisms like *Aeromonas spp.* may also hydrolyze gelatin (Dhiman Barua and William B. Greenough III, 1992).

Vibrio species grow best under alkaline conditions (Kaysner, Charles and DePaola, 2004). While many *Vibrios* are halophilic, *V. cholerae* and *V. mimicus* do not require addition of NaCl in culture media; these organisms can utilize sodium ions from other constituents that make up most media (Kaysner C. and DePaola A. 2004). Multiple strains of *V. cholerae* demonstrate optimal growth at NaCl levels between 0.5% and 5% NaCl (Griffitt & Grimes, 2013).

Selective Agents

Selective media for *Vibrio spp.* incorporate selective agents and differential components that inhibit gram-positive organisms and distinguish *Vibrios* from other gram-negative organisms. Incubation in alkaline conditions and under high concentrations of NaCl may improve the selectivity of culture media for *Vibrios*.

Bile salts. Bile salts may be incorporated into culture media for the selective growth of gram-negative, enteric organisms. Originating from the gastrointestinal tract, these animal-origin agents inhibit most gram-positive organisms and some gram-negative organisms. Among the most common bile salts used for selective culture of *V. cholerae* are: oxgall/oxbile, sodium cholate, sodium deoxycholate, and sodium taurocholate. Ox bile is a mixture of conjugated bile salts. Sodium deoxycholate is a conjugated bile salt and the most potent of the animal-origin inhibitors (EMD Millipore Corporation, 2008). While gram-negative bacteria are relatively resistant to bile salts, sodium deoxycholate may exhibit an initial killing effect on gram-negative bacteria (Paul D 'Mello, 1980).

Tellurite. Tellurium (Te) compounds have been used as antimicrobial agents (Taylor, 1999). Microbes that are resistant to tellurite may convert the agent to a less toxic form, tellurium. These microbes produce black colonies on selective media. Potassium tellurite has been described as a selective agent for the isolation of *Corynebacterium diphtheriae*, *Staphylococcus aureus,* and *Shigella spp*. Tellurite resistance has been occasionally reported for *Pseudomonas spp*. Potassium tellurite has no inhibitory effect on *V. cholerae* at a concentration of 1:200,000 (Monsur, 1961).

Media prepared with potassium tellurite must be monitored for quality assurance, as tellurite converts to tellurite over time and diminishes the potency of the medium (Bolinches, Romalde, & Toranzo, 1988; Thompson et al., 2006). It is best practice to use media with potassium tellurite within 24 hours of preparation (Thompson et al., 2006). This reagent is filter sterilized (Atlas, 1997).

Detergents. Anionic detergents, such as teepol, sodium dodecyl sulfate, and sodium lauryl sulfate, are effective in the selective inhibition of gram-positive bacteria. These detergents

may be used in place of bile salts (Jameson, J.E., 1956). Cationic detergents are more effective against gram-positive bacteria, but also inhibit gram-negative organisms (Baker, Harrison, & Miller, 1941; Salton, 1960). A killing effect of gram-negative organisms has been observed for anionic detergents at concentrations exceeding 1:30,000 (Baker et al., 1941).

Selective Dyes. Selective dyes may be incorporated into culture media for the inhibition of non-target microorganisms. Crystal violet is a basic dye that inhibits growth of gram-positive organisms, while still allowing growth of gram-negative organisms (Fung & Miller, 1973). Bromothymol blue is an acid dye that inhibits gram-positive organisms (Fung and Miller 1973). This dye may be implemented as a pH indicator in Vibrio media.

Sodium Citrate. Sodium citrate has antimicrobial properties for the inhibition of gram-positive bacteria, but shows little activity against gram-negative bacteria (Lee, Cesario, Owens, Shanbrom, & Thrupp, 2002).

Antibiotics. Additional antimicrobials may improve the selectivity of a culture medium, depending on the antibiogram of the target organism and competing non-target organisms. Polymixin B and colistin may be implemented in selective culture media for *V. cholerae*, but some serogroups of *V. cholerae* (like the Classical biovars) are susceptible to colistin (Dhiman Barua and William B. Greenough III, 1992; Thompson et al., 2006). *V. cholerae* has demonstrated resistance to ampicillin and streptomycin (Kitaoka, Miyata, Unterweger, & Pukatzki, 2011). Recently, fosfomycin and vancomycin have been incorporated into selective media for *V. cholerae* (Briquaire et al., 2017). Fosfomycin has antimicrobial activity against many gram-positive and gram-negative bacteria. Of interest, fosfomycin is selective against *Eneterococcus faecalis*, *E. coli*, *Proteus spp*, and *Pseudomonas aeruginosa*, bacterial species

known to cause false-positives in media for *V. cholerae* (Donovan & van Netten, 1995; Monsur, 1961; Popovic, Steinort, Pillai, & Joukhadar, 2010).

Selective Culture Conditions

High pH. *Vibrio* species grow best under alkaline conditions (Centre for Disease Control and Prevention (CDC);, 2004; Dhiman Barua and William B. Greenough III, 1992; Thompson et al., 2006). Optimal growth is observed at pH between 7.6 and 8.6, but may be supported at a pH range between 5.6 and 9.6 (Dhiman Barua and William B. Greenough III, 1992). High pH is thought to improve the selectivity of culture media for *Vibrio spp.* but this assertion has also been contested (Donovan & van Netten, 1995; Thompson et al., 2006).

Incubation Temperature. *Vibrio cholerae* grows optimally at incubation temperatures between 20°C and 45°C (Martinez, Megli, & Taylor, 2010). Increased incubation temperature may select against non-target environmental or enteric organisms. *V. cholerae* does not persist at 4°C for extended periods; refrigeration or cold storage of samples may result in *V. cholerae* becoming VBNC (Martinez et al., 2010; USEPA, 2010).

Growth Indicators and Specific Substrates

Chromogenic and fluorogenic indicators are chemical agents and metabolizable substrates that improve the selection for and visualization of bacterial growth in culture and may be implemented as differential agents to distinguish target from non-target organisms. In culture media for vibrios., pH indicators are often used to identify biochemical reactions specific to certain species. *Vibrio cholerae* ferments sucrose; the acid-base indicators bromothymol and thymol blue change from blue to yellow when fermentation products lower the pH of the culture

(Kaysner, Charles and DePaola, 2004). Phenol red turns yellow in acidic conditions. However, sucrose fermentation is not specific to *V. cholerae* or *Vibrio spp.* The vibrios *V. alginolyticus*, *V. fluvialis*, *V. furnissii*, and *V. metschnikovii* are all sucrose-positive, as are the Enterobacteriaceae *Klebsiella pneumonia* and *Proteus vulgaris* (Dhiman Barua and William B. Greenough III, 1992; Kaysner, Charles and DePaola, 2004; Koneman et al., 1997). *V. mimicus* is a non-fermenting vibrio, and may be distinguished from *V. cholerae* based on an indicator for sucrose fermentation (Dhiman Barua and William B. Greenough III, 1992; Kaysner, Charles and DePaola, 2004).

Colwell et al. described that 4-methylumbelliferyl-β-D-galactoside (4-MU-Gal) may be incorporated into selective and differential media for the culture of *V. cholerae* (1985). The substrate fluoresces when cleaved by enzymatically-active β-D-galactosidase in viable cells of *V. cholerae*. The β-galactosidase enzyme is not specific to *V. cholerae*, however, and may contribute to high numbers of false-positive results when examining contaminated water samples (Tryland & Fiksdal, 1998). The vibrios *V. fluvialis*, *V. furnissii* and *V. mimicus* are all capable of β-gal activity (Kaysner, Charles and DePaola, 2004). The β-galactosidase enzyme has been identified in several families and genera of gram-negative organisms including the Enterobacteriaceae, Pseudomonadaceae, and Neisseriaceae (Tryland & Fiksdal, 1998).

The commercial development of chromogenic substrates may improve the specificity of culture media for *V. cholerae.* Regrettably, many of these chromogenic substrates are proprietary and nondisclosed for general use. However, some insights into the key chromogenic substrates and other specific ingredients that distinguish *V. cholerae* from other *Vibrio* species have been identified in patents for these media. An example is the US patent assigned to Biomereaux for detection of key *Vibrio* species (US 7,892,783 B2, 2011). The commercial media,

HardyCHROM Vibrio, CHROMagar Vibrio, chromID, and HiChrome Vibrio utilize such proprietary chromogenic substrates (**Table 1**).

Criteria for Cholera CBT Medium

An appropriate medium for quantification of *V. cholerae* with the CBT should enrich the growth of *V. cholerae* to the exclusion non-target microorganisms. An appropriate medium may differentiate between *V. cholerae* and organisms of non-concern. This medium must perform as a broth medium that can be applied to an MPN format to enrich the growth of *V. cholerae* and accurately estimate the concentration of organisms in a drinking water sample. Such a broth medium should incorporate selective agents such as bile salts, detergents, selective dyes, or antibiotics to inhibit gram-positive and other gram-negative organisms. The medium may maintain a pH at or above 8.6 to improve selectivity. A chromogenic or fluorogenic substrate and indicator should be incorporated for clear visualization of *V. cholerae* in the CBT.

Culture media were included in this study based on the above criteria, and the ease of access of media components. Media selected for examination are described in **Table 2**. Samples of the commercial media HardyCHROM Vibrio Agar, HiChrome Vibrio Agar, and CHROMagar Vibrio Agar were donated by their manufacturers. These media were examined for their exclusivity against non-target organisms, and their plating efficiency for growth of *V. cholerae*. Media were examined according to manufacturers' instructions, and with adaptations appropriate for field use with the CBT.

Table 1. Composition of selective culture media described for *V. cholerae*. Adapted in part from Donovan & van Netten, 1995 and Thompson et al., 2006.

Medium	Selective Agents	Indicators (Reactions)	Reference(s)
Alkaline peptone water	pH	(turbidity)	Furniss, Lee, & Donovan, 1978
Cellobiose, polymyxin, colistin agar (CPC)	Colistin, polymixin B	pH indicators Bromothymol blue and Cresol red (green)	Massad & Oliver, 1987
CHROMagar Vibrio	Salts mixture pH 8.6	Chromogenic substrates (blue)	CHROMagar
chromID Vibrio	Not disclosed	Chromogenic substrate (blue-green)	bioMerieux
Fe-EDTA enrichment broth	Fe-EDTA pH 9.0	(turbidity)	Kida, Suzuki, & Taguchi, 1995
Gelatin phosphate saline broth (GPS)	-	(turbidity)	Madden, McCardell, & Morris, 1989
Glucose salt teepol broth (GSTB)	Methyl violet, teepol, 3% NaCl pH 9.4	(turbidity)	Akiyama et al., 1963
Glucose-salt-tellurite-crystal violet medium (GSTC)	crystal violet, potassium tellurite, 3% NaCl pH 8.4	pH indicator Bromothymol blue (yellow)	Bolinches, Romalde, & Toranzo, 1988
HardyCHROM Vibrio agar	Sea salt mixture, oxbile, sodium citrate, sodium pyruvate	Chromogenic mixture (purple)	Hardy Diagnostics
HiChrome Vibrio agar	Sodium citrate, sodium cholate pH 8.5	Chromogenic mixture (purple)	HiMedia Labs
Modified taurocholate tellurite gelatin agar (TTGA)	Sodium taurocholate, potassium tellurite pH 8.6	4-MU-Gal (blue fluorescence)	Monsur, 1961; O'Brien & Colwell, 1985

Table 1 (Continued). Composition of selective culture media described for *V. cholerae*. Adapted in part from Donovan & van Netten, 1995 and Thompson et al., 2006.

Medium	Selective Agents	Indicators (Reactions)	Reference(s)
Modified Vibrio agar	Sodium citrate, sodium deoxycholate, oxbile, SDS	Bromothymol blue, cresol red (blue-gray)	Tamura, Shimada, & L.M., 1971
Polymixin mannose tellurite agar (PMT)	Polymixin, tellurite, SDS	pH indicators Bromothymol blue, cresol red (green)	Shimada et al., 1990
SDS polymixin sucrose agar (SPS)	SDS, polymixin	(halo production)	Kitaura et al., 1983
Sucrose teepol tellurite agar (STT)	Teepol, tellurite	pH indicators Bromothymol blue (yellow)	Chatterjee & De, 2017
Starch gelatin polymixin broth (SGP)	Polymixin pH 7.6	(turbidity)	Kitaura et al., 1983
SV Medium	Sodium citrate, oxbile	(opaque)	Salles, Voros, Marbell, & Amenuvor, 1976
Taurocholate tellurite peptone broth (TTGB)	Sodium taurocholate, potassium tellurite pH 8.6	(turbidity or fluorescence with 4-MU-Gal)	Monsur, 1961
Thiosulfate-citrate-bile salts-sucrose agar (TCBS)	Sodium thiosulfate, sodium citrate, ferric citrate, oxgall pH 8.6	pH indicators Bromothymol blue and thymol blue (yellow)	Kobayashi, Enomoto, Sakazaki, & Kuwahara, 1963
Thiosulfate-chloride iodide agar (TCI)	Potassium iodide	(colorless colonies)	Beazley, 1992
***V. parahaemolyticus* agar (VP or Modified TCBS)**	Sodium taurocholate, SDS pH 8.5	pH indicators Bromothymol blue and thymol blue (yellow)	De, Sen, De, P. C. Ghosh, & Pal., 1977
***V. cholerae* chromogenic agar medium**	Animal origin inhibitors, sodium citrate, oxbile, vancomycin and fosfomycin pH 8.6	Glycosidase enzyme substrate (blue)	Briquaire et al., 2017

CHAPTER 3: METHODS

Sample Collection and Preparation

1% Primary Effluent-Surface Water Sample

Primary sewage effluent collected from the Mason Farm Wastewater Treatment Plant (Orange County, NC) was combined at a volume ratio of 1:100 (1% final concentration) with natural surface water from Morgan Creek (Chapel Hill, NC). This combined water matrix was prepared to achieve a diversity of microorganisms in a small-volume inoculum and to simulate samples from impaired watersheds and fecally-contaminated drinking water sources. Cholera is not endemic in the United States and neither primary effluent nor surface water samples were expected to contain *V. cholerae*. The effluent and surface water may contain other non-target organisms that interfere with culture-based methods to detect *V. cholerae*. Therefore, any organisms cultured from the primary effluent are presumptive non-vibrios and non-*V. cholera*e.

Primary sewage samples were collected in sterile polypropylene bottles by the staff at the wastewater treatment facility. Samples were stored at 4°C overnight. A 1mL sample of primary effluent was spiked into 99mL of natural surface water before each experiment.

Natural Surface Waters

Natural surface water samples were collected from Morgan Creek, the receiving stream for the Mason Farm Wastewater Treatment Plant. Tertiary treated sewage effluent enters Morgan Creek after treatment and UV disinfection. Samples were collected alongside the Morgan Creek

Trail, a public green space with paved and wooden walking trails. Samples were collected in sterile polypropylene bottles and stored at 4°C overnight. Morgan Creek is an inland stream that is not expected to have a population of *Vibrio spp.* Therefore, any organisms cultured from Morgan Creek are presumptive non-vibrios and non-*Vibrio cholerae*.

Media Preparation

Enrichment broths and plating agars examined for this study are described in **Table 2**. Media were prepared according to *The Handbook of Microbiological Media* (1997). Media that were commercially-sourced were prepared according to manufacturer's instructions. Broth and agar adaptations of Taurocholate-Tellurite-Gelatin Agar (TTGA) were prepared according to modifications of Monsur's agar described by Colwell et al. (1986), which incorporates 4-methylumbelliferyl-β-D-galactopyranoside (4-MU-Gal) for detection of β-Gal activity. A homemade Thiosulphate-Citrate-Bile Salts-Sucrose (TCBS) broth was prepared according to R. Atlas (1997), without the agar component. Thiosulphate-Chloride-Iodide (TCI), was prepared according to C. Pfeffer and J.D. Oliver (2003).

In preparing Glucose Salt Teepol Broth (GSTB), gelatin was substituted for glucose as a carbon source. Teepol and sodium dodecyl sulfate were substituted with sodium lauryl sulfate. This altered composition will be referred to as Gelatin Salt Lauryl Sulfate (GSLS) broth. The concentration of gelatin in TTGA was reduced from 30g to 20g in both agar and broth compositions.

Broth adaptations of TCI, TCBS, and CV were prepared by removing the agar component through centrifugation. Each medium was prepared first as 3x concentrated solution. Before boiling, these solutions were centrifuged at 3000 RPM for 15 minutes at 4°C. During

centrifugation, the insoluble agar component sediments out of the suspension and the remaining liquid medium can be recovered. The liquid medium was removed and DI water was added in equal parts. The solution was centrifuged again, and once more DI was added in equal parts to the recovered liquid medium for a final 1x concentration (approximate). A 2x broth medium was prepared in the same manner, but without the final DI water dilution.

The pH of each medium was adjusted prior to sterilization through boiling or autoclaving. Media containing sucrose or other heat-sensitive components were brought to a boil not exceeding 100°C. All other media were autoclaved at 115 psi and 121°C for 15 minutes. All antibiotics and enzyme substrates were added aseptically after media cooled. Potassium tellurite was filter-sterilized and added aseptically after media cooled. All media were prepared and stored at 4°C. Selective media were stored no more than 24 hours before use.

Table 2. Vibrio-specific media examined.

Non-Selective Media	Luria-Bertani Broth (Difco) Luria-Bertani Agar (Broth with Bacto Agar)
Vibrio-Specific Agar Media	Cellobiose, Polymyxin, Colistin (CPC) Agar (HiMedia) CHROMagar Vibrio Agar (CHROMagar) HardyCHROM Vibrio Agar (Hardy Diagnostics) HiCrome Vibrio (HiMedia) Sucrose Teepol Tellurite (STT) Agar Taurocholate Tellurite Gelatin Agar (TTGA) Thiosulphate Chloride Iodide (TCI) Agar Thiosulphate-Citrate-Bile Salts-Sucrose (TCBS) Agar (Difco) Vibrio parahaemolyticus (VP) Agar
Vibrio-Specific Enrichment Broths	Gelatin Phosphate Saline (GPS) Gelatin Salt Lauryl Sulfate (GSLS) Taurocholate Tellurite Peptone (TTGB)
Broth Adaptations of Vibrio-Specific Agar Media	CHROMagar Vibrio (CHROMagar) Thiosulphate-citrate-bile Salts-Sucrose (TCBS) (Difco) Thiosulphate-chloride-iodide (TCI)

Examining the Exclusivity of Media for Culturing *V. cholerae*

Enumeration of Non-Target Organisms from 1% Primary Effluent-Surface Water Matrix on Solid Agar Media

An appropriate medium for quantification of *V. cholerae* with the CBT should enrich the growth of *V. cholerae* as evidence of sensitivity by exclude non-target microorganisms as evidence of specificity. An appropriate medium should differentiate between *V. cholerae* and organisms of non-concern, with both high sensitivity (detects all of the target organisms) and specificity (does not detect any of the non-target organisms).

Plating agars were examined for their inhibition or differentiation of non-target organisms as evidence of their specificity from a 1% primary effluent-natural water matrix. Duplicate plates of each agar medium were inoculated with 100µL of 1% primary effluent-natural water sample by the spread plate method that uniformly distributes the inoculum over the surface of the agar medium plate. Plates were incubated for 18-24 hours at 37°C and 42°C. Any colony growth was reported if colonies presented the same chromogenic or fluorogenic change as *V. cholerae* on the solid agar medium. *Vibrio cholerae* O1, El Tor Ogawa (ATCC BAA-2163) was used as the positive control. **Table 3** describes the criteria applied for enumerating colonies based on chromogenic or fluorogenic changes.

Table 3. Criteria for enumerating colonies appearing the same as *V. cholerae* from the 1% primary effluent matrix on solid agar media.		
Agar Medium	Colony Color of *V. cholerae* O1, El Tor Ogawa	Color of Colonies Enumerated
LBA	Clear	Clear
VP	Yellow	Yellow
STT	Yellow	Yellow
CPC	Green	Green
HiCHROM	Purple	Purple
TTGA	Blue Fluorescence	Blue Fluorescence
HardyCHROM	Purple	Purple
TCBS	Yellow	Yellow
CV	Blue	Blue (Light to Turquoise)
TCI	Clear	Clear

Quantification of Non-Target Organisms from 1% Primary Effluent-Surface Water Matrix in Broth Media

Selective broths were examined for their inhibition or differentiation of non-target organisms from a 1% primary effluent-natural water matrix as evidence of specificity. Culturable organisms from 1% primary effluent in natural water were quantified by an adapted MPN method, using 24-well plates to perform a 3-well, 3-dilution MPN assay. The 1% primary effluent-natural water sample was serially diluted to 10^{-4} in Standard Methods phosphate buffer. Wells were prepared with 0.9mL of the appropriate selective broth medium and inoculated with 100µL of the diluted sample. Well plates were incubated for 24 and 48 hours at 37°C and 42°C. Wells were scored for growth; wells were considered positive if presented any visible chromogenic/fluorogenic change, regardless of the color. Such growth may interfere with *V. cholerae* growth and detection in a broth culture medium. *Vibrio cholerae* O1, El Tor Ogawa was used as the positive control. **Table 4** describes the criteria applied for scoring wells based on chromogenic or fluorogenic changes.

Table 4. Criteria for scoring wells for growth of all non-target organisms from the 1% primary effluent-surface water matrix.		
Broth Medium	Color of Wells with *V. cholerae* O1, El Tor Ogawa	Color of Wells Scored
LB	Turbid	Turbid
GPS	Turbid	Turbid
GSLS	Turbid	Turbid
TTGB	Blue Fluorescence	Turbid and Blue Fluorescence
CV	Blue	Turbid, White, Purple, and Blue
TCBS	Yellow	Green to Yellow

Quantification of Non-Target Organisms from Surface Waters

To examine the exclusivity of Vibrio-specific broth media with large-volume samples, a 10-tube MPN assay was prepared to quantify non-target organisms cultured from 50mL natural water samples. To prepare the assay, 5mL volumes of 2X culture media were added to sterile glass culture tubes. Each of these tubes was inoculated with 5mL of natural surface water for a 10-tube MPN assay of a total 50 ml of sample water, analyzed in duplicate (100 ml of sample water in total). If necessary, natural surface water was diluted serially in phosphate buffer to ensure that results were within a reliable and readable range to achieve some positive and some negative tubes for growth and make it possible to estimate an MPN concentration. Tubes were incubated at 37°C for 18-24 hours on a shaker tray, and then scored for growth. Tubes were considered positive were if they presented any visible chromogenic or fluorogenic change, regardless of the color. Such growth may interfere with *V. cholerae* growth and detection in a broth culture medium. *Vibrio cholerae* O1, El Tor Ogawa was used as the positive control. **Table 5** describes the criteria applied for scoring tubes based on chromogenic or fluorogenic changes.

Table 5. Criteria for scoring tubes for growth of all non-target organisms from the natural surface waters.		
Broth Medium	Color of Tube with *V. cholerae* O1, El Tor Ogawa	Color of Tubes Scored
LB	Turbid	Turbid
TTGB	Blue Fluorescence	Turbid and Blue Fluorescence
CV	Blue	Turbid, White, Purple, Blue
TCBS	Yellow	Green to Yellow

Identification of Non-Target Organisms in Taurocholate Tellurite Peptone (TTGB)

To further examine the lack of exclusivity of the TTGB medium, non-target organisms cultured from natural surface waters were isolated and purified as individual colonies and then identified by MALDI-TOF Mass Spectrometry. Natural surface waters were collected and 0.5mL of the sample was spiked into 15 sterile tubes each containing 8mL of TTGB. Tubes were incubated at 37°C for 18-24 hours. The contents of tubes positive for fluorescence under long-wavelength UV light were touched with a loop and streaked onto LBA plates. Plates were incubated at 37°C for 18-24 hours. Colonies recovered on LBA were differentiated based on colony morphology and streaked to isolation of pure colonies. These pure cultures were submitted to North Carolina State University Clinical Microbiology Lab for species identification by MADI-TOF MS.

Examining the Efficiency of Media for Culturing *V. cholerae*

Quantification and Enumeration of *V. cholerae* (Multiple Tube Format)

An appropriate medium for the Cholera CBT must perform as a broth medium that can be applied to an MPN format to enrich the growth of *V. cholerae* and accurately estimate the concentration of organisms in a drinking water sample. To examine the efficiency of selective broth media, pure cultures of *V. cholerae* were quantified by multiple-tube test (MTT) and also enumerated by direct spread plate. Initial experiments quantified pure cultures of *V. cholerae* by MTT using the TTGB medium. The MPN results were compared to Colony-Forming Units (CFU) from direct spread plate on non-selective LBA, selective TCBS, and selective TTGA agar media.

Overnight cultures of *V. cholerae* O139 (ATCC 51395), *V. cholerae* O1, El Tor Ogawa (ATCC BAA-2163) and *V. cholerae* Non-O1 (ATCC 35971) were prepared separately in alkaline peptone water (APW) and incubated at 37°C for 24 hours. Each overnight culture was serially-diluted in phosphate buffer. A 5-tube, 3-dilution MTT assay was prepared by inoculating 100µL sample into sterile tubes with 10mL of TTGB. Multiple Tube Tests were prepared in triplicate. A 100µL inoculum was plated in duplicate on LBA, TTGA, and TCBS by spread plate. Tubes and plates were incubated at 37°C for 18-24 hours. Tubes were incubated on a shaker tray.

Quantification and Enumeration of *V. cholerae* (Multiple Well Format)

Broth adaptations of TTGB, TCBS and CV were examined as selective and sensitive culture media for the quantification of *V. cholerae* in an adapted MPN format. The MPN results

were compared to the enumeration of colonies by agar versions of the original media. These candidate media were compared to the enumeration and quantification of *V. cholerae* by non-selective LBA and LB. An adapted MPN assay was prepared in 48-well plates for a 3-well, 4-dilution MPN assay. Overnight cultures of *V. cholerae* O139, *V. cholerae* O1, El Tor Ogawa and *V. cholerae* Non-O1 were incubated in APW at 37°C for 18-24 hours. Each overnight culture was serially-diluted in phosphate buffer. A 100µL sample was inoculated into 0.9mL of selective broth media in the corresponding well. The multiple-well assays were prepared in duplicate. A 100µL sample was also plated in duplicate on solid agar media by spread plate. A set of duplicate well plates and solid agar plates were incubated at both 37°C and 42°C for 18-24 hours.

Examining the Efficiency of Basal Media for Culturing *V. cholerae*

Screening Candidate Basal Media for Growth of *V. cholerae*

An appropriate broth medium for the CBT should consist of a basal medium that will enrich the growth of *V. cholerae* to provide reliable results when quantifying organisms from drinking water samples. Brain Heart Infusion Broth (BHI Broth), peptone, and a combination of peptone, yeast and beef extract were evaluated as basal media for quantification of *V. cholerae* by MPN assay. These media were evaluated at different concentrations of NaCl (**Table 6**).

Table 6. Constituents of basal media.

Basal Medium (g/L)	Added NaCl (% weight/volume)
Brain Heart Infusion Broth (37g/L)	+0% NaCl +1% NaCl +3%NaCl +5% NaCl
Peptone (10g/L)	+0% NaCl +1% NaCl +3% NaCl +5% NaCl
Peptone (10g/L) + Yeast Extract (5g/L) + Beef Extract (5g/L)	+0% NaCl +1% NaCl +3% NaCl +5% NaCl

Each basal medium was screened for growth of pure cultures of *V. cholerae*. Overnight cultures of *V. cholerae* O139, *V. cholerae* O1, El Tor Ogawa and *V. cholerae* Non-O1 were incubated in APW at 37°C for 18-24 hours. The overnight cultures were touched with a loop and transferred to a well containing 0.9mL of the basal medium. Well plates were incubated at 42°C for 18-24 hours and visualized for changes in turbidity. Turbid wells indicated positive growth.

Efficiency of Basal Media for Quantification of *V. cholerae*

Basal media were examined for plating efficiency by quantifying *V. cholerae* in an MPN format. The MPN assay was prepared using a 48-well plate, each well containing 0.9mL of broth for a 3-well, 4-diltuion assay prepared in duplicate. Overnight cultures of *V. cholerae* O139, *V. cholerae* O1, El Tor Ogawa and *V. cholerae* Non-O1 were incubated in APW at 37°C for 18-24 hours. The overnights were serially diluted 10-fold in phosphate buffer to 10^{-9}. An inoculum of 100µL was plated into the corresponding wells of media. Well plates were incubated at 42°C for 18-24 hours and visualized for changes in turbidity. Turbid wells indicated positive growth.

Antibiotic Susceptibility Testing of *V. cholerae* and Non-*V. cholerae* Bacteria

Antibiotics may be incorporated into culture media to improve selectivity and specificity by preventing the growth of non-target bacteria. Four antibiotics – fosfomycin, streptomycin, ampicillin, and vancomycin – were evaluated for their selective properties in broth culture media. The minimum inhibitory concentration (MIC) was determined for three strains of *V. cholerae*, one strain of *V. mimicus*, and seven non-Vibrio bacteria suspected to interfere in Vibrio-specific media (**Table 7**). The MIC values were obtained following the EUCST methods for determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution (EUCAST Discussion Document E. Dis 5.1, March 2003).

Table 7. Organisms included in antibiotic susceptibility testing for the MIC determination of fosfomycin, streptomycin, ampicillin, and vancomycin.

Vibrio spp.
Vibrio cholerae 0139 (ATCC 51395)
Vibrio cholerae O1, El Tor Ogawa (ATCC BAA-2163)
Vibrio cholerae Non-O1 (ATCC 35971)
Vibrio mimicus (ATCC 33653)
Gram-Negative Organisms
Aeromonas hydrophila (ATCC 7966)
Proteus mirabilis (ATCC 9921)
Pseudomonas aeruginosa (ATCC 12175)
Escherichia coli (ATCC 25922)
Gram-Positive Organisms
Bascillus pumilus (Environmental)
Enterococcus casseliflavus (Environmental)
Paenibacillys pabuli (Environmental)

Preparation of Antibiotics

Solid salts of each antibiotic were dissolved in distilled water at a final concentration of 5120mg/L and stored at -20°C. Antibiotic stock solutions were diluted in Muller-Hinton Broth (MHB) to final concentrations of: 1mg/L, 2mg/L, 4mg/L, 8mg/L, 16mg/L, 32mg/L, 64mg/L, 128mg/L, 256mg/L, 512mg/L. Of these solutions, 175µL was transferred into 96-well plates. The antibiotics were plated in order of increasing concentration.

When mixed with an equal volume of inoculum in the wells, the final concentrations of each antibiotic were: 0.5mg/L, 1mg/L, 2mg/L, 4mg/L, 8mg/L, 16mg/L, 32mg/L, 64mg/L, 128mg/L, 256mg/L. The solution with 256mg/L of antibiotic was plated as the antibiotic control, mixed with an equal volume of sterile MHB. Stock solutions were discarded after use.

Preparation of Inoculum

Pure cultures of each test organism were streaked onto LBA plates and incubated at 37°C for 18-24 hours. Pure cultures of *Aeromonas hydrophila* were streaked onto LBA and incubated at 28°C for 18-24 hours. From each plate, four or five colonies were suspended into 8mL of MHB in sterile glass culture tubes. Cultures were then incubated at 37° for 2-4 hours until the turbidity was equal to that of a 0.5 McFarland standard, or the absorbance at 625nm was in the range of 0.08-0.10 as measured by a UV-VIS Spectrophotometer. Within 30 minutes of this standardization, 0.1mL of the culture was transferred into 9.9mL of MHB. Of this dilution, 175µL was transferred into each well of the 96-well plate, excluding the antibiotic control wells. Positive control wells contained 175µL of the bacterial strain as well as 175µL of the MHB. The final inoculum concentration was approximately $3 – 7 \text{ X } 10^5$ CFU/mL. For quality control, a 10µL inoculum of the positive control well was transferred into 10mL of MHB, and 100µL of this solution was spread onto LBA plates and incubated at 37°C to ensure that the inoculum concentration was within this range.

MIC Determination

All test organisms were plated in duplicate to determine the minimum inhibitory concentration of four antibiotics in the range of 0.5 – 256 mg/L. Well plates were incubated at

37°C for 18-24 hours. Wells were observed for turbidity compared to the positive control. The MIC_{100} was recorded as the lowest concentration of the antibiotic that completely inhibited growth.

Examining the Exclusivity of Newly-Proposed Culture Media for *V. cholerae*

Six new compositions of culture media were proposed for the selective culture of *V. cholerae* (**Table 8**). These compositions incorporate new selective agents to existing culture media (CV Plus), modify previously described media (modified TTGB), or incorporate different selective agents than previously described media. Detailed compositions of these media are described in Appendix A.

To examine the exclusivity of these media, three strains of *V. cholerae*, nine gram-negative organisms, six gram-positive organisms, and *V. mimicus* were tested for growth in these broth media (**Table 9**). In addition, growth was tested in LB, TCBS, TTGB, and CV.

An overnight culture of each test organism was incubated in LB at 37° for 18-24 hours. One loopful of this culture (10µL) was inoculated into a well containing 1.0mL of broth medium. Well plates were incubated at 37° and 42°C for 24h and 48h. Growth was observed at both 24h and 48h, and reported after 48h incubation. Growth was indicated by turbidity, chromogenic change, or fluorogenic change appearing the same as *V. cholerae* in the culture medium. **Table 10** describes the criteria applied for scoring wells based on chromogenic or fluorogenic changes.

Table 8. Composition of novel culture media for the selective culture of *V. cholerae*.

Medium	Selective Agents	Indicators
CV Plus	Salts mixture, streptomycin, fosfomycin pH 8.6	Chromogenic Substrate
Cholera CBT Medium A	Sodium taurocholate, sodium citrate, sodium lauryl sulfate, crystal violet pH 8.6	pH indicator (Bromothymol Blue)
Cholera CBT Medium A-Plus	Sodium taurocholate, sodium citrate, sodium lauryl sulfate, crystal violet streptomycin, fosfomycin pH 8.6	pH indicator (Bromothymol Blue)
Cholera CBT Medium B	Oxgall, sodium taurocholate, sodium citrate, sodium desoxycholate Streptomycin, ampicillin pH 8.6	4-MU-Gal
Cholera CBT Medium C	Sodium citrate, oxgall, sodium taurocholate, potassium tellurite pH 8.6	pH indicator (Bromothymol Blue)
Cholera CBT Medium D	Sodium taurocholate, sodium carbonate, potassium tellurite Streptomycin, ampicillin pH 8.6	4-Mu-Gal

Table 9. Test organisms to examine the exclusivity of culture media for Vibrio spp. and *V. cholerae*.
Vibrio spp.
Vibrio cholerae 0139 (ATCC 51395)
Vibrio cholerae O1, El Tor Ogawa (ATCC BAA-2163)
Vibrio cholerae Non-O1 (ATCC 35971)
Vibrio mimicus (ATCC 33653)
Gram-Negative Organisms
Aeromonas hydrophila (ATCC 7966)
Proteus mirabilis (ATCC 9921)
Pseudomonas aeruginosa (ATCC 12175)
Escherichia coli (ATCC 25922)
Shigella flexneri (ATCC 12661)
Shigella spp. (ATCC 23354)
Klebsiella pneumoniae (ATCC 23357)
Salmonella typhimurium LT2
Raoultella terrigena
Gram-Positive Organisms
B. cereus (ATCC 1778)
S. aureas (ATCC 29213)
E. faecalis (ATCC 29212)
Bascillus pumilus (Environmental)
Enterococcus casseliflavus (Environmental)
Paenibacillys pabuli (Environmental)

Table 10. Criteria for scoring wells for growth of known, relevant test organisms.		
Broth Medium	Color of Well with *V. cholerae* O1, El Tor Ogawa	Color of Wells Scored
LB	Turbid	Turbid
TTGB	Blue Fluorescence	Blue Fluorescence
CV	Blue	Blue
CV Plus	Blue	Blue
TCBS	Yellow	Yellow
Cholera CBT Medium A	Yellow	Yellow
Cholera CBT Medium A Plus	Yellow	Yellow
Cholera CBT Medium B	Blue Fluorescence	Blue Fluorescence
Cholera CBT Medium C	Yellow	Yellow
Cholera CBT Medium D	Blue Fluorescence	Blue Fluorescence

Data Analysis

The data collected was visualized using Microsoft Excel and analyzed using R software. All data were analyzed using non-parametric tests, including Kruskal-Wallis, Wilcoxon Rank-Sum, and Wilcoxon Signed Rank Tests. All tests were performed using direct counts of colony forming units (CFU) or most probable number (MPN). An alpha value of 0.05 ($P < 0.05$), was used to establish statistical significance.

CHAPTER 4: RESULTS

Examining the Exclusivity of Media for Culturing *V. cholerae*

Enumeration of Non-Target Organisms from 1% Primary Effluent-Surface Water Matrix on Solid Agar Media

The exclusivity of Vibrio-specific agar media was examined by observing the growth of naturally occurring, non-target organisms from a 1% primary effluent-surface water matrix, when plated on the different selective media. These non-target organisms were enumerated on the selective agar media. Colonies were counted if they presented the same morphology and color as *V. cholerae* on the medium. The enumeration of these non-target organisms is described in **Figure 5**.

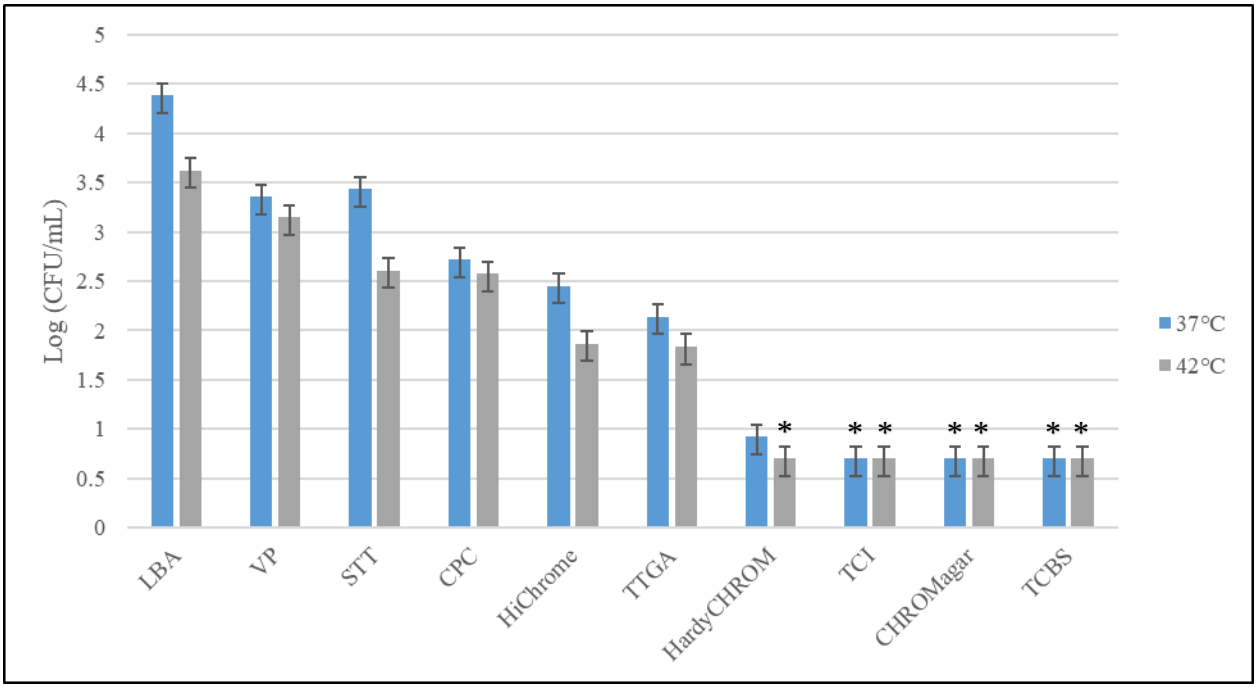

Figure 5. Enumeration of naturally-occurring, non-target organisms with Vibrio appearance cultured from 1% primary effluent in surface water on selective agar media for Vibrio spp. as determined by direct spread plate (n = 3). Values marked with (*) were estimated from plates with no exhibited growth from an undiluted inoculum. Examination of TTGB and LB were conducetd seperately from those other media; these experiments are not paired.

Of the nine Vibrio-specific media examined, TCBS (Difco), CV (CHROMagar), and TCI agars completely inhibited the growth of *any* non-target organisms from the plated inoculum of surface water with 1% primary effluent. Other media allowed the growth of non-target microbes appearing the same as *V. cholerae*, with STT and VP allowing the highest levels of growth. Incubation at 42°C gave less growth of non-target organisms and thereby improved inhibition of non-target organisms.

To further examine the exclusivity of these agar media, we describe the frequency of observing non-target growth on each selective agar medium that appears the same as colonies of *V. cholerae* on the medium. This frequency is described across the three replicate trials. These frequencies are described in **Table 11**. The most appropriate selective medium would give no growth across any of the three trials. Based on these results, TCBS, CV and TCI are effective

solid agar media for the inhibition of non-target organisms from the 1% primary effluent matrix at incubation of 37°C or 42°C. HardyCHROM agar is additionally effective at 42°C incubation.

Table 11. Frequency of observing non-target growth appearing the same as *V. cholerae* on selective agar media for across three replicate trials. Frequency is reported below as # Trials (out of three total). Values represent the number of trials for which growth appearing the same as *V. cholerae* was observed on selective agar media, when inoculated with a 1% primary effluent in surface water matrix.

Medium	**37°C**	**42°C**
LBA	3	3
VP	3	3
STT	3	3
CPC	3	3
HiCHROM	3	3
TTGA	3	3
HardyCHROM	3	0
TCBS	0	0
CHROMagar	0	0
TCI	0	0

Quantification of Non-Target Organisms from 1% Primary Effluent-Surface Water Matrix in Broth Media

The exclusivity of Vibrio-specific broth media was examined by quantifying the non-target organisms from a 1% primary effluent in surface water matrix plated in a multiple-well MPN assay. Growth was indicated by turbidity, chromogenic change, or flourogenic change. Wells were scored if *any* growth was observed in the broth medium. The quantification of these organisms is reported in **Figure 6**.

Of the seven broth media examined, TTGB was the most selective. The broth adaptation of the proprietary TCBS medium was also effective at inhibiting the growth of background organisms, although the homemade adaptation of TCBS was less selective than the medium

acquired from Difco. Although GSLS was among the most selective media, bacterial growth was difficult to visualize, especially at 42°C incubation. This medium does not incorporate any chromogenic or fluorogenic indicators to aid in visualization of bacteria growth. Selective agents from the most effective of these media may be considered for new adaptations of selective Vibrio media.

CHROMagar Vibrio ranks among the more selective media examined, limiting non-target growth. Across all media, incubation at 42°C improved exclusivity, reducing the growth of non-target organisms.

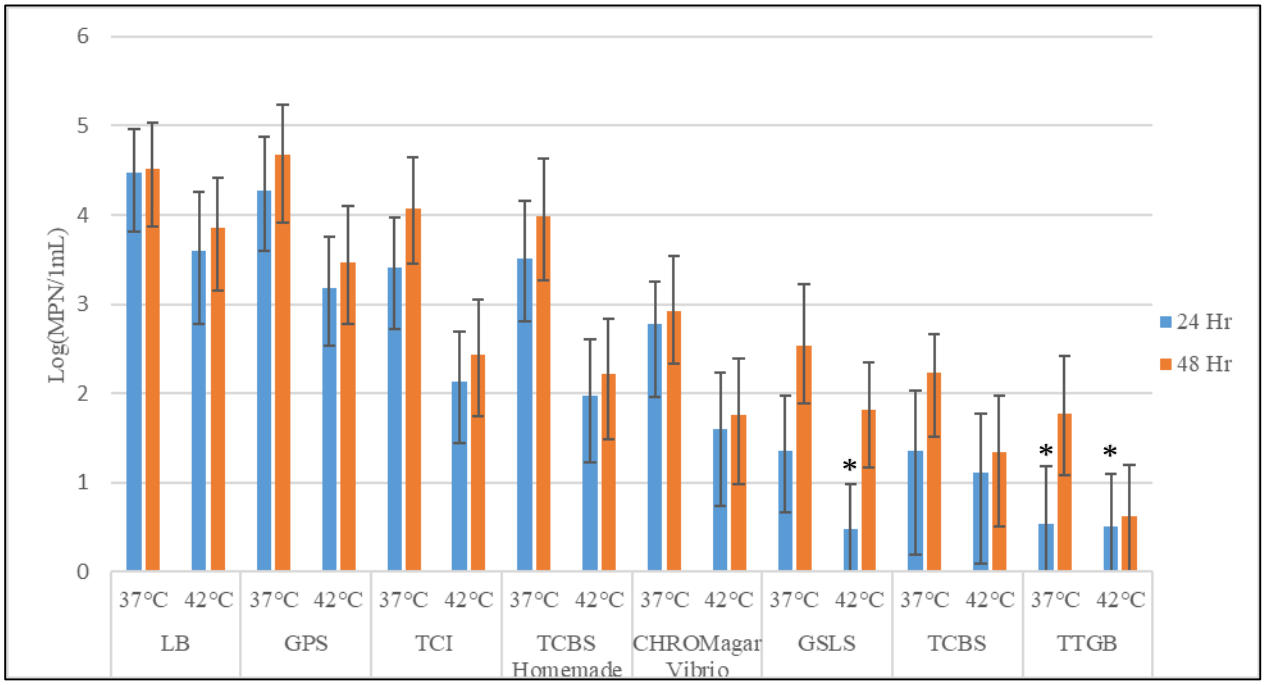

Figure 6. Quantification of all non-target organisms from 1% primary effluent in surface water in selective broth media as determined by multiple well MPN assay (n = 3). Log MPN values marked with (*) were estimated from plates with no exhibited growth from an undiluted inoculum. Examination of TTGB and LB were conducted separately from the other media; so, these experiments are not paired.

To further examine the exclusivity of these broth media, we describe the frequency of observing *any* non-target growth in each selective broth medium. Growth was observed and reported based on any changes in turbidity, or chromogenic or fluorogenic changes in the

medium. This frequency is described across the three replicate trials. These frequencies are described in **Table 12**. The most appropriate selective medium would give no growth of any bacteria across any of the three trials. Only one medium, GSLS was consistently effective at inhibiting non-target organisms from the 1% primary effluent-surface water matrix across three replicate trials, at an incubation of 42°C for 24 hours. The TTGB medium was moderately effective at inhibition of non-target organisms at 42°C for 24 or 48 hours; non-target organisms were detected in only one of the three replicate trials.

Table 12. Frequency of observing *any* non-target growth in selective broth media for *Vibrio* spp. across three replicate trials. Values represent the number of trials for which growth was observed on selective broth media, when inoculated with a 1% primary effluent in surface water matrix. Frequency is reported below as number of trials (out of three total trials).

Medium	37°C - 24 Hr	37°C - 48 Hr	42°C - 24 Hr	42°C - 48 Hr
LB	3	3	3	3
GSP	3	3	3	3
TCI	3	3	3	3
GSLS	3	3	0	3
CV	3	3	3	3
TCBS	3	3	3	3
TCBS Homemade	3	3	3	3
TTGB	2	3	1	1

Quantification of Non-Target Organisms from Surface Waters

The exclusivity of Vibrio-specific broth media was examined by quantifying the extent of growth of all non-target organisms cultured from 50mL samples of natural surface waters, when cultured in duplicate ten-tube MPN assays with 5 mL/tube). Growth was observed and reported based on any changes in turbidity, or chromogenic or fluorogenic changes in the broth medium. The quantification of these organisms is shown in **Figure 7**. This MPN assay of a total 100mL volume was intended to quantify the background organisms that might interfere with results from

an adapted CBT while attempting to quantify *V. cholerae* from water samples. An ideal candidate medium would exclude or inhibit the growth of all non-target organisms in a 100-mL sample volume.

Of the three selective broth media examined, CV was most selective in inhibiting the growth of non-target microorganisms from a 100-mL-volume surface water sample. The average MPN and 95% confidence interval of interfering from undiluted 100 mL water samples was 4.6 (0.74, 16.2) MPN/100mL (n = 3). The mean concentration includes an estimated MPN from a trial where no growth was observed in either set of the duplicate ten-tube assays. Growth of non-target organisms appeared white or turbid in most tubes of CV, and this growth was concentrated at the bottom of tubes. No tubes appeared blue, the chromogenic change of the positive control *V. cholerae* El Tor, Ogawa. Growth and color change due to metabolism of chromogenic substrates of the *V. cholerae* positive control were concentrated at the top of the tube, even though the cultures were incubated on a shaker tray.

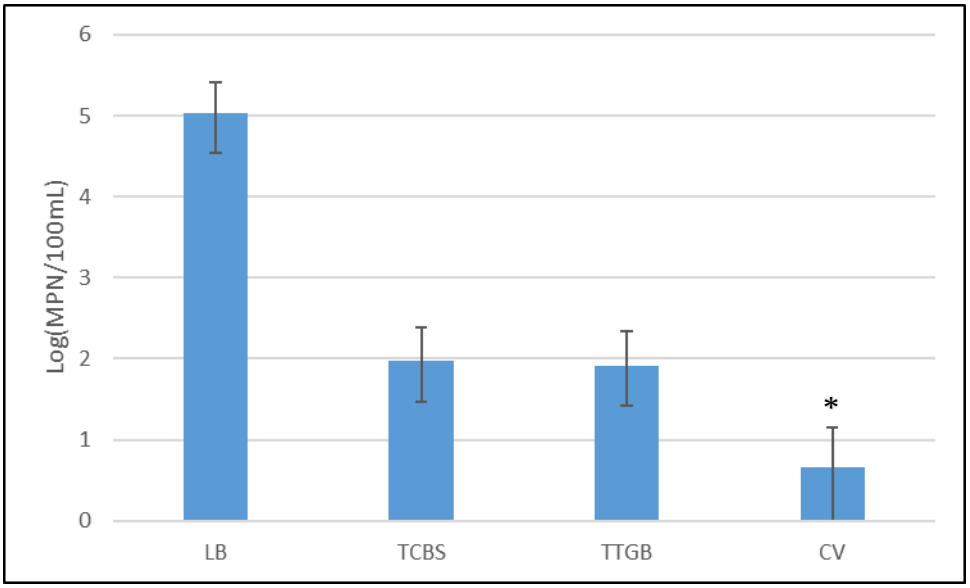

Figure 7. Quantification of all non-target organisms from natural surface waters as determined by ten-tube MPN assay of duplicate 50-mL sample volumes (n = 3). Values marked with (*) were estimated from tubes with no exhibited growth from an undiluted inoculum. Values are determined from different sampling events; so, these data are not paired.

Based on this ten-tube assay of 100 mL volumes of surface water, the CV medium gave the greatest specificity by either preventing the growth in 1 of 3 samples or allowing the least amount of growth. The extent of exclusivity was comparable between TCBS and TTGB based on the extent of growth in this ten-tube MPN assay. The average concentrations of non-target organisms were 102 (32, 260) MPN/100mL and 33 (4.2, 140) MPN/100mL respectively. In order to quantify interfering organisms, the original surface water sample was diluted 10-fold to ensure that scores were based on both positive and negative tubes.

Analysis by the Kruskal-Wallis Test suggests there is a significant difference between the median concentrations of non-target organisms detected by any of the four media (p-value = 0.02374, n = 3). The difference in median concentration between the selective media only (TCBS, TTGB, CV) was moderately significant (p = 0.06081, n = 3). There was no difference in

median concentration between LB, TCBS, TTGB or CV based on the step-down Wilcoxon Rank-Sum Test, most likely due to limited sample size.

Identification of Non-Target Organisms in Taurocholate Tellurite Peptone (TTGB)

Non-target organisms, cultured from natural surface waters in TTGB, and then isolated as individual colonies on agar media were identified by MALDI-TOF MS. Of the fifteen tubes spiked with natural surface waters, six tubes fluoresced under long-wave UV light as a false positive result. Organisms from these tubes were streaked to isolation and differentiated based on colony morphology. Sixteen isolates were submitted for identification by MALDI-TOF MS. Of these isolates, five were identified as *Bacillus pumilus*, nine were identified as *Enterococcus casseliflavus*, and one was identified as *Paenibacillus pabuli*. One isolate could not be identified.

Examining the Plating Efficiency of Media for Culturing *V. cholerae*

Quantification and Enumeration of V. cholerae (Multiple Tube Format)

The efficiency of selective media for *V. cholerae* was examined by comparing the quantification of *V. cholerae* in a multiple tube MPN assay to the enumeration of *V. cholerae* by spread plate on agar media. TTGB served as the broth medium for quantal methods in a multiple tube format. MPN results were compared to CFU results from spread plate on LB, TCBS, and TTGA. These assays were conducted in parallel; the results are matched-paired estimations of the concentration of each overnight culture. Estimated concentrations are described separately for each strain of *V. cholerae* and presented in **Figure 8**.

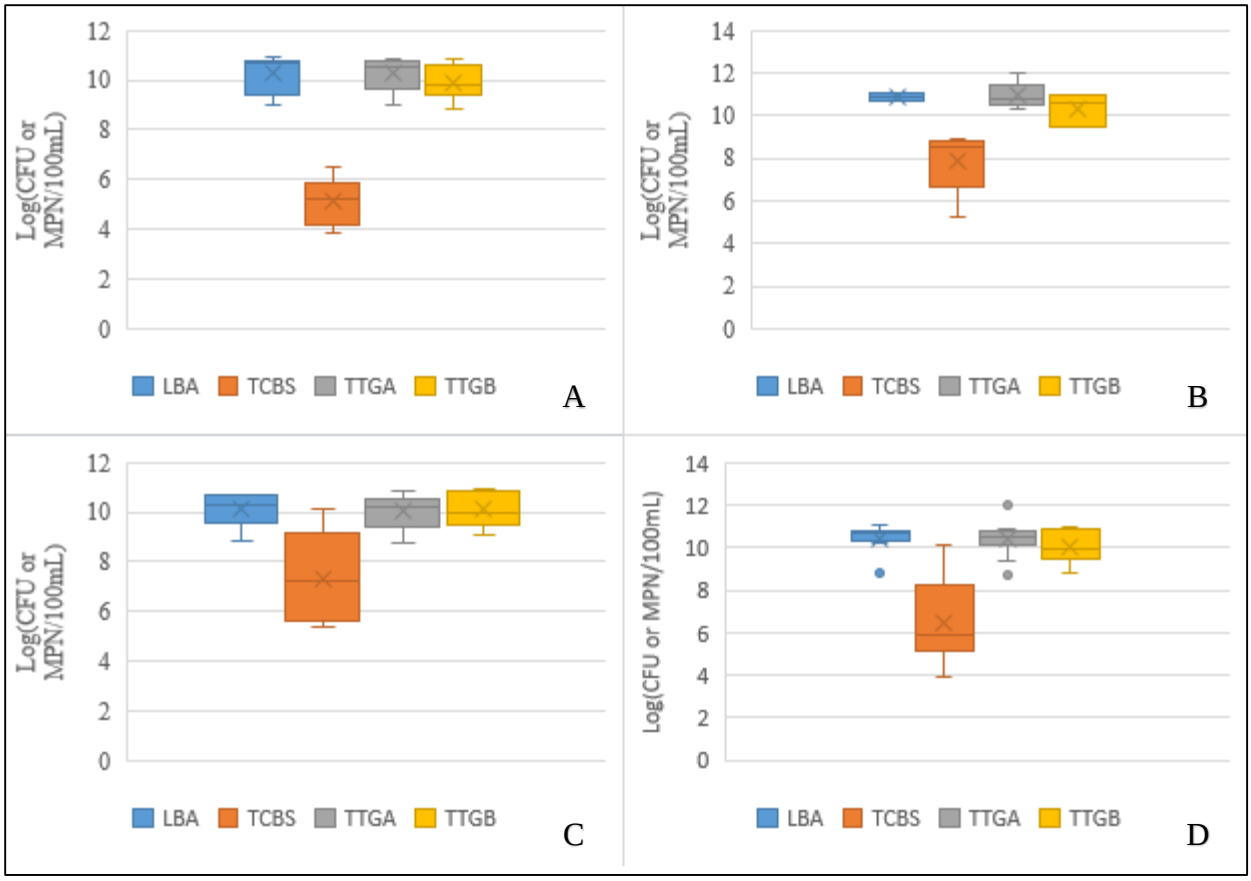

Figure 8. Plating efficiency of TTGB for the quantification of *V. cholerae* by multiple tube test. **A.** Quantification and enumeration of *V. cholerae* O139 (n = 8). **B.** Quantification and enumeration of *V. cholerae* O1 El Tor Ogawa (n = 5). **C.** Quantification and enumeration of *V. cholerae* Non-O1 (n = 5). **D.** Pooled concentrations to compare the efficiency of selective and non-selective media for quantification and enumeration of *V. cholerae* (n = 18).

Quantification of *V. cholerae* O139 by the MTT assay with TTGB was comparable to enumeration of *V. cholerae* O139 by spread plate on selective TTGA and non-selective LBA. The average concentrations and 95% confidence intervals were $2.17*10^{10}$ ($6.06*10^{9}$, $6.84*10^{10}$) MPN/100mL, $3.41*10^{10}$ ($2.98*10^{10}$, $3.84*10^{10}$) CFU/100mL, and $4.28*10^{10}$ ($3.75*10^{10}$, $4.82*10^{10}$) CFU/100mL. The efficiency of TCBS was limited for enumeration of *V. cholerae* O139; the average concentration was $6.85*10^{5}$ ($6.00*10^{5}$, $7.71*10^{5}$) CFU/100mL.

Median concentrations of *V. cholerae* O139 were compared by the Wilcoxon Signed-Rank Test, appropriate for non-parametric and paired data. The results of this statistical analysis

are presented in **Table 13**. Concentrations estimated by TCBS were significantly different than those estimated by LBA, TTGA, and TTGB. There were no significant differences between the concentrations estimated by LBA, TTGA, or TTGB.

Table 13. Results of the Wilcoxon Signed-Rank Test comparing median concentrations for *V. cholerae* O139 as determined by multiple tube test with TTGB and by spread plate on LBA, TCBS, and TTGA (n = 8).

Medium 1	Medium 2	P-Value
LBA	TCBS	0.007813
LBA	TTGA	0.4609
LBA	TTGB	0.3125
TCBS	TTGA	0.007813
TCBS	TTGB	0.007813
TTGA	TTGB	0.3125

Quantification of *V. cholerae* O1 El Tor Ogawa by the MTT assay with TTGB was comparable to enumeration of *V. cholerae* Non-O1 by spread plate on selective TTGA and non-selective LBA. The average concentrations were $4.57*10^{10}$ ($9.63*10^{9}$, 1.59^{11}) MPN/100mL, $2.47*10^{11}$ ($1.98*10^{11}$, $2.96*10^{11}$) CFU/100mL, and $8.07*10^{10}$ ($6.45*10^{10}$, $9.68*10^{10}$) CFU/100mL respectively (n = 5). The efficiency of TCBS was limited for enumeration of *V. cholerae* O1 El Tor Ogawa; the average concentration was $3.41*10^{8}$ ($2.73*10^{8}$, $4.09*10^{8}$) CFU/100mL. The wide distribution of CFU results on TCBS can be observed in the box-and-whisker plot in **Figure 8**.

Median concentrations of *V. cholerae* O1 El Tor Ogawa were compared by the Wilcoxon Signed-Rank Test. The results of this statistical analysis are presented in **Table 14**. There were no significant differences in median concentration across the different media, though the power of this analysis is limited by the number of replicates (n = 5). The difference in median

concentration between TCBS and all other media is nearly significant, with a p-value of 0.0625. The difference between LB and TTGB is nearly significant as well, with a p-value of 0.0625.

Table 14. Results of the Wilcoxon Signed-Rank Test comparing median concentrations for *V. cholerae* O1 El Tor Ogawa as determined by multiple tube test with TTGB and by spread plate on LBA, TCBS, and TTGA (n = 5).

Medium 1	Medium 2	P-Value
LB	TCBS	0.0625
LB	TTGA	0.625
LB	TTGB	0.0625
TCBS	TTGA	0.0625
TCBS	TTGB	0.0625
TTGA	TTGB	0.3125

Quantification of *V. cholerae* Non-O1 by the MTT assay with TTGB was comparable to enumeration of *V. cholerae* Non-O1 by spread plate on selective TTGA and non-selective LBA. The average concentrations and 95% confidence intervals were $3.45*10^{10}$ ($7.36*10^{9}$, $9.09*10^{10}$) MPN/100mL, $2.41*10^{10}$ ($1.93*10^{10}$, $2.90*10^{10}$) CFU/100mL, and 2.76 ($2.21*10^{10}$, $3.32*10^{10}$) CFU/100mL respectively (n = 8). The efficiency of TCBS was limited for enumeration of *V. cholerae* Non-O1; the average concentration was $2.82*10^{9}$ ($2.26*10^{9}$, $3.39*10^{9}$) CFU/100mL. respectively (n = 5).

Median concentrations of *V. cholerae* Non-O1 were compared by the Wilcoxon Signed-Rank Test. The results of this statistical analysis are presented in **Table 15**. There were no significant differences in median concentration across the different media, though the power of this analysis is limited by the number of replicates (n = 5). The difference in median concentration between TCBS and all other media is nearly significant, with a p-value of 0.0625.

Table 15. Results of the Wilcoxon Signed-Rank Test comparing median concentrations for V. cholerae Non-O1 as determined by multiple tube test with TTGB and by spread plate on LBA, TCBS, and TTGA (n = 5).

Medium 1	Medium 2	P-Value
LB	TCBS	0.0625
LB	TTGA	0.4375
LB	TTGB	0.625
TCBS	TTGA	0.0625
TCBS	TTGB	0.0625
TTGA	TTGB	0.625

All estimated concentrations were pooled to compare the efficiencies of each plating medium. The results are depicted in Figure 8D. A Kruskal-Wallis analysis was conducted to compare the plating efficiency of LBA, TCBS, TTGA, and TTGB. The Kruskal-Wallis test is a non-parametric equivalent to an ANOVA. The Kruskal-Wallis test assesses differences in a continuous dependent variable (median concentration), given a categorical independent variable (strain type, medium type). The results, reported in **Table 16**, demonstrate that there is no significant difference between the overnight concentrations of *V. cholerae* O139, *V. cholerae* O1 El Tor Ogawa, or *V. cholerae* Non-O1. There is a significant difference between the plating efficiencies of these four media.

Table 16. Results of the Kruskal-Wallis analysis comparing pooled median concentrations of *V. cholerae* by strain type and by medium type (n = 18).

Comparison by:	p-value
Strain (O139, El Tor Ogawa, Non-O1)	0.0951
Medium (LBA, TCBS, TTGA, TTGB)	3.236e-08

To evaluate these differences, a Wilcoxon Signed-Rank Test was conducted to compare the efficiencies of LBA, TCBS, TTGA and TTGB. This paired analysis compares the paired data on the median concentrations of each overnight, as determined by different culture media. The results of this analysis are described in **Table 17**. Concentrations estimated by TCBS were significantly different than those estimated by LBA, TTGA, and TTGB. There were no significant differences between the concentrations estimated by LBA, TTGA, or TTGB.

Table 17. Results of the Wilcoxon Signed-Rank Test to compare pooled median concentrations of *V. cholerae* by medium type (n = 18).

Medium 1	Medium 2	p-value
LB	TCBS	7.629e-06
LB	TTGA	0.154
LB	TTGB	0.08143
TCBS	TTGA	7.629e-06
TCBS	TTGB	7.629e-06
TTGA	TTGB	0.2121

Quantification and Enumeration of *V. cholerae* (Multiple Well Format)

The efficiency of selective media for *V. cholerae* was examined by comparing the quantification of *V. cholerae* in a multiple well MPN assay to the enumeration of *V. cholerae* by spread plate. Broth adaptations of LB, TCBS, TTGB and CV were employed in a multiple well MPN assay. The MPN results were compared to CFU results from spread plate on solid agars of LBA, TCBS, TTGA, and CV. These assays were conducted in parallel; the results are matched-paired estimations of the concentration of each overnight culture. Estimated concentrations are reported separately for each strain of *V. cholerae* and presented in **Figure 9**.

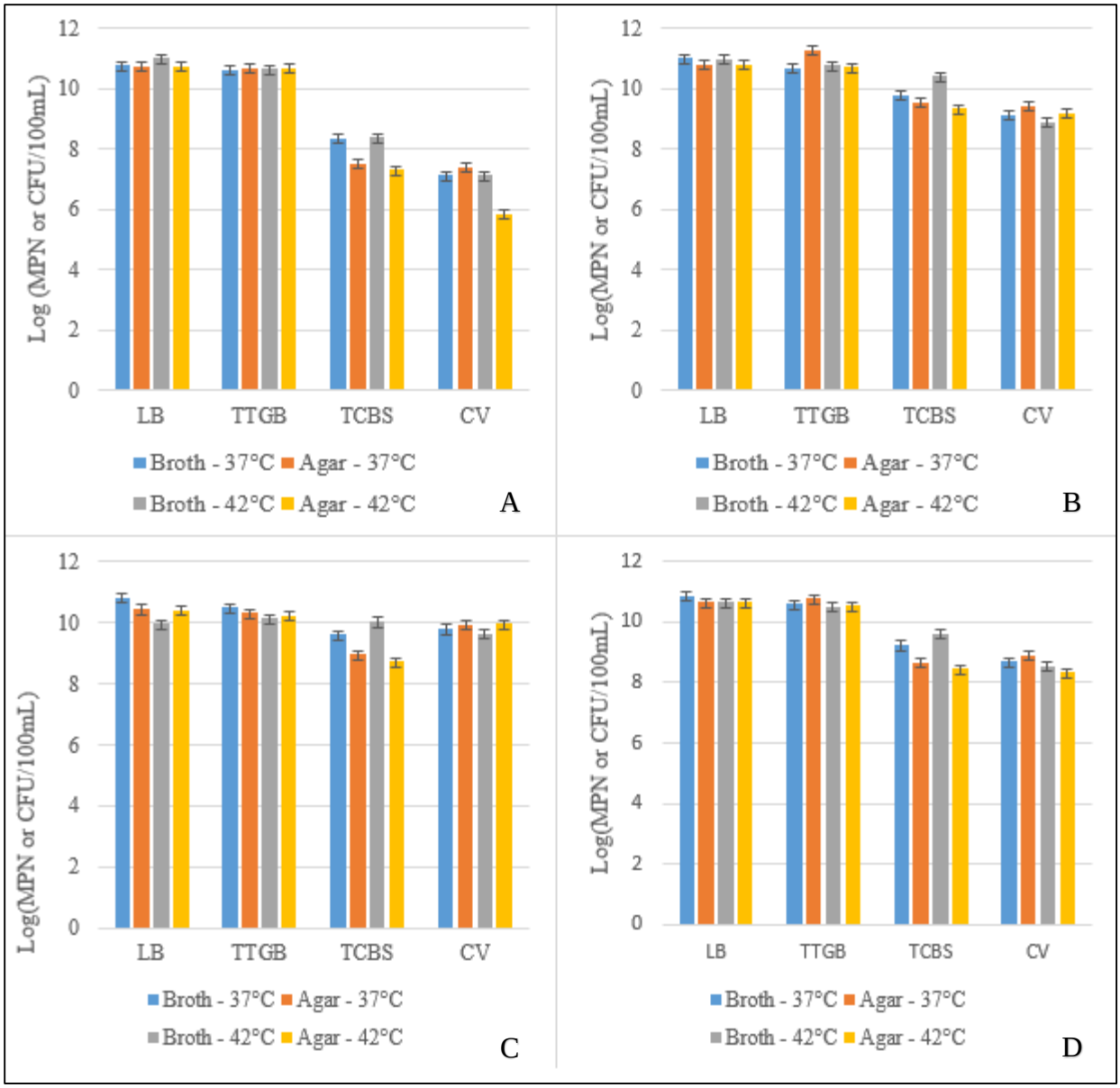

Figure 9. Plating efficiency of selective culture media for the quantification of *V. cholerae* by multiple well test and compared to corresponding agar media enumeration. **A.** Quantification and enumeration of *V. cholerae* O139 (n = 3). **B.** Quantification and enumeration of *V. cholerae* O1 El Tor Ogawa (n = 3). **C.** Quantification and enumeration of *V. cholerae* Non-O1 (n = 3). **D.** Pooled concentrations to compare the efficiency of media for quantification and enumeration of all *V. cholerae* types tested (n = 9).

Median concentrations of *V. cholerae* O139 were compared by the Kruskal-Wallis test to assess differences based on medium type, incubation temperature, and medium format (broth or

agar) as presented in **Table 18**. There are no significant differences in estimated concentrations based on incubation temperature (37 or 42^{o}C), or medium format (broth or agar). There was a statistically significant difference in the estimated concentrations based on medium type; therefore, a statistically significant difference in plating efficiency between the different culture media.

Table 18. Results of the Kruskal-Wallis test comparing median concentrations of *V. cholerae* O139 by medium type, incubation temperature, and medium format.

Category	p-value
Medium (LB, TTGB, TCBS, CV) (n = 12)	4.001e-08
Temperature (37°C, 42°C) (n = 24)	0.8447
Format (Broth, Agar) (n = 24)	0.6061

To evaluate the difference in efficiency between these culture media, a Wilcoxon Signed-Rank Test was conducted to compare the median concentrations of LBA, TCBS, TTGB and CV. The results of this statistical analysis are presented in **Table 13**. There were significant differences between the non-selective medium, LB, and each selective medium. There were significant differences between TTGB and the two selective agar media; but between TCBS and CV, the median concentrations were not quite significant, with a p-value of 0.064.

Table 19. Results of the Wilcoxon Signed-Rank Test comparing median concentrations of *V. cholerae* O139 by medium type (n = 12).

Medium 1	Medium 2	P-Value
LB	TTGB	0.01656* estimated with ties
LB	TCBS	0.0004883
LB	CV	0.0004883
TTGB	TCBS	0.0004883
TTGB	CV	0.0004883
TCBS	CV	0.06396

Median concentrations of *V. cholerae* O1 El Tor Ogawa were compared by the Kruskal-Wallis test to assess differences based on medium type, incubation temperature, and medium format. The results of this analysis are presented in **Table 20**. There were no differences in estimated concentrations based on incubation temperature, or medium format. There was a significant difference in the estimated concentrations based on medium type; therefore, a significant difference in plating efficiency between the different culture media.

Table 20. Results of the Kruskal-Wallis test comparing median concentration of *V. cholerae* O1 El Tor Ogawa by medium type, temperature, and medium format.

Category	p-value
Medium (LB, TTGB, TCBS, CV) (n = 12)	1.092e-07
Temperature (37°C, 42°C) (n = 24)	0.8365
Format (Broth, Agar) (n = 24)	0.6631

To evaluate the difference in efficiency between these culture media, a Wilcoxon Signed-Rank Test was conducted to compare the median concentrations of LBA, TCBS, TTGB and CV. The results of this statistical analysis are presented in **Table 21**. There were significant differences in median concentration between LB and TCBS as well as between LB and CV but not between LB an TTGB. There were also significant differences in median concentrations between all pairs of selective media.

Table 21. Results of the Wilcoxon Signed-Rank Test comparing median concentrations of *V. cholerae* O1 El Tor Ogawa as determined by LB, TTGB, TCBS and CV (n = 12).

Medium 1	Medium 2	p-value
LB	TTGB	0.1259
LB	TCBS	0.0004883*
LB	CV	0.0004883
TTGB	TCBS	0.002441
TTGB	CV	0.0004883
TCBS	CV	0.03418

*p-values estimated with ties

Median concentrations of *V. cholerae* Non-O1 were compared by the Kruskal-Wallis test to assess differences based on medium type, incubation temperature, and medium format. The results of this analysis are presented in **Table 22**. There were no differences in estimated concentrations based on incubation temperature, or medium format. There was a significant difference in the estimated concentrations based on medium type; indicating that there were differences in plating efficiencies between the different culture media.

Table 22. Results of the Kruskal-Wallis test comparing median concentration of *V. cholerae* Non-O1 by medium type, temperature, and medium format.

Category	p-value
Medium (LB, TTGB, TCBS, CV) (n = 12)	0.009505
Temperature (37°C, 42°C) (n = 24)	0.5918
Format (Broth, Agar) (n = 24)	0.7993

To evaluate the difference in plating efficiency between these culture media, a Wilcoxon Signed-Rank Test was conducted to compare pairwise the median concentrations of LBA, TCBS, TTGB and CV. The results of this statistical analysis are presented in **Table 23**. There were statistically significant differences in median concentrations between all pairs of culture media except between TCBS and CV, which was not quite significant with a p-value of 0.064.

Table 23. Results of the Wilcoxon Signed-Rank Test comparing median concentrations of *V. cholerae* Non-O1 as determined by LB, TTGB, TCBS and CV (n = 3).

Medium 1	Medium 2	p-value
LB	TTGB	0.03667**
LB	TCBS	0.0004883**
LB	CV	0.002441
TTGB	TCBS	0.009277
TTGB	CV	0.003857**
TCBS	CV	0.06396

**p-values estimated with zeros

The results of all assays were pooled to compare the median concentrations of *V. cholerae*. A Kruskal-Wallis test was conducted to compare the median concentrations of *V. cholerae* O139, *V. cholerae* O1 El Tor Ogawa, and *V. cholerae* Non-O1. As shown in **Table 24**, there was a significant difference in median concentrations between the overnight samples based on strain type and based on medium type. There was no significant difference in median concentration of *V. cholerae* by incubation temperature or medium format (broth or agar).

Table 24. Results of the Kruskal-Wallis test comparing pooled estimates of the median concentration of *V. cholerae* by strain type, medium type, temperature, and medium format.

Category	p-value
Strain (O139, O1, Non-O1) (n = 48)	0.01677
Medium (LB, TTGB, TCBS, CV) (n = 36)	< 2.2e-16
Temperature (37°C, 42°C) (n = 72)	0.6617
Format (Broth, Agar) (n = 72)	0.3684

The pooled estimated concentration of *V. cholerae* are reported by medium type in **Figure 10**. The median concentration was compared between the different media with a Wilcoxon Signed-Rank Test. The results are reported in **Table 25**. There is no agreement between the median concentration of *V. cholerae* determined by LB/LBA and any of the selective media. There is no agreement between TTGB and TCBS or CV. There is only agreement between median concentrations estimated by TCBS and CV.

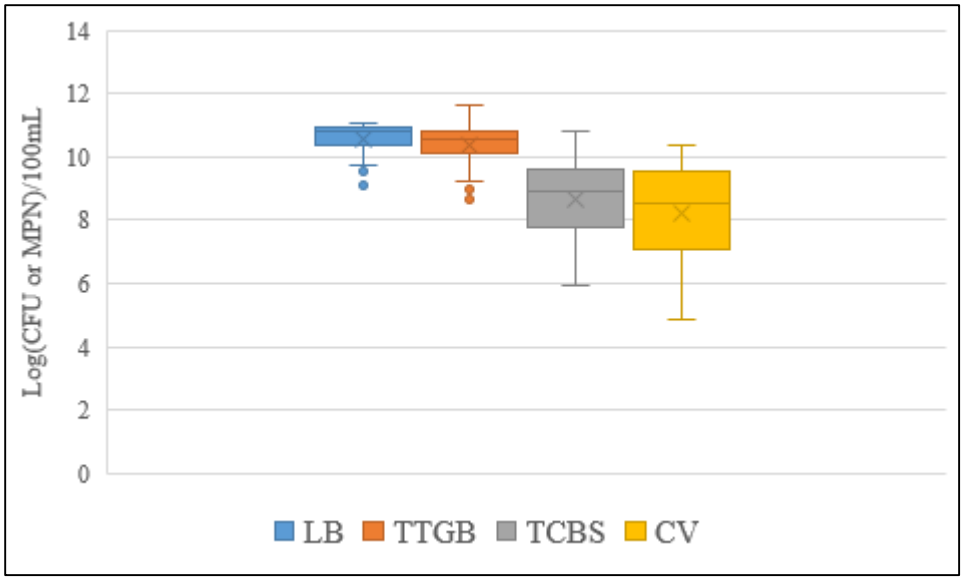

Figure 10. Estimated concentrations of *V. cholerae* as determined by LBA/LB, TTGA/TTGB, TCBS/TCBS Broth, and CV/CV Broth. Plot includes counts across three strain types, medium format and incubation temperatures.

Table 25. Results of the Wilcoxon Signed-Rank Test comparing median concentrations of *V. cholerae* as determined by LB, TTGB, TCBS and CV (n = 36).

Medium 1	Medium 2	p-value
LB	TTGB	0.0005976
LB	TCBS	9.604e-10
LB	CV	1.455e-10
TTGB	TCBS	2.631e-08
TTGB	CV	2.588e-07**
TCBS	CV	0.7386

**estimated with zeros

The candidate selective media were then compared by examining the productivity ratio of each medium in relationship to the non-selective LBA or LB medium. The productivity ratio is defined as a measure of the relative plating efficiency of the selective media, by benchmarking their concentrations against those from a non-selective medium. The productivity ratio was determined with the following equation:

$$PR = \frac{Count\ by\ Selective\ Medium}{Count\ by\ Non-Selective\ Medium}$$

The average productivity ratio of each medium is reported in **Figure 11**. This average considers the productivity ratio of each medium, across three strains of *V. cholerae*, broth and agar versions of the selective medium, and both incubation temperatures. The average productivity ratio of TTGB (0.86) is greater than the average productivity ratios of TCBS or CV (0.077 and 0.079 respectively).

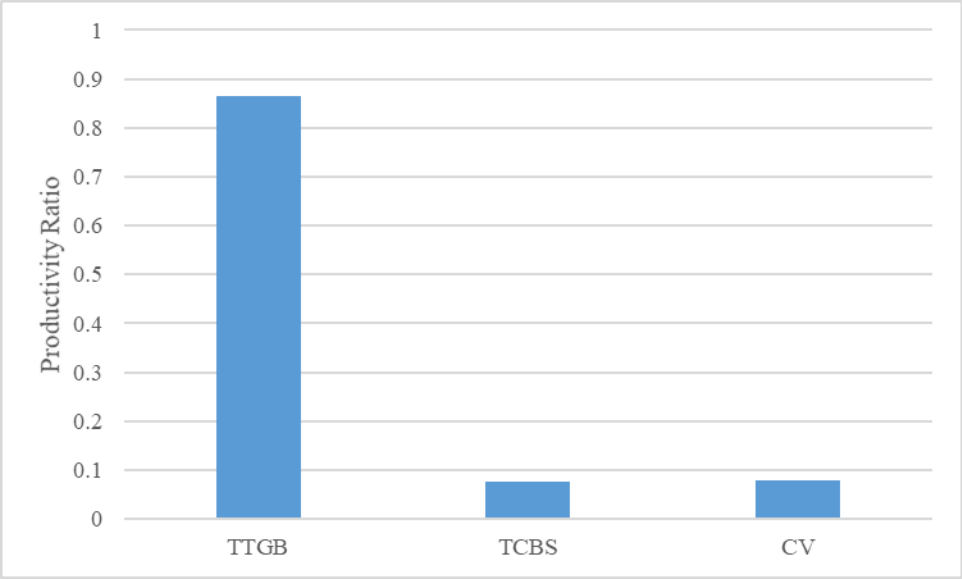

Figure 11. Average productivity ratio (relative plating efficiency) of each selective medium. The productivity ratio compares selective media counts to those from either LBA or LB (n = 36).

The average productivity ratio of each medium was compared by an ANOVA analysis, by strain type, medium type, medium format, and incubation temperature. The results of the ANOVA analysis are reported in **Table 26**. There were no significant differences in productivity ratio between the strain types, between incubation temperature, or between format (broth or agar). There was a significant difference in average productivity ratio between the three selective media.

Table 26. Results of the ANOVA test comparing the productivity ratio of each medium by strain type, medium type, temperature, and medium format.

Category	p-value
Strain (O139, O1, Non-O1) (n = 36)	0.375
Medium (TTGB, TCBS, CV) (n = 36)	1.66e-05
Temperature (37°C, 42°C) (n = 54)	0.647
Format (Broth, Agar) (n = 54)	0.274

These productivity ratios were compared statistically by paired t-tests; the results are reported in **Table 27**. The productivity ratio of TTGB differs significantly from the productivity ratios of TCBS and CV. The productivity ratios of TCBS and CV do not differ significantly from one another.

Table 27. Results of the Paired T-Test comparing productivity ratios of TTGB, TCBS and CV (n = 36).

Medium 1	Medium 2	p-value
TTGB	TCBS	0.001273
TTGB	CV	0.001295
TCBS	CV	0.9352

Examining the Plating Efficiency of Basal Media for Culturing *V. cholerae*

Screening Basal Media for Growth of *V. cholerae*

Basal media were screened for overnight growth of *V. cholerae* to quantify plating efficiency. Growth of *V. cholerae* El Tor Ogawa was inhibited at the highest concentration of NaCl, regardless of the nutrient base medium (**Table 28**). Peptone alone in the absence of NaCl was insufficient for growth. However, the addition to peptone of either NaCl at 1 or 3% or yeast and beef extract promoted growth at 0% NaCl as well as at 1 and 3% NaCl. The base composition of Brain Heart Infusion Broth incorporates 0.5% NaCl, therefore no additional NaCl is necessary for growth of *V. cholerae*. Only basal media that supported growth of all strains were considered for further evaluation.

Table 28. Screening of basal media for overnight growth of *V. cholerae*. Overnight cultures of pure strains of *V. cholerae*. Wells were observed for turbidity, indicating growth (+), or absence of growth (-).

Medium	*V. cholerae* O139	*V. cholerae* El Tor Ogawa	*V. cholerae* Non-O1
Brain Heart Infusion	+	+	+
BHI + 1% NaCl	+	+	+
BHI + 3% NaCl	+	+	+
BHI + 5% NaCl	+	-	+
Peptone	-	-	-
Peptone + 1% NaCl	+	+	+
Peptone + 3% NaCl	+	+	+
Peptone + 5% NaCl	+	-	+
Peptone, Yeast Extract and Beef Extract	+	+	+
Peptone, YE, BE + 1% NaCl	+	+	+
Peptone, YE, BE + 3% NaCl	+	+	+
Peptone, YE, BE + 5% NaCl	+	-	+

Plating Efficiency of Basal Media for Quantification of *V. cholerae*

Basal media that supported growth of all three *V. cholerae* strains were further examined for the efficient quantification and results are presented in **Figure 12**. Growth of *V. cholerae* may be supported by Brain Heart Infusion Broth, an alkaline peptone solution, or peptone supplemented with yeast and beef extract. The Log_{10} concentration ranged from a high of >11.0 to a low of about 9.0.

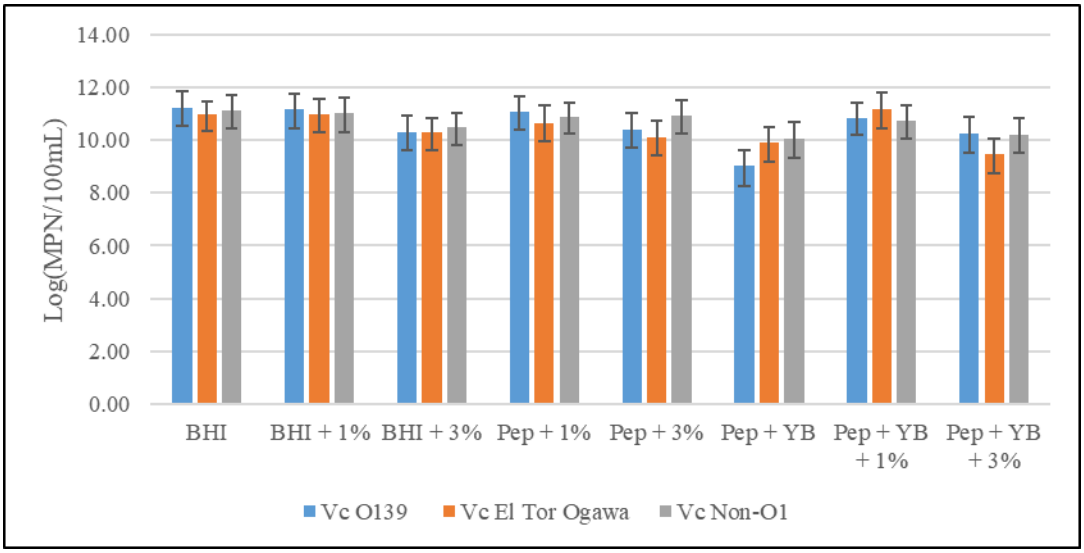

Figure 12. Efficiency of basal broth media for the quantification of *V. cholerae* (n = 3).

The plating efficiencies of these basal media were compared with the Kruskal-Wallis test to compare median concentrations by strain type, base medium type, added NaCl, and by final medium composition. The results of this analysis are summarized in **Table 29**. There was no difference in median concentration when compared by strain type. There was a significant difference, however, in the median concentration of *V. cholerae* when categorized by base medium type, by percentage of added NaCl, and by media composition.

Table 29. Results of the Kruskal-Wallis test comparing median concentrations of *V. cholerae* by strain type, basal medium type, added NaCl, and complete composition of the basal medium (n = 9).	
Category	p-value
Strain (O139, El Tor Ogawa, Non-O1)	0.5699
Basal Media - Type (BHI, Peptone, Peptone + YB)	0.007606
Basal Media - Salinity (0%, 1%, 3% added NaCl)	0.0001927
Basal Media – Complete Composition (BHI, Pep, Pep + YB at Varying %NaCl)	5.285e-06

The results of these MPN assays were pooled to compare basal media for optimal growth of *V. cholerae*. These pooled concentrations are presented in **Figure 13** and reported based on the complete basal medium composition.

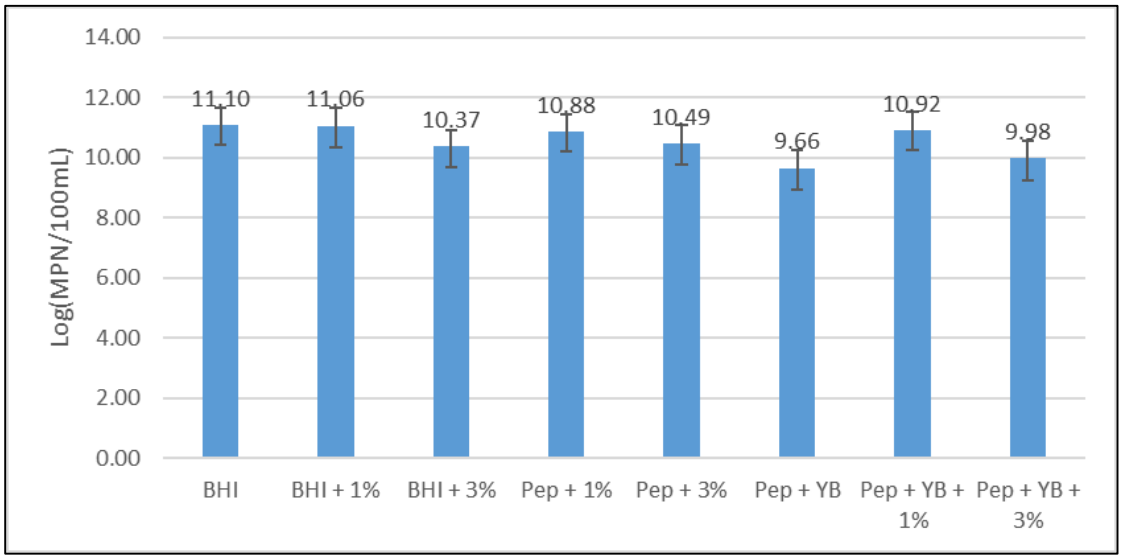

Figure 13. Comparison of basal broth media for the quantification of *V. cholerae* (n = 9).

Basal media were ranked by mean concentration to compare the plating efficiency. The median concentrations of six basal media were compared by the Wilcoxon Signed-Rank Test. Results of this analysis are reported in **Table 30**. The only significant difference in median

concentration was between BHI and BHI + 3% NaCl; however, BHI vs. Peptone + 3% NaCl was nearly significant at p = 0.055. Optimal growth of *V. cholerae* was supported similarly by the following basal media: BHI; BHI + 1% NaCl; Peptone + 1% NaCl; Peptone + Yeast and Beef Extract + 1% NaCl and perhaps Peptone + 3% NaCl.

Table 30. Results of the Wilcoxon Signed-Rank Test comparing the median concentration of *V. cholerae* (pooled) by basal medium (n = 9).

Medium 1	Medium 2	p-value
BHI	BHI + 1% NaCl	0.5533*
BHI	BHI + 3% NaCl	0.009091*
BHI	Pep + 1% NaCl	0.1829*
BHI	Pep + 3% NaCl	0.05469
BHI	Pep + YB + 1% NaCl	0.3008
BHI + 1% NaCl	Pep + 1% NaCl	0.1921*
BHI + 1% NaCl	Pep + 3% NaCl	0.1073
BHI + 1% NaCl	Pep + YB + 1% NaCl	0.5286*
Pep + 1% NaCl	Pep + 3% NaCl	0.1289
Pep + 1% NaCl	Pep + YB + 1% NaCl	0.4768*
Pep + 3% NaCl	Pep + YB + 1% NaCl	0.1641

Antibiotic Susceptibility Testing of *V. cholerae* and Non-*V. cholerae* Bacteria

Four antibiotics - fosfomycin, streptomycin, ampicillin, and vancomycin – were evaluated as antibiotic supplements for selective culture media for *V. cholerae*. The minimum inhibitory concentrations of each antibiotic are reported in **Table 31**. MIC Values were determined by broth microdilution assays in MHB, using antibiotic concentrations in the range of 0.5 – 256 mg/L.

Table 31. Average minimum inhibitory concentration (MIC) values of four antibiotics (n = 3). MICs shown in bold for each antibiotic are lower than the MICs of all *V. cholerae* strains tested.

Strain	Average MIC (mg/L)			
	Fosfomycin	Streptomycin	Ampicillin	Vancomycin
V. cholerae Serotype O139	64	203	32	213
V. cholerae O1 El Tor Ogawa	48	>256	53	>256
V. cholerae Non-O1	16	37	21	>256
V. mimicus	32	53	32	192
Bacillus pumilus	43	**7**	**0.75**	**0.33**
Enterococcus casseliflavus	75	**27**	**4**	**23**
Paenibacillus pabuli	203	>256	**16**	**96**
Escherichia coli	**14**	**16**	27	**192**
Pseudomonas aeruginosa	27	**15**	>256	>256
Proteus mirabilis	**4**	45	**13**	>256
Aeromonas hydrophila	**8**	117	>256	**149**

Susceptibility to all four antibiotics varied between the three strains of *V. cholerae* tested. Between the three strains, average MICs for the *V. cholerae* strains ranged from 16-64 mg/L for fosfomycin from 37 mg/L to >256 mg/L for streptomycin, from 21-53 mg/L for ampicillin and from 213->256 mg/L for vancomycin. *Vibrio cholerae* was most resistant to vancomycin (all average MIC concentrations >200 mg/L) and was relatively susceptible to ampicillin (all average MIC concentrations < 50 mg/L). Variation in MIC values was observed between replicate assays; all MIC values are reported in Appendix H. This variation was considered when

determining an antibiotic supplement for selective culture media. The lowest MIC values reported against *V. cholerae* are reported in **Table 32**, alongside the highest MIC values reported against non-target organisms. Among the three strains of *V. cholerae*, *V. cholerae* Non-O1 was the most susceptible to fosfomycin, streptomycin and ampicillin. This strain was the most resistant to vancomycin.

Table 32. Lowest MIC values observed for three strains of *V. cholerae* and highest MIC values observed for non-target test organisms. MIC values in bold were lower than that of the lowest MIC observed for any *V. cholerae* strain.

Strain	Lowest MIC (mg/L)			
	Fosfomycin	Streptomycin	Ampicillin	Vancomycin
V. cholerae O139	32	64	32	128
V. cholerae O1 El Tor Ogawa	32	64	32	256
V. cholerae Non-O1	8	16	16	>256
	Highest MIC (mg/L)			
V. mimicus	32	128	32	256
Bacillus pumilus	64	**8**	**2**	**1**
Enterococcus casseliflavus	128	32	**4**	**64**
Paenibacillus pabuli	256	>256	16	128
Escherichia coli	32	16	32	256
Pseudomonas aeruginosa	32	16	>256	>256
Proteus mirabilis	16	64	16	>256
Aeromonas hydrophila	16	256	>256	256

All four of the antibiotics tested may be effective antibiotic supplements for a selective culture media for *V. cholerae*. Of the seven non-target organisms examined, vancomycin inhibited five organisms at an average MIC value lower than those reported for *V. cholerae*. Ampicillin inhibited four organisms at an average MIC value lower than those reported for *V. cholerae.* Fosfomycin inhibited three organisms, and streptomycin inhibited two organisms at concentrations lower than the average MIC values reported for *V. cholerae*.

Vancomycin and ampicillin may be effective antibiotic supplements for inhibiting the growth of gram-positive organisms. Based on average MIC values, these antibiotics would inhibit all three environmental strains of gram-positive organisms - *Bacillus pumilus*, *Enterococcus casseliflavus*, and *Paenibacillus pabuli* – at lower concentrations than would inhibit *V. cholerae*.

Fosfomycin may be an effective antibiotic supplement for inhibiting the growth of non-vibrio gram-negative organisms. When compared to the average MIC for fosfomycin against *V. cholerae*, the average MIC values against *A. hydrophila*, *P. mirabilis*, and *E. coli* were lower than the MIC values for three strains of *V. cholerae*. The average MIC for fosfomycin against *P. aeruginosa* was greater only than the average MIC for *V. cholerae* Non-O1.

Streptomycin was the only antibiotic that inhibits *Pseudomonas aeruginosa* at a concentration lower than the average MIC for the three strains of *V. cholerae*. *Pseudomonas aeruginosa* is a gram-negative bacterium that is unaffected by bile salts, high pH, high incubation temperature or other growth conditions used for selective culture of *V. cholerae*.

The four antibiotics examined may be incorporated individually or in combination to improve the specificity of a culture medium for *V. cholerae*. If incorporated at 16 mg/L, ampicillin may inhibit *B. pumilus E. casseliflavus*, *Paenibacillus pabuli*, and *P. mirabilis*, but may inhibit the growth of *V. cholerae* Non-O1. Similarly, vancomycin could be incorporated at 64 mg/L to inhibit *B. pumilus* and *E. casseliflavus*. Fosfomycin may be incorporated into a selective culture medium at 8 mg/L to inhibit *A. hydrophila* and *P. mirabilis*, but may this may ultimately inhibit the growth of the *V. cholerae* Non-O1 strain. Streptomycin may be incorporated at 16 mg/L to inhibit *E. coli* and *P. aeruginosa*, but this may also inhibit *V. cholerae* Non-O1.

In developing new broth culture media for *V. cholerae*, fosfomycin and streptomycin were incorporated at 8 mg/L and 16mg/L respectively. The combination of these supplements may inhibit the growth of gram-negative organisms that would not be inhibited by other selective agents in the culture media. These selective agents most often target gram-positive organisms.

Examining the Exclusivity of Newly-Proposed Culture Media for Culturing *V. cholerae*

The exclusivity of newly-proposed and previously described broth culture media is presented in **Table 33**. Non-target test organisms were cultured in these selective media, along with *V. cholerae* O139, *V. cholerae* O1 El Tor Ogawa, and *V. cholerae* Non-O1. A detailed examination of the growth of these organisms is described in Appendix I.

Table 33. Growth of test organisms in selective broth culture media for *V. cholerae*. Values indicate the percentage of relevant strains that grew and appeared the same as *V. cholerae* in the culture media after 48h incubation.

Medium	Temp.	*V. cholerae* (3 Strains)	*V.mimicus* (1 Strain)	Gram-Negative (9 Strains)	Gram-Positive (6 Strains)
LB	37°C	100%	100%	100%	100%
	42°C	100%	100%	100%	100%
TCBS	37°C	100%	0%	0%	33%
	42°C	100%	0%	0%	33%
TTGB	37°C	100%	100%	11%	83%
	42°C	100%	0%	11%	83%
CV	37°C	67%	0%	11%	0%
	42°C	67%	0%	0%	0%
CV Plus*	37°C	67%	0%	0%	0%
	42°C	67%	0%	0%	0%
Cholera CBT Medium A	37°C	100%	0%	56%	33%
	42°C	0%	0%	33%	33%
Cholera CBT Medium A-Plus*	37°C	100%	0%	0%	0%
	42°C	0%	0%	0%	0%
Cholera CBT Medium B*	37°C	67%	0%	0%	0%
	42°C	33%	0%	0%	0%
Cholera CBT Medium C	37°C	100%	0%	0%	33%
	42°C	67%	0%	0%	0%
Cholera CBT Medium D*	37°C	67%	100%	22%	50%
	42°C	67%	100%	22%	50%

*denotes media that incorporate the antibiotics fosfomycin and streptomycin at 8 mg/L and 16 mg/L, respectively.

Growth of the three strains of *V. cholerae* varied across the candidate media. Cultures of *V. cholerae* Non-O1 appeared white in the broth adaptation of CV, at either incubation temperature. Growth of *V. cholerae* Non-O1 was inhibited entirely by CV Plus, Cholera CBT Medium B and Cholera CBT Medium D. These media included the addition of antibiotics. Cholera Medium A and Cholera Medium A-Plus (with antibiotics) supported growth of *V. cholerae* Non-O1, but only at the incubation temperature of 37°C.

Growth of *V. cholerae* O139 and O1, El Tor Ogawa was supported by all media at 37°C incubation. Incubation at 42°C inhibited growth of these two strains when cultured in Medium A and Medium A-Plus. Growth of *V. cholerae* O139 was also inhibited at 42°C incubation by Medium B and Medium C. Growth of all three strains of *V. cholerae* was delayed in Medium A and Medium A-Plus. Growth was observed after 48-hour incubation, but not at 24 hours.

Vibrio cholerae could be differentiated from *V. mimicus* by all media expect for CV and those that incorporated an indicator for beta-galactosidase activity (TTGB and Modified TTGB). The blue fluorescence of cleaved 4-MU-Gal was obscured by the Brain Heart Infusion Broth. Optimizing the concentration of 4-MU-Gal may improve visual examination of results from a broth culture medium.

The most selective and exclusive media against non-target gram-positive and gram-negative organisms were CV (42°C), CV Plus (37°C, 42°C), Cholera CBT Medium A-Plus (37°C), Medium B (37°C) and Medium C (42°C). When incubated at 42°C, CV inhibited or differentiated *V. cholerae* from all non-target gram-positive and gram-negative test organisms. With the addition of fosfomycin and streptomycin antibiotic supplements, CV inhibited or differentiated *V. cholerae* from all non-target organisms at either incubation temperature. Similarly, the newly-proposed Cholera CBT Medium A-Plus inhibited or differentiated all non-

target organisms from *V. cholerae* at 37°C incubation while allowing the growth of all strains of *V. cholerae* after 48 hours. The Cholera CBT Medium B inhibited all non-target organisms entirely at 37°C, but did not permit the growth of *V. cholerae* Non-O1. At 37°C incubation, Cholera CBT Medium C inhibited all gram-negative organisms but did not inhibit or distinguish the growth of two gram-positive organisms. Incubation at 42°C improved the specificity of Cholera CBT Medium C by inhibiting all non-target organisms entirely; however, these conditions inhibited the growth of *V. cholerae* O139.

CHAPTER 5: DISCUSSION

The results of this study demonstrate that previously-described and newly-composed culture media may be adapted into broth media for the direct culture of *V. cholerae*. These broth adaptations were effective to varying degrees for the one-step quantification of *V. cholerae* by MPN methods under laboratory conditions. These results warrant the further optimization of a broth culture medium that is sensitive, specific, efficient, and appropriate for field use with an adapted Cholera CBT.

Of the previously-described and commercially-available culture media examined, CHROMagar Vibrio (CV) was among the most selective and exclusive agar medium for *V. cholerae.* The exclusivity of this agar medium was comparable to TCBS when examining the inhibition of non-target organisms from a 1% primary effluent-natural surface water matrix. Previous work by Nigro et al. 2015 demonstrated that there was no difference in false-positive rates between CV or TCBS when identifying *V. cholerae* from environmental samples. Terio et al. (2010) reported, in contrast, that CV demonstrated improved accuracy and specificity over TCBS when culturing *V. parahaemolyticus* from environmental samples. When adapted into a broth medium, CV maintained exclusivity even against highly-contaminated and large-volume samples. These results suggest that the CV broth medium may be appropriate for culturing *V. cholerae* from field samples, to the exclusion of other naturally-occurring, non-target organisms.

CHROMagar Vibrio is advantageous for examining environmental samples, because this medium incorporates multiple chromogenic indicators that help distinguish between different species of Vibrio (Di Pinto, Terio, Novello, & Tantillo, 2010; Nigro & Steward, 2015). Based on

observations in this study, the chromogenic indicator of the CV medium distinguishes between pathogenic *V. cholerae* and non-pathogenic *V. cholerae*. Strains of *V. cholerae* O139 and O1 El Tor, Ogawa, considered pathogenic, appeared light blue, while *V. cholerae* Non-O1, considered non-pathogenic, appeared white. Some culture media, such as the polymixin mannose tellurite (PMT) agar, have been designed specifically for the differentiation between O1 and Non-O1 strains in epidemiologic investigations (Shimada et al., 1990). The PMT medium differentiates the serotypes based on mannose fermentation; all strains of *V. cholerae* O1 ferment mannose, but few cultures of *V. cholerae* Non-O1 do so (Shimada et al., 1990). This differentiation may be useful to identify microbial contamination of the pathogenic *V. cholerae* strains that are of public health concern in drinking water. However, researchers have asserted that Non-O1 strains of *V. cholerae* may cause diarrheal illness similar to cholera, or may produce enterotoxins similar to CT (Drasar, B.S and Forrest, 1996; Li et al., 2002). Therefore, these Non-O1 strains may have public health importance and may be monitored through microbial surveillance.

Even though TCBS is used most often for isolation and quantification of *V. cholera*e, this medium performed poorly as a selective broth medium in this study. This medium was limited in exclusivity against naturally-occurring, non-target organisms from surface water samples. Several researchers have recognized the lack of selectivity, exclusivity or differentiation between *V. cholerae* and non-vibrios (Donovan & van Netten, 1995). Organisms such as *A. hydrophila*, *Proteus vulgaris*, *Bacillus spp*., and *Klebsiella spp.* may appear yellow in color, just as *V. cholerae* do (Lotz, Tamplin, & Rodrick, 1983). The quality of the TCBS medium may differ between manufacturers (Dhiman Barua and William B. Greenough III, 1992; Donovan & van Netten, 1995; West, Russek, Brayton, & Colwell, 1982). Perishable constituents, such as animal digests and bile salts, may limit the selectivity or productivity of the TCBS medium and

compromise or undermine quality control measures (Dhiman Barua and William B. Greenough III, 1992; Donovan & van Netten, 1995; Nicholls, Lee, & Donovan, 1976). This variability in performance may explain the differences in exclusivity reported here between the lab–made TCBS broth medium and the broth medium adapted from the BD TCBS medium.

Based on the results of this study, TTGA and TTGB were the most productive media for direct plating of *V. cholerae*. These results are comparable to those from previous researchers, who have described TTGB as productive or more productive than APW for supporting growth of *V. cholerae* (Furniss et al., 1978). The ISO standard (2003c) states that the productivity ratio of a selective culture medium should be at least 0.50 when compared with a non-selective medium (Corry, Curtis, & Baird, 2011). The TTGB and TTGA media met this criterion (productivity ratio of 0.86) while the TCBS and CV media failed to do so (productivity ratios of 0.077 and 0.079 respectively).

The results of this research indicate that the broth adaptation of TTGB may be more selective against non-target organisms than the agar formulation. The exclusivity of the TTGB medium was comparable to the TCBS broth medium, which is consistent with results described by Morris et al. 1979 that described TTGA and TCBS as having comparable and excellent selectivity. Other researchers have described TCBS as more specific and more efficient for recovery than TTGB (Bolinches et al., 1988). The TTGB culture medium performed poorly in excluding and differentiating non-target organisms from a natural surface water when 50mL undiluted samples were examined in duplicate. The application of the broth TTGB medium may not be appropriate for use with the Cholera CBT without further improving the specificity for culturing *V. cholerae*.

The culture media examined in this study vary in their applicability for use an adapted CBT. **Table 34** compares CV, TCBS, and TTGB based on the desired criteria for a Cholera CBT broth medium. Presently, CV may be the most appropriate medium for use in an adapted Cholera CBT, as this medium is specific for *V. cholerae* and provides clear visualization of growth of *V. cholerae* O139 and O1 El Tor Ogawa. The chromogenic change associated with *V. cholerae* Non-O1 was inconsistent and varied between white to light blue. The medium does not differentiate between *V. cholerae* and *V. mimicus* when cultures are incubated at 37°C, because *V. mimicus* produces the same light blue chromogenic change as *V. cholerae* O139 and *V. cholerae* El Tor Ogawa. The plating efficiency of the CV medium is limited, as the productivity ratio is only 0.079, less than the ISO standard for selective culture media (Corry et al., 2011). The CV medium may be easily obtained from the manufacturer, but it is not currently available in a broth format. The medium does not require autoclaving, so may be appropriate for field applications.

Table 34. Comparison of existing culture media based on selection criteria for a Cholera CBT broth medium.

Characteristic of the Broth Medium	CV	TCBS	TTGB
Productivity*	Poor	Poor	Excellent
Visual Indicator	Yes (Chromogenic Substrate)	Yes (Bromothymol/ Thymol Blue)	Yes (4-MU-Gal)
Visualization of *V. cholerae*	Variable	Yes	Yes
Inhibits or differentiates between *V. cholerae* and *V. mimicus*	No	Yes	No
Commercially Available	Yes	Yes	Yes
Requires Autoclaving	No	No	Yes
Shelf Life	3 years	5 years	4 years

*Productivity qualified by comparing the productivity ratio to the ISO standard of 0.5 for selective culture media.

An appropriate medium for the Cholera CBT should be specific for *V. cholerae* while retaining high plating efficiency to accurately estimate the concentration of microbial contamination in drinking waters. Currently-available media may be improved upon to tailor their use for an adapted Cholera CBT. Incubation at 42°C improved the exclusivity of CV, TCBS, and TTGB media. These results are consistent with the findings of DePaola et al. (1988) who described that elevated enrichment at 42°C may improve recovery and specificity when culturing *V. cholerae* from oysters. In this present study, incorporating vancomycin in combination with streptomycin improved the exclusivity of CV by inhibiting non-target organisms appearing the same morphologically as *V. cholerae* in the culture medium.

In addition to these adaptations of existing media, five new broth media were proposed for the selective culture of *V. cholerae*. These media incorporated BHI as a basal medium, along with selective agents that target gram-positive organisms and some gram-negative organisms. Three of these new compositions incorporated antibiotics that target gram-negative organisms known to interfere by their growth in media specific for *V. cholerae*. The new media candidates called Cholera CBT Medium A-Plus, Medium B, and Medium C demonstrated improved exclusivity over other previously-described culture media. The efficiency of these newly-described media may be limited, however because growth of *V. cholerae* was delayed in Medium A and A-Plus. Growth was observed only after 48 hours of incubation, but not after 24 hours. This delayed growth may be a result of the action of selective agents, acting independently or synergistically to inhibit the growth of *V. cholerae* as well as the non-target organisms. Use of these media would require 48-hour incubation of the CBT assay in the field, delaying results. The use of other selective culture media, such as CV, may be useful for specific culture of *V.*

cholerae within 24 hours of sampling, therby allowing for more timely intervention and remediation if *V. cholerae* is detected in drinking water or water reservoirs.

The improved exclusivity of Cholera CBT Medium A-Plus and Medium B may be attributed to the incorporation of the antibiotics fosfomycin and streptomycin. While these agents may improve the specificity of a culture-based medium, these antibiotics may increase the cost of the medium by approximately $0.51 per liter (Sigma-Aldrich). These antibiotics would require a cold-chain, which may not be accessible in field or low-resource settings. Of the candidate antibiotics incorporated into the new culture media, fosfocymin should be stored at 2-8^{o}C, and streptomycin should be stored at -20^{o}C for any extended period of time (Sigma-Aldrich). Furthermore, the efficacy of these culture media may vary because spontaneous resistance to fosfomycin may occur in broth cultures (Popovic et al., 2010). Incorporation of these two antibiotics may select against or inhibit the growth of *V. cholerae* Non-O1; growth of the Non-O1 strain was inhibited in Cholera CBT Medium B and D.

Additional improvements to these adapted broth media may enhance the specificity of the Cholera CBT assay. The development of new chromogenic substrates allows for improved differentiation between organisms. Substrates can be incorporated in combination to improve the specificity of selective culture media. The chromogenic substrates in CV are believed to distinguish *V. parahaemolyticus* from other organisms based on β-glucosidase activity (US 7,892,783 B2, 2011). *Vibrio cholerae* and *V. vulnificus* are distinguished based on β -galactosidase activity. A newly-proposed proprietary medium, chromID Vibrio (bioMerieux), incorporates chromogenic substrates (5-bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside) and pH indicators (neutral red) in combination to detect *V. cholerae* and distinguish growth from other species of Vibrio (US 7,892,783 B2, 2011). A novel culture method described by Richards

et al. (2006) incorporates the substrate L-lysyl-7-amino-4-trifluoromethylcoumarin to visualize growth of Vibrio and Aeromonas species. Incorporation of these specific enzymatic substrates may improve the specificity of a broth culture medium, while providing clear visualization of the growth of *V. cholerae*.

To further improve the specificity of this direct culture method, field samples may be examined by two or three media in parallel. Previous research suggests that double-plating or triple-plating with different selective media may improve the specificity of culture assays for culturing Vibrios (Nigro & Steward, 2015; Williams, Froelich, & Oliver, 2013).

Standard protocols for the enumeration of *V. cholerae* recommend that environmental samples be enriched before isolating *V. cholerae* (Donovan & van Netten, 1995; Kaysner, Charles and DePaola, 2004; Thompson et al., 2006; USEPA, 2010). We propose that *V. cholerae* can instead be quantified from drinking water and its sources by a one-step culture method employing the CBT. The results of this study demonstrate that enumeration of *V. cholerae* by direct plating on a selective agar medium is comparable to quantification of *V. cholerae* by direct plating in an MPN assay utilizing the broth adaptation of the same selective medium. These two direct plating methods require that all organisms are viable and culturable. However, it is often difficult to recover *V. cholerae* from environmental samples (USEPA, 2010). Stressed cells of *V. cholerae* can enter into a VBNC state in which they may not be cultured by typical culture methods (Huq, 2013; Thompson et al., 2006). The Cholera CBT culture method should be further optimized for the efficient recovery of damaged or stressed cells of *V. cholerae*. The incorporation of the BHI Broth was chosen for the efficient recovery of overnight cultures and should be further evaluated in its efficacy for recovering damaged cells. Previous studies have demonstrated that enhanced recovery of VBNC cells of *V. cholerae* may be achieved by storing

samples at ambient air temperatures for 20 hours prior to processing (Alam et al., 2006). This recovery step may be optimized further for the examination of environmental samples with the Cholera CBT.

Because culture-based methods are subject to false-negative results or may underestimate the concentration of *V. cholerae* in environmental samples if they are present in a VBNC or injured state and at low cell densities (Huq, 2013), their effective monitoring in drinking water requires the analysis of sufficient sample volumes to improve their culture-based detection. One recommendation is that environmental samples of up to 1000mL be concentrated by filtration using polycarbonate membranes prior to processing and isolation of *V. cholerae* (Huq, 2013). Multiple 100mL volumes may be analyzed using the Cholera CBT to increase the total volume examined with this method. Sample dilutions should be optimized in the field in order to achieve reliable results that are within the detection limits of the CBT (up to 48.3 organisms per 100mL).

This research aims to optimize a low-cost, field-ready, self-contained portable test kit for microbial surveillance of *V. cholerae* in drinking waters and their sources. Such a test kit may be implemented to identify contaminated source waters that require treatment prior to use. This method may provide a means to evaluate point-of-use drinking water treatments in field settings. Routine monitoring of freshwater reservoirs and ground waters used as drinking water sources may improve the understanding of the complex ecology of *V. cholerae* and may identify hotspots that may lead to cholera outbreaks. A highly-specific culture-based method may also be adapted for clinical applications. This field-ready kit may be utilized as a diagnostic tool to identify and track sources of drinking water linked to cholera cases during an outbreak. Such practical field assays require the application of a specific and efficient broth culture medium. This research

provides a proof-of-concept that selective broth media may be adapted for direct culture of *V. cholerae* and should be further optimized for the adapted Cholera CBT.

Limitations

This research demonstrates that broth adaptations of existing culture media may be appropriate for quantification of *V. cholerae*. However, there are limitations to this study which can be addressed by further research for the evaluation and validation of the Cholera CBT.

This project aimed to identify an appropriate selective and differential medium for use with an adapted CBT. Existing media were first screened based on their selectivity by examining the media's exclusivity based on preventing the growth of non-*V. cholerae* bacteria. This screening was appropriate for an efficient examination of the extent of non-target bacteria growth to identify those candidate media that may be most appropriate for further consideration, modification and adaptation. However, exclusivity may not be a sufficiently robust measure to identify the most effective candidate media based on quantitative statistical comparisons of media performance for *V. cholerae* and non-*V. cholerae* bacteria. Relative accuracy, relative specificity, sensitivity and selectivity also need to be determined for quantitative statistical comparisons of culture media and can be compared to values reported in literature (Corry et al., 2011). While these additional criteria and performance metrics were not examined in this study, they should be considered for further examination of the Cholera CBT.

In this study, the effectiveness of each culture medium was examined on the basis of relative plating efficiency using three reference strains of *V. cholerae*. These three strains were used as the test organisms for the development of a new culture medium for *V. cholerae*. The strains selected were intended to be representative of those that are of public health concern or

may otherwise be detected in drinking waters. Standard guidelines recommend that microbial methods be validated using at least 50 strains of the target organism. There are more than 140 strains of *V. cholerae*. Therefore, a more thorough and robust analysis would examine the detectability of multiple strains of pathogenic *V. cholerae* in a broth culture medium paired with the CBT and a comparison of its performance with other *V. cholerae* culture methods considered to be "gold standard" or reference methods.

This study examined the use of antibiotics to improve the selectivity and exclusivity of a broth culture medium for the Cholera CBT by determining the concentrations of candidates that were able to prevent the growth of non-target bacteria and allow the growth of *V. cholerae* strains. The MIC assays were limited in scope and scale, as they examined the susceptibility of only eight non-target organisms to four antibiotics. The susceptibility of these non-target organisms was examined because these organisms are known to interfere in selective culture media for Vibrio spp. Therefore, targeted inhibition of their growth may improve the specificity of a *V. cholerae* culture medium.

The goal of this research is to design, evaluate and implement an adapted CBT for the quantification of *V. cholerae* from drinking water sources. However, this research has not yet examined this culture-based method and its candidate media using natural surface waters with naturally-occurring background non-target organisms. The results described here indicate that concentrations of *V. cholerae* can be estimated by direct broth culturing in an MPN assay in a model system used in a laboratory setting. However, this research does not yet consider the implications of culturing stressed or damaged cells from environmental waters or the performance of the method in diverse samples of drinking water in field settings.

This research has examined the use of selective *V. cholerae* broth culture media for use with multiple-tube and multiple-well MPN assays. Parallel research conducted in this lab has demonstrated that quantification of *V. cholerae* by multiple-tube assay is comparable to quantification of *V. cholerae* using the adapted CBT with the TTGB medium (results not shown). However, this research has not yet compared the performance of this CBT culture-based method to "gold standard" or reference methods, such as the EPA Standard Protocol. The CBT method should be validated in comparison to these standard methods to demonstrate the feasibility of a low-cost, field-ready test kit for examination of *V. cholerae* from drinking water and other environmental water samples.

Future Directions

The results of this study suggest that commercially-available media and newly-proposed culture media may be adapted for selective and differential, direct culture of *V. cholerae* by quantal methods, although further optimization and evaluation is needed. In order to optimize a medium appropriate for the Cholera CBT, we plan to determine the minimum inhibitory concentration (MIC) of candidate selective agents used in the culture medium against *V. cholerae* strains and types to optimize their concentrations. Specifically, crystal violet, sodium lauryl sulfate, sodium citrate, oxgall and others will be tested. These agents should be examined in parallel and as combinations to identify any synergetic or inhibitory effects that limit the growth of *V. cholerae*. Several combinations of antibiotic supplements may be considered to improve the exclusivity and plating efficiency of broth culture media. Any newly-proposed medium will be examined for exclusivity against non-target organisms, and the non-target *Vibrio* spp. that may be encountered in freshwaters.

The plating efficiency of any newly-proposed medium should be examined in parallel with previously-described media to determine if detection is at maximum levels for each medium, compared to a benchmark medium. Chromogenic or fluorogenic indicators should be optimized for visualization of *V. cholerae* in broth culture with the CBT. The CBT method for quantifying *V. cholerae* should be examined and compared to the ISO methods and the USEPA Standard Analytical Protocol for *V. cholerae* O1 and O139 in Drinking and Surface Waters. Once validated under laboratory conditions, the Cholera CBT method should be validated with field-studies to determine exclusivity, specificity, sensitivity and robustness for culturing *V. cholerae* from stored household drinking water, community drinking water supplies, and drinking water sources.

CHAPTER 6: CONCLUSION

The Compartment Bag Test is a field-ready, low-cost and portable tool that may be appropriate for the microbial surveillance of drinking water resources. The CBT may be adapted for detection and quantification of *V. cholerae* by incorporating a broth medium that is both selective and efficient. The results of this study indicate that previously-described and commercially-available culture media may be adapted for selective broth culture of *V. cholerae,* although some media may be limited in exclusivity, efficiency, or clear visualization of *V. cholerae* growth in broth culture. In light of these limitations, five new culture media were proposed and examined based on their exclusivity. These media demonstrated comparable or improved exclusivity over previously-described culture media. These media should be further optimized and examined for the direct culture and quantification of *V. cholerae* with the CBT.

APPENDIX A: Composition of Newly Proposed Media for Selective Culture of *V. cholerae*

Cholera CBT Medium A and A-Plus: This media incorporates selective agents predominately for the inhibition of gram-positive organisms. Medium A-Plus incorporates additional antibiotics.	
Component	**Concentration (g/L)**
Brain Heart Infusion Broth	37
Sucrose	10
Sodium Taurocholate	5
Sodium Citrate	5
Sodium Carbonate	1
Sodium Lauryl Sulfate	0.2
Crystal Violet	0.002
Bromothymol Blue	0.04
Antibiotics (A-Plus)	Streptomycin: 16mg/L Fosfomycin: 8mg/L

Cholera CBT Medium B: Inspired by the paper-based culture method described in (Briquaire et al., 2017)	
Component	**Concentration (g/L)**
Brain Heart Infusion Broth	37
Oxgall	35
Sucrose	6
Sodium Taurocholate	5
Sodium Citrate	5
Sodium Desoxycholate	1
4-MU-Gal	0.15
Antibiotics	Streptomycin: 16mg/L Fosfomycin: 8mg/L

Cholera CBT Medium C: This media incorporates selective agents from TTGB and TCBS.	
Component	**Concentration (g/L)**
Brain Heart Infusion Broth	37
Sucrose	10
Sodium Citrate	10
Oxgall	8
Sodium Taurocholate	5
Sodium Carbonate	1
Potassium Tellurite	0.5mL of 1% solution
Bromothymol Blue	0.04

Cholera CBT Medium D: Modified from Monsur's Agar to incorporate BHI and antibiotics.	
Component	**Concentration (g/L)**
Brain Heart Infusion Broth	37
Sodium Taurocholate	5
Sodium Carbonate	1
Potassium Tellurite	0.5mL of 1% solution
4-MU-Gal	0.15
Antibiotics	Streptomycin: 16mg/L Fosfomycin: 8mg/:

APPENDIX B: Raw Data for Enumeration of Non-Target Organisms from 1% Primary Effluent

Counts in CFU/1mL	Trial 1		Trial 2		Trial 3	
	37°C	42°C	37°C	42°C	37°C	42°C
VP	2.94E+03	1.87E+03	1.45E+03	1.11E+03	2.40E+03	1.22E+03
STT	1.46E+03	4.40E+02	4.10E+03	3.15E+02	2.60E+03	4.55E+02
CPC	3.70E+02	2.65E+02	4.50E+02	5.65E+02	7.50E+02	2.95E+02
HiCHROM	2.20E+02	1.30E+02	2.75E+02	4.50E+01	3.50E+02	4.50E+01
TCBS	5.00E+00	5.00E+00	5.00E+00	5.00E+00	5.00E+00	5.00E+00
HardyCHROM	7.50E+00	5.00E+00	7.50E+00	5.00E+00	1.00E+01	5.00E+00
CHROMagar	5.00E+00	5.00E+00	5.00E+00	5.00E+00	5.00E+00	5.00E+00
TCI	5.00E+00	5.00E+00	5.00E+00	5.00E+00	5.00E+00	5.00E+00

Counts in CFU/1mL	Trial 1		Trial 2		Trial 3	
	37°C	42°C	37°C	42°C	37°C	42°C
LBA	3.38E+04	3.00E+03	2.45E+04	5.50E+03	1.45E+04	4.00E+03
TTGA	7.00E+01	9.00E+01	2.30E+02	7.50E+01	1.15E+02	4.00E+01

APPENDIX C: Raw Data for Quantification of Non-Target Organisms from 1% Primary Effluent

Counts in MPN/1mL		TCI	GSP	GSLS	CV	TCBS	TCBS Homemade
Trial 1	37C - 24 Hours	2400	8400	23	1665	22.6	5850
	37C - 48 Hours	26500	12150	335	360	47.5	5850
	42C - 24 Hours	161.5	965	3	6.4	6.4	93
	42C - 48 Hours	235	1950	16.1	43	33	240
Trial 2	37C - 24 Hours	1950	19500	23	123	22.6	3350
	37C - 48 Hours	4950	19500	460	1650	445	6800
	42C - 24 Hours	92	2700	3	22.6	9.2	133
	42C - 48 Hours	161	6800	68	39.5	9.2	133
Trial 3	37C - 24 Hours	2520	29000	23	28.5	22.6	610
	37C - 48 Hours	4300	110000	240	511	21.5	16650
	42C - 24 Hours	150	840	3	92	22.6	57.5
	42C - 48 Hours	430	150	112.5	92	22.6	126.5

Counts in MPN/1mL		TTGB	LB
Trial 1	37C - 24 Hours	3	23000
	37C - 48 Hours	43	33000
	42C - 24 Hours	3	3600
	42C - 48 Hours	3	9200
Trial 2	37C - 24 Hours	3.6	21150
	37C - 48 Hours	68	21150
	42C - 24 Hours	3.6	6800
	42C - 48 Hours	6.4	9650
Trial 3	37C - 24 Hours	3.6	45000
	37C - 48 Hours	68	45000
	42C - 24 Hours	3	1610
	42C - 48 Hours	3	2610

APPENDIX D: Raw Data for Quantification of Non-Target Organisms in Surface Water

Count in MPN/100mL	LB	TTGB	TCBS	CV
Trial 1	87000	ND	44	7.2
Trial 2	62000	ND	ND	2.2
Trial 3	171000	22	138	4.4
Trial 4	ND	189	ND	ND
Trial 5	ND	ND	102	ND
Trial 6	ND	33	ND	ND

*ND – Not Done

APPENDIX E: Raw Data for Quantification and Enumeration of *V. cholerae* (Multiple Tube Test)

Vibrio cholerae O139				
Counts in MPN/100mL	LBA	TCBS	TTGA	TTGB
Trial 1	1.00E+09	3.50E+06	1.04E+09	9.53E+09
Trial 2	1.00E+09	7.50E+05	1.68E+10	2.73E+09
Trial 3	8.95E+10	7.50E+03	4.80E+10	4.22E+09
Trial 4	3.30E+10	5.75E+05	2.75E+10	2.68E+09
Trial 5	6.03E+10	5.50E+04	4.73E+10	6.93E+10
Trial 6	5.53E+10	2.25E+04	7.18E+10	7.53E+10
Trial 7	4.33E+10	1.25E+04	2.50E+09	6.50E+08
Trial 8	5.95E+10	5.60E+05	5.80E+10	8.93E+09

Vibrio cholerae O1 El Tor Ogawa				
Counts in MPN/100mL	LBA	TCBS	TTGA	TTGB
Trial 1	5.80E+10	1.00E+08	5.08E+10	3.87E+10
Trial 2	8.10E+10	7.50E+08	7.33E+10	2.97E+09
Trial 3	1.12E+11	1.85E+05	6.13E+10	8.13E+10
Trial 4	1.03E+11	4.90E+08	1.03E+12	1.02E+11
Trial 5	4.98E+10	3.65E+08	2.00E+10	3.50E+09

Vibrio cholerae Non-O1				
Counts in MPN/100mL	LBA	TCBS	TTGA	TTGB
Trial 1	5.30E+10	1.40E+10	7.48E+10	8.87E+10
Trial 2	1.85E+10	1.25E+08	1.58E+10	7.03E+09
Trial 3	7.35E+08	7.50E+05	5.70E+08	1.13E+09
Trial 4	4.65E+10	2.48E+05	1.76E+10	6.67E+10
Trial 5	1.95E+10	1.72E+07	1.20E+10	9.10E+09

APPENDIX F: Raw Data for Quantification and Enumeration of *V. cholerae* (Multiple Well Test)

V. cholerae O139					
Counts in MPN/100mL		LB	TTGB	TCBS	CV
Trial 1	37C - Broth	4.60E+10	9.30E+09	5.85E+06	3.35E+06
	37C - Agar	1.30E+10	4.00E+09	1.00E+07	7.75E+06
	42C - Broth	1.10E+11	9.65E+09	6.80E+06	3.30E+06
	42C - 48 Agar	2.40E+10	4.00E+09	8.25E+06	7.50E+04
Trial 2	37C - Broth	6.70E+10	3.50E+10	5.85E+08	1.57E+06
	37C - Agar	7.00E+10	6.75E+10	8.30E+07	1.25E+06
	42C - Broth	1.10E+11	7.80E+10	6.80E+08	9.65E+05
	42C - 48 Agar	5.70E+10	6.20E+10	5.30E+07	1.30E+06
Trial 3	37C - Broth	6.70E+10	7.80E+10	6.80E+07	3.50E+07
	37C - Agar	8.80E+10	7.65E+10	7.50E+06	6.45E+07
	42C - Broth	7.80E+10	4.60E+10	2.15E+07	3.50E+07
	42C - 48 Agar	8.65E+10	8.30E+10	9.25E+05	6.50E+05

V. cholerae O1 El Tor Ogawa					
Counts in MPN/100mL		LB	TTGB	TCBS	CV
Trial 1	37C - Broth	1.10E+11	4.60E+10	1.22E+10	2.40E+08
	37C - Agar	6.55E+10	5.70E+10	5.10E+09	7.15E+09
	42C - Broth	1.10E+11	3.50E+10	6.70E+10	4.30E+07
	42C - 48 Agar	6.65E+10	3.95E+10	3.95E+09	3.70E+09
Trial 2	37C - Broth	7.80E+10	2.40E+10	3.50E+09	4.30E+08
	37C - Agar	6.48E+10	6.15E+10	7.85E+08	7.55E+08
	42C - Broth	6.70E+10	1.10E+11	3.50E+09	5.80E+08
	42C - 48 Agar	6.43E+10	6.38E+10	1.10E+08	6.23E+08
Trial 3	37C - Broth	1.10E+11	7.80E+10	2.40E+09	3.50E+09
	37C - Agar	5.50E+10	4.60E+11	4.55E+09	2.65E+08
	42C - Broth	1.10E+11	3.50E+10	7.80E+09	1.67E+09
	42C - 48 Agar	6.45E+10	5.31E+10	2.50E+09	1.90E+08

V. cholerae Non-O1					
Counts in MPN/100mL		LB	TTGB	TCBS	CV
Trial 1	37C - Broth	7.80E+10	1.50E+10	2.37E+09	4.30E+09
	37C - Agar	2.15E+10	1.80E+10	1.32E+09	5.00E+09
	42C - Broth	1.42E+09	1.67E+10	2.40E+10	2.65E+09
	42C - 48 Agar	2.28E+10	1.35E+10	8.00E+08	5.05E+09
Trial 2	37C - Broth	1.10E+11	7.80E+10	9.30E+09	1.42E+10
	37C - Agar	5.80E+10	4.33E+10	1.20E+09	2.00E+10
	42C - Broth	2.40E+10	2.40E+10	6.80E+09	9.65E+09
	42C - 48 Agar	5.10E+10	3.58E+10	7.50E+08	2.24E+10
Trial 3	37C - Broth	5.85E+09	4.30E+08	1.90E+08	4.30E+08
	37C - Agar	3.50E+09	1.75E+09	1.25E+08	2.55E+08
	42C - Broth	1.42E+09	5.85E+08	1.25E+09	4.05E+08
	42C - 48 Agar	1.25E+09	1.00E+09	6.00E+07	1.45E+08

APPENDIX G: Raw Data for Efficiency of Basal Media

V. cholerae O139			
MPN/100mL	Trial 1	Trial 2	Trial 3
BHI	1.22E+11	9.3E+10	2.77E+11
BHI + 1% NaCl	2.4E+10	1.95E+11	2.4E+11
BHI + 3% NaCl	2.77E+10	8.3E+09	2.52E+10
Peptone + 1% NaCl	9.3E+10	1.67E+11	1.02E+11
Peptone + 3% NaCl	2.4E+10	1.57E+10	3.65E+10
Peptone + YB	1.42E+08	2.4E+09	7.4E+08
Petone + YB + 1%NaCl	6.8E+10	6.8E+10	7.87E+10
Petone + YB + 3%NaCl	3.5E+10	6.4E+08	1.61E+10

V. cholerae O1 El Tor Ogawa			
MPN/100mL	Trial 1	Trial 2	Trial 3
BHI	1.5E+11	1.22E+11	1.9E+10
BHI + 1% NaCl	1.22E+11	4.3E+10	1.32E+11
BHI + 3% NaCl	5.9E+10	1.68E+09	9.2E+08
Peptone + 1% NaCl	6.8E+10	7.4E+08	6.8E+10
Peptone + 3% NaCl	3.3E+10	3.65E+09	2.26E+09
Peptone + YB	2.3E+10	3.6E+08	3.6E+08
Petone + YB + 1%NaCl	4.3E+10	2.77E+11	1.32E+11
Petone + YB + 3%NaCl	4.55E+09	2.3E+09	2.3E+09

V. cholerae Non-O1			
MPN/100mL	Trial 1	Trial 2	Trial 3
BHI	9.65E+10	1.67E+10	2.77E+11
BHI + 1% NaCl	5.65E+10	9.4E+09	2.4E+11
BHI + 3% NaCl	5.9E+10	9.4E+09	2.52E+10
Peptone + 1% NaCl	9.65E+10	3.5E+10	1.02E+11
Peptone + 3% NaCl	1.95E+11	2.52E+10	4.05E+10
Peptone + YB	2.61E+10	6.8E+09	7.4E+08
Petone + YB + 1%NaCl	5.8E+10	9.3E+09	9.65E+10
Petone + YB + 3%NaCl	4.3E+10	5.8E+09	1.61E+09

APPENDIX H: Raw Data Antimicrobial Susceptibility Testing – MIC Determination

Bacterial Strain Trial Number	MIC (mg/L) (Average of Duplicates)			
Proteus mirabilis	Fosfomycin	Streptomycin	Ampicillin	Vancomycin
1	8.5	64	16	>256
2	0.5	8	8	>256
3	3	64	16	>256
Total Average	4	45.33	13.33	>256
Pseudomonas aeruginosa	Fosfomycin	Streptomycin	Ampicillin	Vancomycin
1	32	16	>256	>256
2	16	12	>256	>256
3	32	16	>256	>256
Total Average	26.67	14.67	>256	>256
Aeromonas hydrophila	Fosfomycin	Streptomycin	Ampicillin	Vancomycin
1	12	192	>256	128
2	6	64	>256	128
3	6	96	>256	192
Total Average	8	117.3333333	>256	149.3333333
V. cholerae Non-O1	Fosfomycin	Streptomycin	Ampicillin	Vancomycin
1	24	16	16	>256
2	16	48	24	256
3	8	48	24	>256
Total Average	16	37.33333333	21.33333333	>256
V. cholerae O1 El Tor Ogawa	Fosfomycin	Streptomycin	Ampicillin	Vancomycin
1	48	96	48	256
2	64	>256	64	>256
3	32	>256	48	>256
Total Average	48	>256	53.33333333	>256
V. cholerae Serotype O139	Fosfomycin	Streptomycin	Ampicillin	Vancomycin
1	128	96	32	128
2	32	256	32	256
3	32	256	32	256
Total Average	64	202.6666667	32	213.3333333
V. mimicus	Fosfomycin	Streptomycin	Ampicillin	Vancomycin
1	32	16	32	128
2	32	96	32	256
3	32	48	32	192
Total Average	32	53.33333333	32	192
Bacillus pumilus	Fosfomycin	Streptomycin	Ampicillin	Vancomycin
1	48	6	0.5	0
2	16	8	0.5	0
3	64	6	1.25	1
Total Average	42.66666667	6.666666667	0.75	0.333333333

Bacterial Strain Trial Number	MIC (mg/L) (Average of Duplicates)			
Enterococcus casseliflavus	Fosfomycin	Streptomycin	Ampicillin	Vancomycin
1	64	24	4	16
2	128	32	4	48
3	32	24	4	6
Total Average	74.66666667	26.66666667	4	23.33333
Paenibacillus pabuli	Fosfomycin	Streptomycin	Ampicillin	Vancomycin
1	160	>256	16	96
2	192	>256	16	96
3	256	>256	16	96
Total Average	202.6666667	>256	16	96
Escherichia coli	Fosfomycin	Streptomycin	Ampicillin	Vancomycin
1	24	16	32	128
3	8	16	16	256
3	10	16	32	192
Total Average	14	16	26.66666667	192

APPENDIX I: Examining the Exclusivity of Newly-Proposed Selective Culture Media

	Luria Broth		**TCBS**		**TTGB**		**CV**		**CV Plus**	
	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C
Vibrio spp.										
Vibrio cholerae 0139	+	+	+	+	+	+	+	+	+	+
Vibrio cholerae O1, El Tor Ogawa	+	+	+	+	+	+	+	+	+	+
Vibrio cholerae Non-O1	+	+	+	+	+	+	White	White	-	-
Vibrio mimicus	+	+	Green	Green	+	-	+	White	-	-
Gram-Negative Organisms										
Aeromonas hydrophila	+	+	-	-	-	-	-	-	-	-
Proteus mirabilis	+	+	Black	Black	-	-	White	White	-	-
Pseudomonas aeruginosa	+	+	-	-	-	-	White	-	-	-
Escherichia coli	+	+	-	-	+	+	+	-	-	-
Shigella flexneri	+	+	-	-	-	-	-	-	-	-
Shigella spp.	+	+	-	-	-	-	-	-	-	-
Klebsiella pneumoniae	+	+	-	-	-	-	-	-	-	-
*Salmonella typhimurium*LT2	+	+	-	-	-	-	White	-	-	-
Raoultella terrigena	+	+	-	-	-	-	-	-	-	-
Gram-Positive Organisms										
Bascillus cereus	+	+	-	-	-	-	-	-	-	-
Staphylococcus aureas	+	+	-	-	+	+	White	White	-	-
Enterococcus faecalis	+	+	+	+	+	+	Purple	Purple	Purple	Purple
Bascillus pumilus	+	+	-	-	+	+	-	-	-	-
Enterococcus casseliflavus	+	+	+	+	+	+	Purple	Purple	Purple	-
Paenibacillys pabuli	+	+	-	-	+	+	-	-	-	-

	Medium A		Medium A-Plus		Medium B		Medium C		Medium D	
	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C
Vibrio spp.										
Vibrio cholerae 0139	+	-	+	-	+	-	+	-	+	+
Vibrio cholerae O1, El Tor Ogawa	+	-	+	-	+	+	+	+	+	+
Vibrio cholerae Non-O1	+	-	+	-	-	-	+	+	-	-
Vibrio mimicus	-	-	Green	-	-	-	-	-	+	+
Gram-Negative Organisms										
Aeromonas hydrophila	Green	-	-	-	-	-	-	-	-	-
Proteus mirabilis	Green	Green	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-	+	+
Shigella flexneri	Green	-	-	-	-	-	-	-	-	-
Shigella spp.	+	+	-	-	-	-	-	-	-	-
Klebsiella pneumoniae	+	+	-	-	-	-	-	-	-	-
Salmonella typhimurium LT2	+	+	Green	-	-	-	-	-	-	-
Raoultella terrigena	+	-	-	-	-	-	-	-	+	+
Gram-Positive Organisms										
Bascillus cereus	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureas	-	-	-	-	-	-	-	-	-	-
Enterococcus faecalis	+	+	Green	Green	-	-	+	+	+	+
Bascillus pumilus	-	-	-	-	-	-	-	-	-	-
Enterococcus casseliflavus	+	+	Green	-	-	-	+	+	+	+
Paenibacillys pabuli	-	-	-	-	-	-	-	-	+	+

WORKS CITED

Akiyama, S., Takizawa, K., Ichinoe, H., Enomoto, S., Kobayashi, T., & Sakazaki, R. (1963). Application of Teepol to isolation of Vibrio parahaemolyticus. *Japan Journal of Bacteriology, 18,* 255–256.

Alam, M., Sadique, A., Nur-A-Hasan, Bhuiyan, N. A., Nair, G. B., Siddique, A. K., … Colwell, R. R. (2006). Effect of transport at ambient temperature on detection and isolation of Vibrio cholerae from environmental samples. *Applied and Environmental Microbiology*, *72*(3), 2185–2190. https://doi.org/10.1128/AEM.72.3.2185-2190.2006

Ali, M., Nelson, A. R., Lopez, A. L., & Sack, D. A. (2015). Updated global burden of cholera in endemic countries. *PLoS Neglected Tropical Diseases*, *9*(6), e0003832. https://doi.org/10.1371/journal.pntd.0003832

Aquagenx. (2018). Water Quality Test Kit | Aquagenx Compartment Bag Test. Retrieved May 28, 2018, from https://www.aquagenx.com/how-to-use-the-cbt/

Atlas, R. M. (1997). *Handbook of Microbiological Media*. (L. Parks, Ed.). Boca Raton, New York, London, Tokyo: CRC Press.

Bain, R., Bartram, J., Elliott, M., Matthews, R., Mcmahan, L., Tung, R., … Gundry, S. (2012). A Summary Catalogue of Microbial Drinking Water Tests for Low and Medium Resource Settings. *Int. J. Environ. Res. Public Health International Journal of Environmental Research and Public Health*, *9*, 1609–1625. https://doi.org/10.3390/ijerph9051609

Baker, Z., Harrison, R. W., & Miller, B. F. (1941). ACTION OF SYNTHETIC DETERGENTS ON THE METABOLISM OF BACTERIA. *The Journal of Experimental Medicine*, *73*(2), 249–271. https://doi.org/10.1084/JEM.73.2.249

Beazley, W. A. (1992). TCI - A new bile free medium for the isolation of Vibrio species. *Australian Journal of Medical Science*, *13*(1), 25–27. Retrieved from http://vb3lk7eb4t.scholar.serialssolutions.com/?ctx_ver=Z39.88-2004&ctx_enc=info%3Aofi%2Fenc%3AUTF-8&rfr_id=info%3Asid%2Fsummon.serialssolutions.com&rft_val_fmt=info%3Aofi%2Ffmt%3Akev%3Amtx%3Ajournal&rft.genre=article&rft.atitle=TCI+-+A+new+bile+free+medi

Bolinches, J., Romalde, J. L., & Toranzo, A. E. (1988). Evaluation of selective media for isolation and enumeration of vibrios from estuarine waters. *Journal of Microbiological Methods*, *8*, 151–160. Retrieved from https://ac.els-cdn.com/0167701288900164/1-s2.0-0167701288900164-main.pdf?_tid=9851a546-d2ec-45cd-852b-bab55ae5a8bf&acdnat=1527624679_0e584cd0ff9a6f41a902f381e4755f23

Briquaire, R., Colwell, R. R., Boncy, J., Rossignol, E., Dardy, A., Pandini, I., … Rozand, C. (2017). Application of a paper based device containing a new culture medium to detect Vibrio cholerae in water samples collected in Haiti. *Journal of Microbiological Methods, 133*, 23–31. https://doi.org/10.1016/j.mimet.2016.12.014

Cecchini, F., Fajs, L., Cosnier, S., & Marks, R. S. (2016). Vibrio cholerae detection: Traditional assays, novel diagnostic techniques and biosensors. *TrAC Trends in Analytical Chemistry*, *79*, 199–209. https://doi.org/10.1016/J.TRAC.2016.01.017

Centre for Disease Control and Prevention(CDC); (2004). Laboratory Methods for the Diagnosis of Vibrio Cholerae: Isolation of Vibrio. *Centers for Disease Control and Prevention*. Retrieved from http://www.cdc.gov/cholera/pdf/laboratory-methods-for-the-diagnosis-of-vibrio-cholerae-chapter-4.pdf

Chatterjee, B. D., & De, P. K. (2017). Sucrose Teepol Tellurite Agar : A New Selective Indicator Medium for Isolation of Vibrio Species Author (s): Barun Deb Chatterjee , Pradip Kumar De and Tarun Sen Published by : Oxford University Press Stable URL : http://www.jstor.org/stable/30107898 Su, *135*(4), 654–658.

Corry, J. E. L., Curtis, G. D. W., & Baird, R. M. (2011). *Handbook of Culture Media for Food and Water Microbiology*. (J. E. L. Corry, G. D. W. Curtis, & R. M. Baird, Eds.). Cambridge: Royal Society of Chemistry. https://doi.org/10.1039/9781847551450

Daniel Mognet, & Denis Robichon. (2011). *US 7,892,783 B2*. Retrieved from https://patentimages.storage.googleapis.com/8a/c0/73/a3add992bc0378/US7892783.pdf

De, S. P., Sen, D., De, P. C. Ghosh, A., & Pal., S. C. (1977). A simple selective medium for isolation of vibrios with particular reference to vibrio parahaemolyticus. *Indian Council of Medical Research.*, *66*(3), 398. Retrieved from http://vb3lk7eb4t.scholar.serialssolutions.com/?ctx_ver=Z39.88-2004&ctx_enc=info%3Aofi%2Fenc%3AUTF-8&rfr_id=info%3Asid%2Fsummon.serialssolutions.com&rft_val_fmt=info%3Aofi%2Ffmt%3Akev%3Amtx%3Ajournal&rft.genre=article&rft.atitle=A+simple+selective+medium+

DePaola, A., Motes, M. L., & McPhearson, R. M. (1988). Comparison of APHA and elevated temperature enrichment methods for recovery of Vibrio cholerae from oysters: collaborative study. *Journal - Association of Official Analytical Chemists*, *71*(3), 584–589. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3391968

Dhiman Barua and William B. Greenough III. (1992). *Current Topics in Infectious Disease, Cholera*. Retrieved from https://books.google.com/books?id=HRwGkfyNv6AC&printsec=frontcover&source=gbs_ge_summary_r&cad=0#v=onepage&q&f=false%0Ahttps://books.google.com/books?id=e4vSBwAAQBAJ&printsec=frontcover&source=gbs_ge_summary_r&cad=0%23v=onepage&q&f=false

Di Pinto, A., Terio, V., Novello, L., & Tantillo, G. (2010). Comparison between thiosulphate-citrate-bile salt sucrose (TCBS) agar and CHROMagar Vibrio for isolating Vibrio parahaemolyticus. *Food Control*. https://doi.org/10.1016/j.foodcont.2010.06.013

Donovan, T. J., & van Netten, P. (1995). Culture media for the isolation and enumeration of pathogenic Vibrio species in foods and environmental samples. *International Journal of Food Microbiology*, *26*(1), 77–91.

Drasar, B.S and Forrest, B. D. (1996). *Cholera and the Ecology of Vibrio cholerae*. (B. D. Drasar, B.S and Forrest, Ed.). London: Chapman & Hall.

EMD Millipore Corporation. (2008). Ox bile , dried.

Forbes, Betty, Sahm, Daniel, Weissfeld, A. (2017). Bailey & Scott's Diagnostic Microbiology (12th ed., pp. 371–379). St. Louis, Missouri: Mosby Elsevier.

Fung, D. Y. C., & Miller, R. D. (1973). Effect of Dyes on Bacterial Growth, *25*(5), 793–799. Retrieved from http://aem.asm.org/content/25/5/793.full.pdf

Furniss, A. L., Lee, J. V., & Donovan, T. J. (1978). The Vibrios. *Monograph Series, Public Health Laboratory Service, HMSO, London*.

Global Task Force on Cholera Control. (2017). *A Global Roadmap to 2030*. *World Health Organization*. Retrieved from http://www.who.int/cholera/publications/global-roadmap.pdf?ua=1

Griffitt, K. J., & Grimes, D. J. (2013). Abundance and Distribution of Vibrio cholerae, V. parahaemolyticus, and V. vulnificus Following a Major Freshwater Intrusion into the Mississippi Sound. *Microbial Ecology*, *65*(3), 578–583. https://doi.org/10.1007/s00248-013-0203-6

Gronewold, A. D., Sobsey, M. D., & McMahan, L. (2017). The compartment bag test (CBT) for enumerating fecal indicator bacteria: Basis for design and interpretation of results. *Science of The Total Environment*, *587–588*, 102–107. https://doi.org/10.1016/J.SCITOTENV.2017.02.055

Hartnell, R. E., Stockley, L., Keay, W., Rosec, J.-P., Hervio-Heath, D., Van den Berg, H., … Baker-Austin, C. (2018). A pan-European ring trial to validate an International Standard for detection of Vibrio cholerae , Vibrio parahaemolyticus and Vibrio vulnificus in seafoods. *International Journal of Food Microbiology*. https://doi.org/10.1016/j.ijfoodmicro.2018.02.008

Huq, A. et al. (2013). *Detection, Isolation, and Identification of Vibrio cholerae from the Environment*. NIH Public Access. https://doi.org/10.1002/9780471729259.mc06a05s26.Detection

ISO. (2017). *Microbiology of the food chain - Horizontal method for the determination of Vibrio spp.* Retrieved from www.iso.org

Jameson, J.E., E. N. W. (1956). A Substitute for Bile Salts in Culture Media. *Journal of General Microbiology*, *15*, 198–204. Retrieved from http://www.microbiologyresearch.org/docserver/fulltext/micro/15/1/mic-15-1-198.pdf?expires=1523728989&id=id&accname=guest&checksum=CFF38ADE1D0B169F029DF712C93A88EA

Kahler, A. M., Haley, B. J., Chen, A., Mull, B. J., Tarr, C. L., Turnsek, M., … Hill, V. R. (2015). Environmental surveillance for toxigenic vibrio cholerae in surface waters of haiti. *American Journal of Tropical Medicine and Hygiene*, *92*(1), 118–125. https://doi.org/10.4269/ajtmh.13-0601

Kaysner, Charles and DePaola, A. J. (2004). Laboratory Methods - BAM: Vibrio. Retrieved from https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070830.htm

Kida, N., Suzuki, S., & Taguchi, F. (1995). Rapid Detection of Vibrio Medium cholerae with a New Chain Selective Reaction Enrichment and Polymerase Center for Inspection of Imported Foods and Infectious Diseases , Yokohama Quarantine Station , Minis. *Kansensho*, 826–834.

Kitaoka, M., Miyata, S. T., Unterweger, D., & Pukatzki, S. (2011). Antibiotic resistance mechanisms of Vibrio cholerae. *Journal of Medical Microbiology*, *60*(4), 397–407. https://doi.org/10.1099/jmm.0.023051-0

Kitaura, T., Doke, S., Azuma, I., Imaida, M., Miyano, K., Harada, K., & Yabuuchi, E. (1983). Halo production by sulfatase activity of V. vulnificus and V. cholerae O 1 on a new selective sodium dodecyl sulfate-containing agar medium: a screening marker in environmental surveillance. *FEMS Microbiology Letters*, *17*, 205–209. https://doi.org/10.1111/j.1574-6968.1983.tb00402.x

Kobayashi, T., Enomoto, S., Sakazaki, R., & Kuwahara, S. (1963). new selective isolation medium for vibrio group on a modified Nakanishi's medium (TCBS agar medium). *Japan Journal of Bacteriology*, (18), 387–392.

Koneman, E. W., Allen, S. D., & Janda, W.M. Schreckenberger, P. C. (1997). *Color Atlas and Textbook of Diagnostic Microbiology*. *Lippicott Williams & Wilkins* (Fourth). Philadelphia, PA: J.B, Lippincott Company.

Lee, Y.-L., Cesario, T., Owens, J., Shanbrom, E., & Thrupp, L. D. (2002). Antibacterial Activity of Citrate and Acetate. *Nutrition (Burbank, Los Angeles County, Calif.)*, (18), 665–666. Retrieved from https://ac.els-cdn.com/S0899900702007827/1-s2.0-S0899900702007827-main.pdf?_tid=67f36cdc-c267-4db6-9123-9de6b557c372&acdnat=1524539053_a9570a2145bdfb40fc229bb3caad2e6a

Li, M., Shimada, T., Morris, J. G., Sulakvelidze, A., & Sozhamannan, S. (2002). Evidence for the emergence of non-O1 and non-O139 Vibrio cholerae strains with pathogenic potential by exchange of O-antigen biosynthesis regions. *Infection and Immunity*, *70*(5), 2441–2453. https://doi.org/10.1128/IAI.70.5.2441-2453.2002

Lotz, M. J., Tamplin, M. L., & Rodrick, G. E. (1983). Thiosulfate-Citrate-Bile Salts-Sucrose Agar and Its Selectivity for Clinical and Marine Vibrio Organisms. *ANNALS OF CLINICAL AND LABORATORY SCIENCE*, *13*(1). Retrieved from http://www.annclinlabsci.org/content/13/1/45.full.pdf

Madden, N. ., McCardell, B., & Morris, J. G. J. (1989). *Foodborne Bacterial Pathogens*. New York.

Martinez, R. M., Megli, C. J., & Taylor, R. K. (2010). Growth and laboratory maintenance of vibrio cholerae. *Current Protocols in Microbiology*, (SUPPL. 17), 1–10. https://doi.org/10.1002/9780471729259.mc06a01s17

Massad, G., & Oliver, J. D. (1987). New selective and differential medium for Vibrio cholerae and Vibrio vulnificus. *Applied and Environmental Microbiology*, *53*(9), 2262–2264.

McMahan, L., Devine, A. A., Grunden, A. M., & Sobsey, M. D. (2011). Validation of the H2S method to detect bacteria of fecal origin by cultured and molecular methods. *Applied*

Microbiology and Biotechnology, *92*(6), 1287–1295. https://doi.org/10.1007/s00253-011-3520-z

McMahan, L., Grunden, A. M., Devine, A. A., & Sobsey, M. D. (2012). Evaluation of a quantitative H2S MPN test for fecal microbes analysis of water using biochemical and molecular identification. *Water Research*, *46*(6), 1693–1704. https://doi.org/10.1016/j.watres.2011.12.037

McMahan, L., Wang, A., Rutstein, S., Sobsey, M. D., Stauber, C., & Reyes, J. (2017). Household Microbial Water Quality Testing in a Peruvian Demographic and Health Survey: Evaluation of the Compartment Bag Test for Escherichia coli. *The American Journal of Tropical Medicine and Hygiene*, *96*(4), 970–975. https://doi.org/10.4269/ajtmh.15-0717

Monsur, K. . (1961). A highly selective gelatin-taurocholate-tellueite medium for the isolation of Vibrio cholerae. *Transactions of the Royal Scoiety of Tropical Medicine and Hygiene*, *55*(5), 440–442.

Nicholls, K. ., Lee, J. ., & Donovan, T. J. (1976). An Evaluation of Commercial Thiosulphate Citrate Bile Salt Sucrose Agar (TCBS). *Journal of Applied Bacteriology*, *41*(2), 265–269. https://doi.org/10.1111/j.1365-2672.1976.tb00629.x

Nigro, O. D., & Steward, G. F. (2015). Differential specificity of selective culture media for enumeration of pathogenic vibrios: Advantages and limitations of multi-plating methods. *Journal of Microbiological Methods*, *111*, 24–30. https://doi.org/10.1016/J.MIMET.2015.01.014

O'Brien, M., & Colwell, R. (1985). Modified taurocholate-tellurite-gelatin agar for improved differentiation of Vibrio species. *Journal of Clinical Microbiology*, *22*(6), 1011–1013.

Paul D 'Mello, A. (1980). *Effects of Sodium Deoxycholate on Selected Gram- Negative Bacteria (Master's Thesis)*. *Loyola University Chicago*. Retrieved from http://ecommons.luc.edu/luc_theses/3083

Popovic, M., Steinort, D., Pillai, S., & Joukhadar, C. (2010). Fosfomycin: An old, new friend? *European Journal of Clinical Microbiology and Infectious Diseases*, *29*(2), 127–142. https://doi.org/10.1007/s10096-009-0833-2

Rashid, M. U., Rahman, Z., Burrowes, V., Perin, J., Mustafiz, M., Monira, S., … George, C. M. (2017). Rapid dipstick detection of Vibrio cholerae in household stored and municipal water in Dhaka, Bangladesh: CHoBI7 trial. *Tropical Medicine and International Health*, *22*(2), 205–209. https://doi.org/10.1111/tmi.12797

Richards, G. P., & Watson, M. A. (2006). A simple fluorogenic method to detect Vibrio cholerae and Aeromonas hydrophila in well water for areas impacted by catastrophic disasters. *The American Journal of Tropical Medicine and Hygiene*, *75*(3), 516–521. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16968933

Righetto, L., Zaman, R. U., Mahmud, Z. H., Bertuzzo, E., Mari, L., Casagrandi, R., … Rinaldo, A. (2015). Detection of Vibrio cholerae O1 and O139 in environmental waters of rural Bangladesh: A flow-cytometry-based field trial. *Epidemiology and Infection*, *143*(11), 2330–2342. https://doi.org/10.1017/S0950268814003252

Salles, C. A., Voros, S., Marbell, E. C., & Amenuvor, L. (1976). Colony Morphology of Vibrio cholerae on SV medium, *40*, 213–216. Retrieved from https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1365-2672.1976.tb04167.x

Salton, M. R. J. (1960). Disaggregation of Bacterial Cell Walls by Anionic Detergents. *J . Gcn. Mimobiol*, *22*, 187–141. Retrieved from http://www.microbiologyresearch.org/docserver/fulltext/micro/23/1/mic-23-1-137.pdf?expires=1519743163&id=id&accname=guest&checksum=E4AB7B2975D9309961074A22C6D084D3

Sharma, M. K., Goel, A. A. K., Singh, A. L., & Rao, A. V. K. (2006). Immunological biosensor for detection of Vibrio cholerae O1 in environmental water samples. https://doi.org/10.1007/s11274-006-9156-y

Shimada, T., Sakazaki, R., Fujimura, S., Niwano, K., Mishina, M., & Takizawa, K. (1990). A new selective, differential agar medium for isolation of Vibrio cholerae O1: PMT (polymyxin-mannose-tellurite) agar. *Japanese Journal of Medical Science & Biology*, *43*(2), 37–41. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2175000

Sigma-Aldrich. (n.d.). Streptomycin sulfate salt | Sigma-Aldrich. Retrieved June 19, 2018, from https://www.sigmaaldrich.com/catalog/substance/streptomycinsulfatesalt72869381074011?lang=en&region=US

Sneath, P. H. A., Mair, N. S., & Sharpe, M. E. (1986). *Bergey's manual of systematic bacteriology*.

Stauber, C., Miller, C., Cantrell, B., & Kroell, K. (2014). Evaluation of the compartment bag test for the detection of Escherichia coli in water. *Journal of Microbiological Methods*, *99*, 66–70. https://doi.org/10.1016/J.MIMET.2014.02.008

Tamura, K., Shimada, S., & L.M., P. (1971). Vibiro Agar: A New Plating Medium for Isolation of Vibrio cholerae. *Japan Journal of Medical Science Biology*, (24), 125–127.

Taylor, D. E. (1999). Bacterial tellurite resistance. *TRENDS IN MICROBIOLOGY*, *111*(3). Retrieved from http://www.cell.com/trends/microbiology/pdf/S0966-842X(99)01454-7.pdf

Thompson, F. ., Austin, B., & Swings, J. (2006). *The Biology of the Vibrios*. (B. and S. J. Thompson, F.L., Austin, Ed.). Washington, D.C.: ASM Press.

Thomson, A. A., & Gunsch, C. K. (2015). Evaluation of a field appropriate membrane filtration method for the detection of Vibrio cholerae for the measurement of biosand filter performance in the Artibonite Valley, Haiti. *Environmental Monitoring and Assessment*, *187*(8), 484. https://doi.org/10.1007/s10661-015-4677-1

Tipton, C. C., Stewart, J. R., Sobsey, M. D., & Whalen, S. C. (2017). Evaluation of a Low-Cost Compartment Bag Test to Quantify Hydrogen Sulfide-Producing Bacteria in Drinking Water. Retrieved from https://search.proquest.com/docview/1951781295?pq-origsite=summon

Tryland, I., & Fiksdal, L. (1998). Enzyme characteristics of beta-D-galactosidase- and beta-D-glucuronidase-positive bacteria and their interference in rapid methods for detection of waterborne coliforms and Escherichia coli. *Applied and Environmental Microbiology*,

64(3), 1018–1023. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9501441

USEPA. (2010). Standard Analytical Protocol for Vibrio cholerae O1 and O139 in Drinking Water and Surface Water. *Standard Methods*, (October), 1–56.

Watson, J. T., Gayer, M., & Connolly, M. A. (2007). Epidemics after natural disasters. *Emerging Infectious Diseases*, *13*(1), 1–5. https://doi.org/10.3201/eid1301.060779

West, P. A., Russek, E., Brayton, P. R., & Colwell, R. R. (1982). Statistical evaluation of a quality control method for isolation of pathogenic Vibrio species on selected thiosulfate-citrate-bile salts-sucrose agars. *Journal of Clinical Microbiology*, *16*(6), 1110–1116. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6761362

Williams, T. C., Froelich, B., & Oliver, J. D. (2013). A new culture-based method for the improved identification of Vibrio vulnificus from environmental samples, reducing the need for molecular confirmation. *Journal of Microbiological Methods*, *93*(3), 277–283. https://doi.org/10.1016/J.MIMET.2013.03.023

World Health Organization. (2018). *WHO Annual Report 2017: Yemen*. Retrieved from http://www.who.int/emergencies/crises/yem/yemen-annual-report-2017.pdf?ua=1