Quantitative evaluation of environmental *Vibrio cholerae* population dynamics over temporal and spatial scales

by

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ABSTRACT

Cholera, a severe life-threatening waterborne diarrheal disease, has been endemic to the Ganges delta for centuries. *Vibrio cholerae*, the causative agent of this disease, is a natural inhabitant of brackish water. Amongst the 200 serogroups identified so far, only O1 and O139 were found responsible for most of the cholera endemics. Transmission of cholera mainly occurs through fecal-oral route from compromised water management systems, poor sanitation and personal hygiene. Effective control of cholera outbr eaks relies on timely detection of the pathogen from clinical samples and environmental sources. Although cholera epidemiology is well studied, information on the abundance of *V. cholerae* and other pathogenic *Vibrio* species in environmental sources is minimal and rapid detection is still a challenge. Moreover, *Vibrio metoecus*, closely related to *V. cholerae,* often coexists with the latter within aquatic environments and has recently been described to cause infections in humans. Therefore, the relative abundance of *V. metoecus* and *V. cholera*e, along with their population dynamics in aquatic reservoirs is critical to understand the virulence of these bacteria. Consistent environmental monitoring of pathogenic *Vibrio* species and the pandemic generating (PG) O1 serogroup of *V. cholerae* could facilitate the identification of their actual distributions in aquatic reservoirs, thus would help predict an outbreak before it strikes. The difficulties in substantial temporal and spatial environmental sampling and lack of specific quantitative methods made this goal difficult until now.

I developed a multiplex qPCR assay with a limit of detection (LOD) of three copies per reaction to simultaneously quantify total *V. metoecus* and *V. cholerae* populations, as

well as the toxigenic and O1 serogroup subpopulations of *V. cholerae* in environmental samples by targeting four different genes as specific markers. Analysis of water samples from four different geographic locations, including cholera-endemic (Dhaka, Kuakata and Mathbaria, Bangladesh) and non-endemic (Oyster Pond in Falmouth, Massachusetts, USA), showed that *V. metoecus* was present seasonally in the USA site only. The non-toxigenic O1 serogroup comprised up to 18% of the total *V. cholerae* population in the USA coastal sites. *V. cholerae* toxigenic O1 serogroup was consistently present as a high proportion of the total *V. cholerae* populations in inland waters but rarely present in coastal waters of the cholera-endemic region studied (Bangladesh). Large numbers (>90% of the total) of both the *Vibrio* species were found attached to host/particle (>63 µm fraction size samples). This is the first study that used a culture-independent method to quantify *V. cholerae* or *V. metoecus* directly in environmental samples from cholera endemic and non-endemic areas. This culture-independent multiplex qPCR-based detection and quantification method was validated to allow direct quantification of pathogenic *V. cholerae* in environmental water samples on-site, in low resource settings and obtained a limit of detection (LOD) of 6×10^3 cells/L of water. Analysis of the environmental water samples collected from a site endemic for cholera (Gabtoli area, Dhaka, Bangladesh) showed O1 serogroup comprises 15% of total *V. cholera*e. Portability of the equipment, the stability of the reagents at 4 °C, user-friendly online software and easy set-up make this assay extremely useful for field research and thus fast quantitative analysis of the abundance of these organisms in the environment could be possible.

Finally, spatial and temporal dynamics of the PG lineage and other lineages of the *V.*

cholerae species in Dhaka's waters were evaluated by sequences analysis coupled with real-time qPCR of a species-specific, highly diverse protein-coding gene (vibriobactin utilization protein, *viuB*) amplified from aquatic biomass DNA. This method provided subspecies level resolution of the abundance and lineage composition of *V. cholerae* populations. The total abundance of *V. cholerae* was found to be very stable, ranging from 2 to 5×10^5 cells/L, being highest in the most densely populated site of the seven locations sampled in Dhaka for six consecutive months. The PG lineage of the O1 serogroup comprised 24 to 92% of the total *V. cholerae* population, which was consistently present and showed occasional but sudden reductions in abundance. In these rare instances in which PG O1 lost its dominance, another lineage underwent a rapid expansion while the total *V. cholerae* population size remained unchanged. This suggests there is intra-species competition among the lineages of *V. cholerae* within a niche. Environmental parameters like salinity and total dissolved solids (TDS) were found to correlate with *V. cholerae* lineage richness which positively indicates more diversity at the subspecies level. However, the abundance of PG O1 showed a negative correlation with salinity. Overall this result suggests that population composition can be influenced by intra-lineage interactions as well as by environmental factors.

PREFACE

Most of the research for this thesis is an outcome of collaborative work. In this section contributions of individuals are listed for each chapter. Chapters 2, 3 and 4 have been written as manuscripts before being included in this thesis and have been prepared for submission to peer-reviewed journals. Manuscripts are included as chapters in this thesis in a format different from that of the intended journals.

1. A version of chapter 2 has been prepared for submission to **Applied and Environmental Microbiology** as:

"**Simultaneous quantification of *Vibrio metoecus* and *Vibrio cholerae* with its O1 serogroup and toxigenic subpopulations in environmental reservoirs**"

Author contribution: Y.B. and T.N. designed the project and wrote the manuscript. M.T.I and M.A. helped in sample collection and sample processing during field trips in Bangladesh. T.N. performed all the experimental procedures. S.K.Y. helped in trouble shooting of any experimental problem. F.D.O performed the bioinformatics analysis for *V. metoecus*. P.C.K. helped to find out the unique gene for *V. cholerae*. N.A.S.H., M.T.I., F.D.O., P.C.K., M.A. and S.K.Y. reviewed the manuscript. S.K.Y. and Y.B. supervised the project.

2. A version of chapter 3 has been prepared for submission to **Journal of Microbiological Methods** as:

"Assay for evaluating the abundance of *Vibrio cholerae* and its O1 serogroup subpopulation directly from water without DNA extraction"

Author contribution: Y.B., T.N. and S.K.Y. designed the project. Y.B. and T.N. wrote the manuscript. M.A helped in sample collection during field trips in Dhaka, Bangladesh. T.N. performed all the experimental procedures. S.K.Y. helped in trouble shooting of any experimental problem. M.A. and S.K.Y. reviewed the manuscript. S.K.Y. and Y.B. supervised the project.

3. A version of chapter 4 has been prepared for submission to **Frontiers in Microbiology** (Infectious Diseases). Abstract was submitted and accepted on February 15, 2019.

"Intraspecies interactions and changes in intrinsic environmental factors influence the prevalence of pandemic *Vibrio cholerae* in an urban region endemic for cholera"

Author contribution: Y.B., T.N. and M.A. designed the project. Y.B. and T.N. wrote the manuscript. M.T.I, F.T.J. and M.A. helped in sample collection and sample processing during field trips in Dhaka, Bangladesh. T.N. performed the qPCR and M.T.I did the amplicon sequencing. T.N. and M.T.I. did the analysis. K.L. helped in bioinformatics analysis. M.T.I., K.L., F.T.J. and M.A. reviewed the manuscript. M.A. and Y.B. supervised the project.

In addition to the work done in this thesis, I was involved in other projects conducted in collaboration in our laboratory and co-authored in publications. A complete list of the journal articles that have been published or are in preparation during the tenure of my doctoral degree is provided in Appendix A.

"All that would be required to prevent the disease [cholera] would be such a close attention to cleanliness in cooking and eating, and to drainage and water supply, as is desirable at all times."

John Snow*

*'On the mode of communication of cholera' The Edinburgh Medical Journal (Jan 1856). Vol. 1, No7.670.

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Pursuing Graduate study is not an easy task. It was quite impossible for me to complete my study without the help of my near and dear ones. I am very thankful to all who supported me by alleviating my stress and keeping my smile during the tenure of my doctoral degree.

First, I would like to thank my Supervisor Dr. Yan Boucher who believed in me and gave me this opportunity. I express my gratitude to him for letting me design and execute the plan of my study independently. I will never forget his support during the interesting and fun-filled but laborious fieldwork in the hot summer in Bangladesh. I also like to thank Dr. Boucher for putting a Nespresso coffee machine in our office area. A hot cup of coffee was a real deal for an energetic start in every morning for the lab work and also for a boost during the time of stress.

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I will always remember the support that I received from my friends, here in Edmonton who made this place a little home for me. I am grateful to my family for their patience and love. I am confident that they would be very proud of me to see me at the finish line of this journey.

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Introduction

1.1. Waterborne diseases

Waterborne disease describes infections that are predominately caused by pathogenic microorganisms and transmitted through contact with or ingestion of contaminated water. Diarrhea and vomiting are the two main symptoms for any waterborne infection, but other symptoms like eye, ear and skin irritations are also reported. Waterborne disease outbreaks occur from bathing, washing or drinking water, or by eating food exposed to contaminated water (WHO, 2017). According to the World Health Organization (WHO), waterborne diseases account for an estimated 3.6% of the total disability-adjusted life years (DALY) of global disease burden and cause 1.5 million deaths annually. Lack of safe drinking water supply and poor sanitation and hygiene are responsible for 58% of global disease burden, or 842,000 deaths per year (WHO, 2014).

1.2. Cholera: Past and Present

Cholera is one of the most devastating waterborne diarrheal diseases (Glass et al., 1991; Hughes et al., 1982; Shapiro et al., 1999) that affects an estimated 1.3 to 4 million people worldwide and causes 21,000-143,000 deaths per year (Ali et al., 2015). It is an infection of the small intestine with the bacterium *V. cholerae* (Centers for Disease Control and Prevention) (Finkelstrein, 1996) that causes profuse watery diarrhea and vomiting, leading to a severe and fast progressing dehydration and shock. The disease is prevalent in places where sewage management infrastructure is inadequate or compromised, and personal hygiene is not prioritized (WHO, 2010). Although cholera

affects both children and adults, children under five years are more vulnerable and have a 4 to 6-fold higher risk for cholera (Colombara et al., 2013). Symptoms of cholera appear within 6 hours to 5 days after exposure but typically are observed within 2 to 3 days (Azman et al., 2013). Cholera is treated with rapid rehydration and antibiotic therapy. Cholera vaccines are also available but have an efficacy of 65% (Ali et al., 2011).

Although cholera has been occurring for many centuries, the disease earned notoriety in the 19th century after a deadly outbreak that occurred in India (Barua, 1992). It is still not known precisely when and where the first cholera cases appeared. Descriptions of sporadic cases of a cholera-like malady have been found in ancient documents from India (by Sushruta Samhita in the 5th Century B.C.) and Greece (Hippocrates in the 4th Century B.C. and Aretaeus of Cappadocia in the 1st century A.D.)(Barua, 1992). Gasper Correa, a Portuguese historian and author of *Legendary India* described an outbreak of a disease similar to cholera during the spring of 1563 in the Ganges Delta, South Asian region of Bangladesh and West Bengal, India. 'Moryxy' was the local name of the disease that killed victims within 8 hours of onset and had a high case-fatality rate (Pollitzer, 1954).

Modern knowledge about cholera originated in the early 19th century when progress had been made in better understanding the causes of this disease and treatment strategies. Seven pandemics of cholera have been recorded to date. The 1st pandemic emerged out from the Ganges Delta with an outbreak in India in 1817 and afterwards spread to other countries around the world. The first, and the five following cholera pandemics originated from the same region, spread all over the world and took a heavy toll before receding. In 1961, the 7th and current pandemic began in Indonesia and

spread rapidly across Asia, Europe and Africa and finally in 1991 to Latin America, which had been free of cholera for more than a century (Pollitzer, 1954; WHO).

In recent years, a number of devastating cholera outbreaks have been reported. In 2010, after a catastrophic earthquake, a cholera outbreak occurred in Haiti for the first time in more than a century. It was reported as the worst incidence of cholera in recent history with over 655,000 cases and 8,183 deaths (Centers for Disease Control and Prevention). Recently, cholera outbreaks occurred in Mexico, Congo and many other African countries. Currently, Yemen is experiencing the world's largest cholera outbreak that has been continuing for more than two years, starting in 2016 and has been responsible for >1.2 million suspected cases and >2,510 death (Federspiel and Ali, 2018). Cholera has been epidemic in many countries with or without a history of cholera outbreaks, and thus, it is categorized as an emerging and re-emerging disease (Satcher, 1995).

1.3. *V. cholerae*: An overview

The legendary figure in public health, John Snow, in 1850 first identified the source of cholera during an outbreak in London in 1850 (Richardson, 1936). However, in 1854, an Italian microbiologist Filipo Pacini identified the bacterium and named it cholerigenic vibrios, but it was not recognized until 1883 when German microbiologist Robert Koch proved that the presence of this bacterium in intestines causes cholera (Nardi, 1954).

V. cholerae are halophilic, highly motile curved Gram-negative rods (Weil and Harris, 2015). Besides being a pertinent pathogen, *V. cholerae* is a part of the free-living

marine coastal microbiota, which suggests that the behaviour of this bacterial species in natural (non-clinical) ecosystems might be of relevance for cholera outbreaks, mainly in endemic areas (Martinez, 2013, Charles and Ryan 2011). Different *V. cholerae* populations are identical in biochemical and genetic properties (Krieg and Manual, 1984), but exhibit significant variation in their pathogenic potential. Considering the public health significance, two properties are essential: i) the possession of the cholera toxin (CT) producing gene cluster that is obligatory to cause severe diarrhea; and ii) presence of O1 or O139 antigen which is the determinant of epidemic potential (Weil and Harris, 2015). *V. cholerae* serogroups are determined based on their somatic antigens (O antigens), and currently, there are about 200 known serogroups (Chatterjee and Chaudhuri, 2003). Only O1 or O139 among all the serogroups are responsible for all of the severe epidemics and pandemics. Serogroup O1 has been further classified into two serotypes: Ogawa and Inaba and the latter has been divided into two biotypes, classical and El Tor, based on some biochemical properties and susceptibility to bacteriophages (Sakazaki, 1970). The other serogroups are recognized as non-O1 *V. cholerae*, and have been reported to cause sporadic cases of diarrhea (Janda et al., 1988) or in some instances cause large cholera-like outbreaks (Albert et al., 1993).

1.3.1. Survival and persistence of *V. cholerae* in the environment

V. cholerae is a cosmopolitan aquatic species that inhabits diverse geographical locations from tropical to temperate waters. This worldwide presence of *V. cholerae* emphasizes its ability to adapt and persist in a wide range of environmental conditions. A range of evolutionary-adaptive responses *V. cholerae* to survive environmental stressors

like nutrient depletion, changes in salinity, temperature and resistance to heterotrophic protists and bacteriophages, thus, increasing its fitness to inhabit in such versatile environmental niches. Strategies to overcome environmental hurdles includes transformation to viable but non-culturable (VBNC) cells during unfavorable conditions (Colwell, 2000; Thomas et al., 2006) and biofilm formation by attaching to abiotic and biotic surfaces (Chitinous and gelatinous zoo- and phytoplankton) (Akselman et al., 2010; Huq et al., 1996; Shikuma and Hadfield, 2010). Colonization to a solid surface often increases access to nutrients and also provides means of dispersal when attached to living, mobile hosts (Costerton et al., 1995; Hall-Stoodley et al., 2004). VBNC cells are viable and metabolically active cells but become smaller in size (most likely form a coccoid shape), and unlike starved cells, they fail to grow on typical microbiological medium (Colwell, 2000). These cells are frequently detected as attached to the surface of higher organisms such as crustaceans, algae, plankton, benthos and chironomid egg masses (Alam et al., 2007; Halpern et al., 2007) (FIG 1.1.). The presence of VBNC cells has been also observed in the biofilm consortia attached to biotic and abiotic surfaces. Surface attachment and progressive biofilm formation are advantageous for the persistence and survival of many organisms as it facilitates access to the nutrients that accumulates at the liquid-surface interface (Dawson et al., 1981). Additionally, biofilm on the biotic surface such as chitinaceous organisms serves as a nutrient source of carbon, nitrogen and other elements required for better colonization.

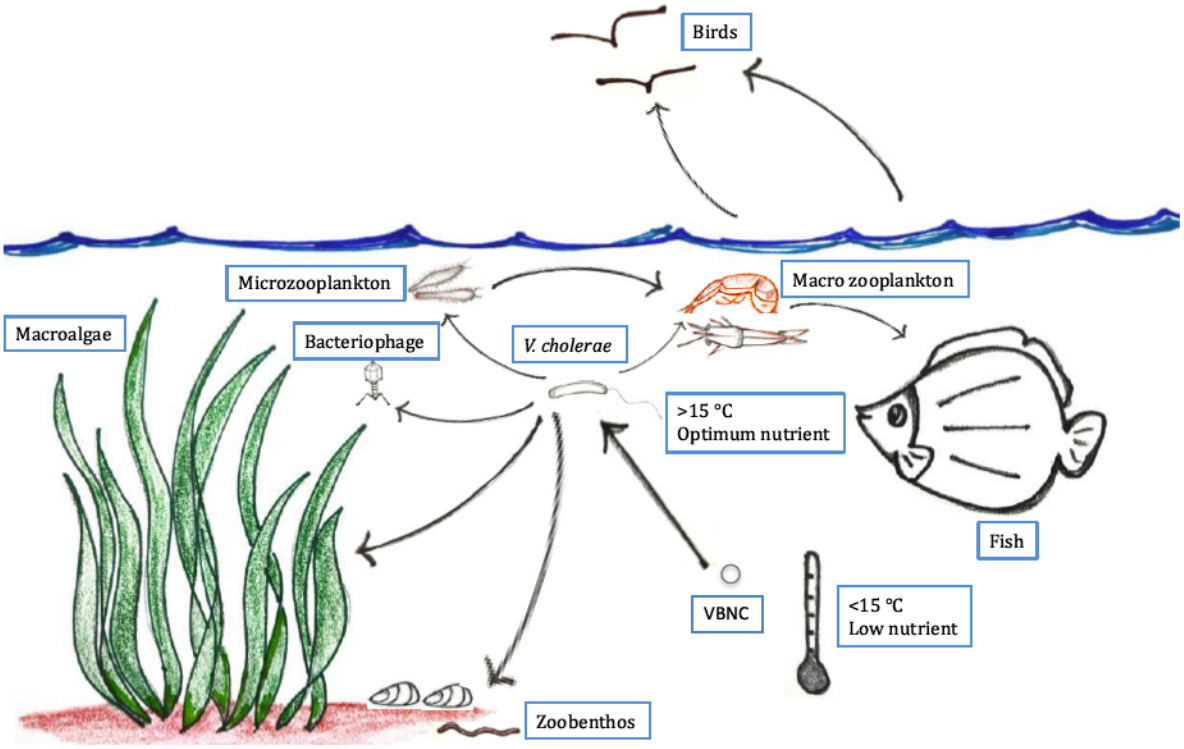

FIG 1.1. *V. cholerae* in the environmental reservoir. *V. cholerae* exists as a part of bacterioplankton in the aquatic environment and usually associates with autotrophic organisms as well as chitinous zooplankton that provide carbon and other nutrients. In the environment, *V. cholerae* undergoes predation pressure by protozoa and infection with bacteriophages. Moreover, unfavorable temperature and nutrient limitation trigger the VBNC state which is resuscitated when the conditions become optimal again. [The author drew the picture and, the idea was adapted from (Lutz et al., 2013)].

Type VI secretion systems (T6SS) are an important molecular weapon for both pathogenic and non-pathogenic *V. cholerae* strains for enhancing persistence within the microbial community in the aquatic environment and to successfully colonize human intestine by challenging commensal host flora (MacIntyre et al., 2010; Pukatzki, 2013). Genes encoding the T6SS have been found in all *V. cholerae*. The T6SS structurally mimics a syringe-like contractile device to deliver effector molecules, which are toxic to particular microorganism and cause lysis of the targeted cell. The T6SS is encoded by

three gene clusters and each of which has effector-immunity proteins combinations. Coexistence of different *V. cholerae* lineages depends on the compatibility of their effector modules. Distant lineages of *V. cholerae* with the identical effector module pairs may occupy the same niche whereas strains with a different effector-immunity combinations are unable to exist together, one must outcompete other, otherwise both will be diminished (Kirchberger et al., 2017; Unterweger et al., 2014). T6SS thus provides competitive advantages in the low nutrient environment as lysis of non-compatible cells provide nutrients and also during cholera infection pathogenic strains have a high level of activated T6SS thus provide better fitness when released to the environment through diarrheal discharge (Pukatzki, 2013).

Persistence of *V. cholerae* in the environment is influenced by intrinsic environmental factors such as temperature, salinity and organic matters. *V. cholerae* is able to withstand a wide range of temperatures. In most instances, they are detectable by culture when the temperature remains >15 °C and show preferences toward warmer temperature (Blackwell and Oliver, 2008; Huq, 1986). Seasonal variation of *V. cholerae* abundance indicates that temperature is a determining factor for its persistence and growth. Higher temperature initiates seasonal bloom of flora and fauna in the aquatic environment and eventually encourages attachment of *V. cholerae* to plankton as well as the chitinous surface with progressive biofilm formation (Huq, 1986). Fluctuations in temperature due to seasonal changes, may have an impact on the salinity of estuarine water. *V. cholerae* is a natural inhabitant of estuarine water bodies and has a tolerance to a wide range of salinity (0 to 35ppt) and thus persist in fresh water as well as in coastal water (Lutz et al., 2013). Although salinity has no effects on the attachment of *V. cholerae*

to the solid surface, it has a significant effect on their dispersal (Kirchberger et al., 2016). Nutrient concentration in the environmental aquatic system is not uniform and is affected by cell decay as well as waste discharge (Blackburn et al., 1998). The total dissolved solids that include inorganic and organic matter could increase nutrient inflow as well as provide more surface for colonization thus may affect the localized abundance of *V. cholerae*.

Relationship between the abundance of *V. cholerae* in the environment and increase in the frequency of cholera cases correlates in cholera-endemic areas. Toxigenic *V. cholerae* O1 represent approximately 23.8% of the total *V. cholerae* isolates from the environmental water samples during the spring months in Bangladesh and represent 7.1% during the fall season months, whereas there was no *V. cholerae* O1 isolated during the inter-epidemic periods of the year (Sultana et al., 2018). In contrast, in cholera non-endemic area, like the Chesapeake Bay in United States, where no major outbreak is reported in the last 50 years, *V. cholerae* has been frequently isolated during the warmer months and remain non-detectable during winter (Colwell et al., 1977). However, in Bangladesh, where cholera episodes display dual peaks, one major outbreak usually is observed right after the monsoon season and the other one during the spring (Alam et al., 2011). During monsoon, rain run-off increases, resulting in an escalation in phytoplankton followed by zooplankton blooms and which provides more chitinous surface area for *V. cholerae* to colonize and proliferate for *V. cholerae* (Huq et al., 2012). A link between transmission of cholera with zooplankton has been reported previously (Huq et al., 2008), thus removal of particulate matters by filtering water with sari cloth drastically reduces the incidence of cholera cases during outbreaks in rural areas in

Bangladesh and is practiced routinely for preliminary purification of water (Huq et al., 1996).

1.3.2. The lifecycle of pathogenic *V. cholerae*

Although *V. cholerae* exists as a diverse species in aquatics environments, only a single lineage (Please see FIG B.1.) is responsible for human disease, and thus its lifecycle (FIG 1.2.) is different from that of other non-pathogenic lineages of *V. cholerae*. Two distinct phases in cholera-causing *V. cholerae* lifecycle are noticeable: i) in the human host; and ii) outside of the host, in the aquatic environment. Because of the continuous and consistent passage between the physical environment and human and vice versa, the toxigenic strains of *V. cholerae* are most detectable in the environment of cholera-endemic regions.

Persistence in these two distinct environments largely depends on their inherent and acquired adaptability to environmental shifts. Ability of *V. cholerae* to withstand a wide range of temperature, utilize intestinal biopolymers as nutrient and biofilm formation using mucin as well as resistance to intestinal bile are useful to evade host immunity and thus *V. cholerae* are able to survive and infect the human host (Rueggeberg and Zhu, 2016). Moreover, two major virulence factors, cholera toxin (CT) and the toxin co-regulated pilli (TCP), are mandatory for *V. cholerae* to become toxigenic and to cause disease in humans. The TCP is the receptor for CTX phages, thus the presence of bacteriophage in the aquatic environment is also essential for emerging pathogenic *V. cholerae* (Faruque et al., 1998; Jensen et al., 2006).

In the aquatic environment, the CTX phage was found to be inversely correlated with the occurrence of viable *V. cholera*; therefore the number of cholera cases decline with the increase of this lysogenic bacterial phage (Faruque et al., 2005b). However, fecal discharge from infected individuals increases *V. cholera* concentration as well as the coexistence of some natural reservoir of it, for example, *Vibrio mimicus* may influence the advent of PG *V. cholerae* (Boyd et al., 2000).

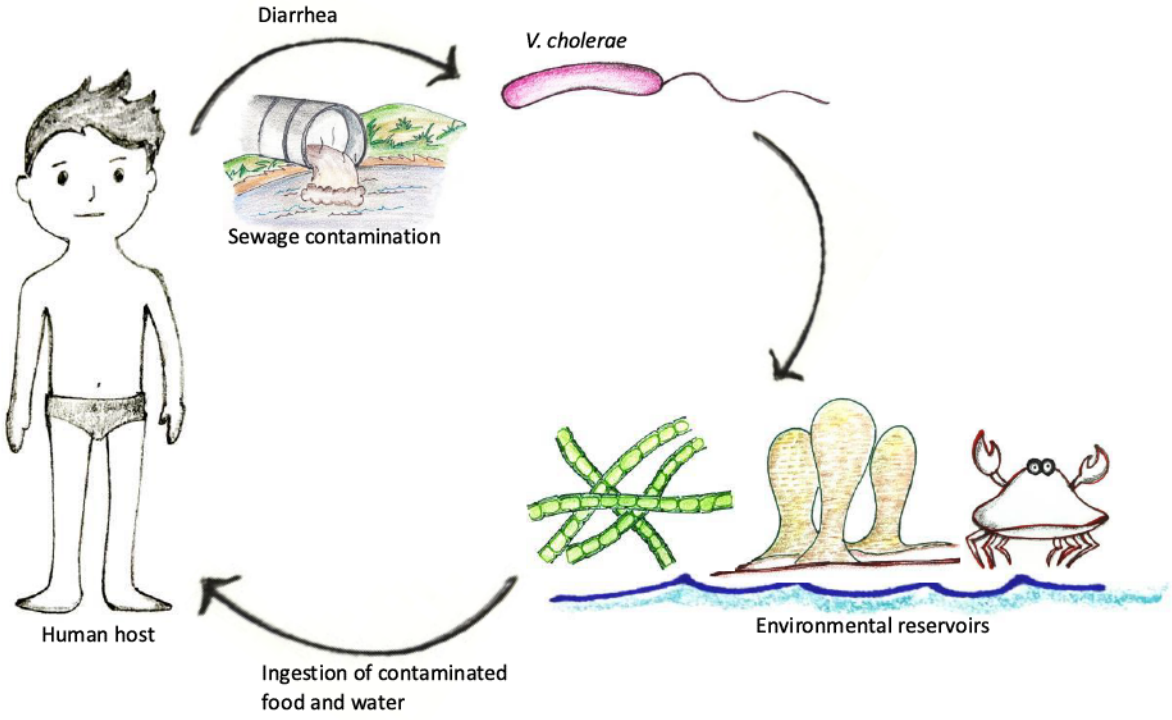

FIG 1.2. The lifecycle of pathogenic *V. cholerae.* Both toxigenic and non-toxigenic *V. cholerae* co-exist in the aquatic environment. Following ingestion of contaminated food or drink, toxigenic strains infect and colonize the host small intestine. Production of cholera toxin leads to profuse diarrhea, and due to improper sewage disposal, toxigenic *V. cholerae* are shed back into the environment.

In addition to CT and TCP, other toxins such as hemolysin/cytolysin (vcc), zonula occludens toxin (Zot) and accessory cholera enterotoxin (Ace) have been identified in *V. cholerae*. Vcc is an 80KDa pore-forming toxins (PFT) and encoded by the *hlyA* gene that is expressed in most *V. cholerae* strains including O1 biotype El Tor, O139 and non-O1 /non-O139 isolates. The effects of vcc on eukaryotic hosts have been documented at both the cellular and organism level; in vitro, vcc is found to be associated with cellular degenerative events such as vacuolization and lysis. In infant mouse and rabbit ileal loop models, toxicity and diarrhea observed due to vcc after the administration of vaccine strains into the gastrointestinal system (Bag et al., 2008; Ichinose et al., 1987).

1.4. *V. metoecus*: A close relative of *V. cholerae*

V. metoecus is the closest known relative of *V. cholerae*, as described by isolates from both clinical and environmental sources from Europe (Italy and Spain) and from the USA (Boucher et al., 2011; Carda-Diéguez et al., 2017; Haley et al., 2010; Kirchberger et al., 2014). *V. metoecus* was originally described as an atypical variant of *V. cholerae* that formed identical yellow colonies on selective thio-sulfate bile sucrose agar and which shared high 16S rRNA sequence identity (98%) with *V. cholerae*. This organism can be differentiated from *V. cholerae* by a few biochemical tests, such as being negative for acetoin in the Voges-Proskauer assay, positive for amylase and lipase production, and able to use N-acetyl-D-galactosamine or D-glucoronic acid as a sole source of carbon (Choopun, 2004; Kirchberger et al., 2014).

V. metoecus was co-isolated with *V. cholerae* from a cholera-free Oyster pond (Falmouth, MA, USA) containing brackish water near the East coast of the USA (Boucher

et al., 2011; Kirchberger et al., 2014). However, their abundance has not been investigated so far, which might be important to predict their interaction with *V. cholerae* and for a possible genetic exchange through horizontal gene transfer (HGT). Antibiotic resistance and virulence genes could be transferred as mobile genetic elements through HGT that could result in the emergence of pathogenic strain. This process plays an essential role in the evolution, adaptation, maintenance and transmission of virulence in between organisms (De la Cruz and Davies, 2000).

Like *V. metoecus*, *V. mimicus* was found to be a closely related species to *V. cholerae*. Phenotypical characteristics of both species are similar except *V. mimicus* is a non-sucrose fermenter (Davis et al., 1981; Desmarchelier and Reichelt, 1984). Due to their clear phylogenetic differentiation, these two species were grouped separately (Thompson et al., 2008).

1.5. Current methods for detection and shortcomings

1.5.1. Conventional culture-based method

Culture of stool specimens is the gold standard for the laboratory diagnosis of cholera (Centers for Disease Control and Prevention), and allows isolation and identification of *V. cholerae* O1 and O139. However, to study the ecology of *V. cholera*e in its natural environment, conventional culture methods are not sufficient (Huq et al., 2012). False negative results can be due to the presence of VBNC *V. cholerae* may not imply an absence of *V. cholerae* in a sample (Roszak and Colwell, 1987; Xu et al., 1982). Molecular techniques coupled with culture methods could be a powerful tool for the detection, quantification and characterization of total populations of this organism. With

the culture method, it is possible to revive only a small portion of the total bacteria (<0.1% of total bacteria) from the original sample (Huq et al., 2012). Conventional culture methods used to isolate *V. cholerae* from an environmental samples requires a pre-enrichment in alkaline peptone water followed by inoculation on a selective medium such as thiosulfate citrate bile salt (TCBS). It was shown that toxigenic *V. cholerae* could be isolated from 2 to 3% of environmental water samples by the culture-based methods after the pre-enrichment procedure (Sultana et al., 2018). Overnight incubation results in yellow colonies due to fermentation of sucrose that results in a pH shift, further confirmed by a set of biochemical tests and serology (Huq et al., 2012). Therefore, the culture-based method allows for detection of viable organisms and is also useful for having in-depth genetic information of the isolates through whole genome sequencing and bioinformatic analysis.

1.5.2. Direct detection of *V. cholerae* from the environmental sample by immunological methods

Microscopic examination with a monoclonal antibody specific to *V. cholerae* has been used to detect *V. cholerae* directly from the environmental samples without carrying out culture. Fluorescence in situ hybridization (FISH) is also used to detect *V. cholerae* by using a gene-specific probes to identify a range of *V. cholerae* irrespective of their serogroup. Fluorescent antibody coupled with viable count (DFA-DVC) has been found to be convenient for detecting *V. cholerae* O1 and O139 serogroups (Huq et al., 2012). This direct fluorescent antibody technique together with direct viable count method developed by Kogure (Kogure et al., 1979) is thought to be more reliable

amongst microscopy techniques because this method alone can identify viable culturable cells and viable but non-culturable cells (VBNC) of both O1 and O139 serogroups of *V. cholerae*. Quantification is possible with these methods if the sample is not pre-enriched (Huq et al., 2012; Schauer et al., 2012).

1.5.3. Molecular methods for the detection and quantification of *V. cholerae* in the environmental reservoir

Direct molecular assays such as conventional polymerase chain reaction (PCR), where species-specific DNA sequences are amplified and visualized by gel electrophoresis is a very useful method for specific identification of *V. cholerae*. The multiplex PCR assay for the detection of *ompW* and *ctxA* genes is routinely practiced in many laboratories to detect *V. cholerae* as well as *ctxA* carrying strains as a part of screening procedure (Huq et al., 2012). Although endpoint PCR is useful for rapid and sensitive identification *V. cholerae* from a wide range of clinical and environmental samples, this procedure is qualitative only and needs the additional step of gel electrophoresis.

An advancement in molecular methods is quantitative real-time PCR (qPCR) which can measure an absolute number of *V. cholerae* cells in environmental samples in a relatively short time. Two different basic qPCR techniques are currently available. A SYBR green assay, which uses SYBR Green I as a dsDNA binding dye that intercalates nonspecifically into dsDNA allows quantification of the amplified product. With the progression of amplification, an increase of the amplified DNA product is detected by the increase of the incorporated SYBR Green molecules into dsDNA. Therefore, the

correlation between fluorescence intensity and the concentration of amplified product can be made to quantify the number of *V. cholerae*. This technique has been used for the detection and quantification of *V. cholerae* from environmental samples and seafood (Gubala, 2006). Another molecular technique exploits a probe-based assay, where 5' exonuclease activity of *taq* polymerase is used to hydrolyze an internal probe labeled with a fluorescent reporter dye (FAM, Cy3, Cy5 and TET) and a quencher. During PCR amplification of the target gene, hydrolysis of the probe separates the reporter dye from the quencher, resulting in an increase of fluorescence proportional to the amount of template DNA in the reaction, thus generating a quantitative estimation. Probe-based quantitative analysis has proven to be a very sensitive and rapid assay for rapid screening for *V. cholerae* in different kinds of seafood like oysters and seawater (Lyon, 2001). Both methods are capable of quantifying *V. cholerae* from direct DNA extracts of environmental samples, with or without pre-enrichment. The major drawback of these qPCR methods is that they cannot differentiate viable and nonviable cells. Moreover, the presence of inhibitors in environmental samples may reduce the sensitivity of the assay. For these methods, multiple reactions must be run to ensure reproducible results.

1.5.4. Next-generation sequencing (NGS): An approach for better surveillance

The massively parallel sequencing technology known as next-generation sequencing (NGS) is a great approach to study microorganisms in detail (illumina). This technology revolutionized sequence-based molecular analysis because of its ultra-high throughput, scalability and speed. This technology provides the researcher with an opportunity to perform a variety of applications and explore information at the genomic

level. Whole genome sequencing provides valuable insights into the genetic makeup of an organism and evolutionary pathways, and thus provides tools for better characterization of microbial strains.

Whole genome sequencing data can provide high-resolution information about single copy genes, species-specific genes and protein-encoding genes other than 16S rRNA. Protein-encoding housekeeping genes often provide better resolution than 16S rRNA gene because of the reduced selection at the third codon position (Case et al., 2007). The major limitation of whole genome sequencing for environmental studies is that it requires isolated strains in a pure culture, which is not always possible.

Amplicon sequencing is a popular approach that enables the researcher to analyze genetic variations in specific genomic regions (illumina) and is useful for analyzing unculturable organisms. The in-depth sequencing of PCR products (amplicons) allows efficient variant identification and characterization, thus useful for demonstrating interspecies and intraspecies diversity. This method uses oligonucleotide primers designed to target regions of interest, which are followed by NGS. Although the price of NGS dropped significantly, it is still considered an expensive approach, yet this powerful tool offers excellent qualitative data and can estimate the relative abundance of the organisms or genes of interest. However, the bias toward high abundant strains compared to low abundant strains in the sample could be a drawback of this method.

1.6. Challenges for the amplification from direct environmental samples

Direct amplicon analysis of environmental samples is mostly impeded by the inhibitor-laden sample. The presence of inhibitors in samples may produce unreliable

data. PCR inhibition and higher limit of detection in qPCR are common causes of incorrect calculation of marker concentration and false negative results. Inhibitors present in the sample may directly interact with DNA, thus prevent the amplification, and interfere with the DNA polymerase, therefore, blocking enzyme activity (Bessetti, 2007). In environmental water, humic and fulvic acids are frequently reported as PCR inhibitors (Abbaszadegan et al., 1993; Ijzerman et al., 1997).

To overcome the effect of inhibitors, purification of extracted DNA using commercial kits could be useful. A wide range of kits are commercially available and remove inhibitors efficiently. Other options that could be advantageous may include the choice of a DNA polymerase which is less prone to inhibitory effects (Katcher and Schwartz, 1994; Wiedbrauk et al., 1995). Moreover, cofactors required for DNA polymerase can be the target of inhibition. In these instances, the most reliable way to avoid inhibitors in the sample is to prevent inhibitors from being processed with the sample. An increased amount of DNA polymerase and the use of additive such as BSA (Bovine Serum Albumin) often provide resistance to inhibitors in samples (Comey et al., 1994). In real-time multiplex qPCR assays, an internal positive control (IPC) provides an opportunity to monitor the effects of inhibitors. Moreover, analyzing the amplification efficiency of a marker gene can also be useful to point out any inhibition effect (Bustin et al., 2009; Kontanis and Reed, 2006).

1.7. Thesis objective and outline

Immediate intervention is often necessary to control a cholera outbreak. Therefore, fast and accurate detection of the causative bacterium *V. cholerae* is critical.

Continuous environmental monitoring of the abundance of *V. cholerae* and the toxigenic O1 subpopulations could contribute knowledge on the distribution of this organism in aquatic reservoirs and thus could help to predict an outbreak before it starts and to take necessary measures to stop it. The lack of substantial temporal and spatial environmental sampling, along with specific quantitative measures, has made this goal elusive so far. Therefore, the main objective of this thesis was to develop tools for efficient and reliable quantitative evaluation of *V. cholerae* abundance in aquatic environments. With a combination of qPCR and amplicon sequencing, I was able to capture the intraspecies diversity of the total *V. cholerae* population and their relative abundance. Initially, I was focused on designing a multiplex qPCR assay for the detection and quantification of toxigenic and nontoxigenic *V. cholerae* along with *V. metoecus*. Then I developed a field ready qPCR assay for rapid surveillance of *V. cholerae* O1 in the environmental water reservoirs. Finally, I carried out a qPCR assay coupled with amplicon sequencing of extracted DNA from environmental biomass and characterized the *V. cholerae* population with a subspecies level resolution.

In Chapter 2, I developed a multiplex qPCR assay to determine the abundance of *V. cholerae* and its toxigenic O1 subpopulation along with *V. metoecus* simultaneously. *V. metoecus*, a recently described species reported to cause opportunistic infections in humans is closely related to *V. cholerae* and often coexists with *V. cholerae* in aquatic environments. The higher specificity of this assay was obtained by designing species-specific markers for four different genes. *viuB*, a gene encoding a vibriobactin utilization protein, was used to quantify the total *V. cholerae* population, the cholera toxin gene *ctxA* provided estimation of toxigenic *V. cholerae* abundance, while the *rfbO1* gene specifically

detected and quantified *V. cholerae* belonging to the O1 serogroup, which includes most lineages of the species responsible for majority of past and ongoing cholera pandemics. To measure the abundance of *Vibrio metoecus*, the gene *mcp*, encoding methyl chemotaxis protein, was used.

In this chapter, I analyzed environmental water samples collected from four different geographic locations including cholera-endemic (Dhaka, Kuakata and Mathbaria, Bangladesh) and non-endemic areas (Oyster Pond in Falmouth, Massachusetts, USA). I hypothesized that *V. cholerae* O1 would be more prevalent in cholera-endemic regions compared to non-endemic regions. There would be noticeable seasonal variation in distribution and abundance of *V. cholerae* as it was reported earlier to be influenced by temperature shifts. Moreover, this is the first study that explores the presence of *V. metoecus* along with *V. cholerae* in cholera endemic and non-endemic region. Despite the fact that cholera has two seasonal peaks in the cholera-endemic region, toxigenic *V. cholerae* O1 was found to be present continually in the inland water reservoir at levels that pose a risk to human health. No detection of *V. metoecus* in this area indicated its different geographical distribution. Furthermore, the sporadic presence of *V. cholerae* O1 at a substantial proportion of the local *V. cholerae* total population in a region not endemic for cholera also signifies its global distribution which could have an impact on the potential for the emergence of novel virulent variants.

In chapter 3, I developed a new on-site method for the detection and quantification of *V. cholerae* O1 (pandemic generating serogroup) and *V. cholerae* non-O1 by qPCR directly from environmental water samples without DNA extraction. The goal was to design and develop a simple, cheap and effective assay, that can be used directly on-site

for the detection and quantification of *V. cholerae* in resource-limiting settings. Although cholera epidemiology is well studied, information on *V. cholerae* abundance in environmental reservoirs are limited and rapid detection is still a challenge. The developed assay was used to analyze environmental water samples collected from a cholera-endemic site (Dhaka, Bangladesh) and revealed that *V. cholerae* O1 is present in a substantial proportion of total *V. cholerae* population that is undetected by conventional culture-based methods. This assay would be extremely useful for field research because of the portability of the low weight thermocycler, easy setup method, reagent stability at refrigerated temperature for a reasonable period and ability to analyze data free via online software.

In chapter 4, I expanded my survey of the abundance of *V. cholerae* in aquatic environments. The objective of this chapter was to acquire information on spatial and temporal dynamics of the O1 toxigenic *V. cholerae* lineage as well as other lineages belonging to the *V. cholerae* species in Dhaka's water reservoirs. Here, I carried out a combined method of high throughput sequencing of a species-specific, highly diverse protein-coding gene (vibriobactin utilization protein, *viuB*) amplified from aquatic biomass DNA and real-time qPCR. With this method, information at subspecies level resolution of the abundance of *V. cholerae* populations and their lineage compositions was obtained. Seven different sites around Dhaka city were included in this study and were sampled for six consecutive months. I hypothesized that abundance and subspecies composition of this organism might vary with the human population density around the sampling sites, the latter directly influencing the physicochemical properties of the water bodies depending on the usage and waste disposal. I also predicted that there might be

variation in the relative abundance of the subspecies due to compatibility or incompatibility among the subspecies. In this study, I measured environmental parameters, and it would be interesting to observe how a minimal change in environmental parameter affects the population dynamic and how they correlate with *V. cholerae* lineage richness. In here, the abundance of total *V. cholerae* population showed consistency but PG lineage displaying the O1 antigen varied in proportion and often showed a sudden reduction. In this instance, other *V. cholerae* lineages took the dominance of the entire *V. cholerae* population. From the observations in this study, it could be predicted that there is a role of the human carrier on circulation of the particular *viuB* allele in a densely populated area in this city. Moreover, small changes in the environmental factors were found to influence the interspecies interaction or survival of specific *V. cholerae* lineages.

1.9. References

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CHAPTER2

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Author contribution: Y.B. and T.N. designed the project and wrote the manuscript. M.T.I and M.A. helped in sample collection and sample processing during field trips in Bangladesh. T.N. performed all the experimental procedures. S.K.Y. helped in trouble shooting of any experimental problem. F.D.O performed the bioinformatics analysis for *V. metoecus*. P.C.K. helped to find out the unique gene for *V. cholerae*. N.A.S.H., M.T.I., F.D.O., P.C.K., M.A. and S.K.Y. reviewed the manuscript. S.K.Y. and Y.B. supervised the project.

CHAPTER 2

Simultaneous quantification of *Vibrio metoecus* and *Vibrio cholerae* with its O1 serogroup and toxigenic subpopulations in environmental reservoirs

2.1. ABSTRACT

Vibrio metoecus is a recently described and little studied causative agent of opportunistic infections in humans, often coexisting with *V. cholerae* in aquatic environments. However, the relative abundance of *V. metoecus* with *V. cholera*e and their population dynamics in aquatic reservoirs is still unknown. We developed a multiplex qPCR assay with a limit of detection of three copies per reaction to simultaneously quantify total *V. metoecus* and *V. cholerae* abundance, as well as the toxigenic and O1 serogroup subpopulations of *V. cholerae* from environmental samples. Four different genes were targeted as specific markers for individual *Vibrio* species or subpopulations; *viuB,* a gene encoding a vibriobactin utilization protein, was used to quantify the total *V. cholerae* population. The cholera toxin gene *ctxA* provided an estimation of toxigenic *V. cholerae* abundance, while the *rfbO1* gene specifically detected and quantified *V. cholerae* belonging to the O1 serogroup, which includes almost all lineages of the species responsible for the majority of past and ongoing cholera pandemics. To measure *V. metoecus* abundance, the gene *mcp*, encoding methyl accepting chemotaxis protein, was used. Marker specificity was confirmed by testing several isolates of *V. cholerae* and *V. metoecus* alongside negative controls of isolates within and outside of the *Vibrio* genus. Analysis of environmental water samples collected from four different geographic locations including cholera-endemic (Dhaka, Kuakata and Mathbaria in Bangladesh) and non-endemic (Oyster Pond in Falmouth, Massachusetts, USA) regions showed that *V.*

metoecus was only present in the USA site, recurring seasonally. Within the coastal USA site, the non-toxigenic O1 serogroup represented up to ~18% of the total *V. cholerae* population. *V. cholerae* toxigenic O1 serogroup was absent or present in low abundance in coastal Bangladesh (Kuakata and Mathbaria) but constituted a relatively high proportion of the total *V. cholerae* population sustained throughout the year in inland Bangladesh (Dhaka). A preference for host/particle attachment was observed, as the majority of cells from both *Vibrio* species (>90%) were identified in the largest water size fraction sampled, composed of particles or organisms >63 μm and their attached bacteria. This is the first study to apply a culture-independent method to quantify *V. cholerae* or *V. metoecus* directly in environmental reservoirs of areas endemic and non-endemic for cholera on significant temporal and spatial scales.

2.2. SIGNIFICANCE

Cholera is a life-threatening disease that requires immediate intervention; it is of prime importance to have fast, accurate and sensitive means to detect *V. cholerae*. Consistent environmental monitoring of the abundance of *V. cholerae* along with its toxigenic and O1 serogroup subpopulations could facilitate the determination of the actual distribution of this organism in aquatic reservoirs and thus help to predict an outbreak before it strikes. The lack of substantial temporal and spatial environmental sampling, along with specific quantitative measures, has made this goal elusive so far. The same is true for *V. metoecus*, a close relative of *V. cholerae* which has been associated with several clinical infections and could likely pose an emerging threat, readily exchanging genetic material with its more famous relative.

2.3. INTRODUCTION

Vibrio cholerae is an autochthonous aquatic bacterium (Colwell et al., 1977) which shows variable physiologies, from non-pathogenic to extremely virulent strains capable of causing a life-threatening diarrheal infection, cholera (Alam et al., 2006). According to the World Health Organization (2016), every year, 1.4 to 4 million people are infected with cholera, and 21,000 to 143,000 people die from this disease (WHO, 2016). This species comprises over 200 serogroups (Thompson et al., 2004), but strains of the O1 and O139 serogroups are distinguished as the most virulent, has caused some of the most devastating pandemics in human history (Ali et al., 2012; Chun et al., 2009; Islam et al., 1994; Kaper et al., 1995). Other serogroups are collectively known as non-O1/non-O139 and can cause sporadic outbreaks of diarrheal disease not severe like cholera (Dutta et al., 2013). *V. cholerae* O139 was only found to be associated with isolated cholera cases after two epidemics occurred in 1993 and in 2002 (Faruque et al., 2003; Ghosh et al., 2016). During 2005 in Bangladesh O139 serogroup have been isolated sporadically from both clinical and environmental samples but there was not any reported largescale outbreak of cholera caused by *V. cholerae* O139. Further study confirmed that four *V. cholerae* isolated from cholera patients identified as *V. cholerae* O139 during 2013 and 2014 in Bangladesh by typing and whole genome sequencing (Alam et al., 2006; Chowdhury et al., 2015; Rashed et al., 2013). However, *V. cholerae* O1 has been prevalent in the Ganges Delta for several hundred years; this remains the only place in the world where cholera has been continually endemic since the first modern pandemic in 1817 (Boucher et al., 2015; Mutreja et al., 2011). Much research has been done to characterize the physiology of pathogenic *V. cholerae* strains, but still, we have

limited understanding of the variation of *V. cholerae* population composition and abundance over time and between areas, which influences where and when epidemics are likely to occur.

Vibrio metoecus is a recently described species, which has been co-isolated with *V. cholerae* from aquatic environments in the USA East coast (Choopun, 2004; Haley et al., 2010; Kirchberger et al., 2014). Based on biochemical, genotypic and phylogenetic evidence, *V. metoecus* is the closest known relative of *V. cholerae* (Kirchberger et al., 2014). It has been recovered not only from the environment but also from a variety of human specimens (blood, stool, ear and leg wounds) in opportunistic infections across the USA (CDC, Atlanta, Georgia, USA)(Kirchberger et al., 2014). Currently, no studies have assessed the abundance of *V. metoecus* in aquatic habitats. Information on the distribution of *V. metoecus* in its natural reservoirs is essential not only to provide insight into transmission routes from the environment to human, but also because of its co-occurrence with *V. cholerae* and the occurrence of possible horizontal gene transfer (HGT) between the species, which could be responsible for producing more virulent strains of *V. metoecus* (Orata et al., 2015).

Most surveys of *V. cholerae* used culture-based methods, requiring significant time and effort, and their reliance on selective enrichment prevents any form of absolute quantification (Baron et al., 2007). Moreover, these culture-based techniques are unable to detect the viable but non-culturable (VBNC) form of *V. cholerae* (Colwell et al., 1996; Colwell and Huq, 1994; Miller et al., 2009) and thus underestimate the abundance of toxigenic and non-toxigenic *V. cholerae* in the environment (Huq et al., 1996). To circumvent this problem, several studies have used antibody or hybridization-based

molecular detection of toxigenic *V. cholerae* from clinical (Lobitz et al., 2000; Qadri et al., 1995) and environmental samples (Baron et al., 2007; Blackstone et al., 2007; Colwell et al., 1985; Huq et al., 1990; Theron et al., 2000), but most are qualitative, and none have been field-tested to comprehensively survey its abundance in natural environments (Gubala, 2006; Neogi et al., 2010). Another molecular tool, real-time quantitative PCR (qPCR), has been used to measure both the presence and abundance of target species (Abdullah et al., 2018; Haugland et al., 2005). This technique can estimate the presence of as few as three bacterial genome equivalents in a sample and provides the high throughput required to investigate the ecological distribution of the organism (Kralik and Ricchi, 2017). Several gene markers have been developed for the detection of the *V. cholerae* species by qPCR, including the *ompW* gene encoding the outer membrane protein (Bliem et al., 2015), the *hlyA* gene encoding for hemolysin (Bliem et al., 2015; Lyon, 2001) and *gbpA* gene encoding the N-acetyl glucosamine-binding protein A (Vezzulli et al., 2015), and the *rtx* gene cluster encoding the Repeats In Toxin protein (Lin et al., 1999). During the assessment of different primers used in previous studies, it was found that the commonly used primers designed for *ompW* also amplified that gene from *V. metoecus* (in this study) and *Vibrio mimicus* (Gubala and Proll, 2006). Copy number is also a confounding factor, as *rtx* (Lin et al., 1999), *hlyA* and *ctxA* can be present in multiple copies in *V. cholerae* genomes (Ghosh et al., 2011; Heidelberg et al., 2000). Although *gbpA* is present as a single copy, it can also be found in environmental strains belonging to other *Vibrio* species including *Vibrio alginolyticus*, *Vibrio metschnikovii*, *Vibrio mimicus*, *Vibrio vulnificus* and *Vibrio parahaemolyticus* (Stauder et al., 2012). Lack of species specificity and the presence of multiple copies in single cells make quantitative

estimates unreliable. Furthermore, given that a single lineage of *V. cholerae* is responsible for all major outbreaks [phylocore genome/pandemic-generating (PG) *V. cholerae*] (Boucher, 2016; Chun et al., 2009) (FIG B.1.), detection of other genotypes which are mostly harmless can be misleading in evaluating the risk of outbreaks. Attempts to accurately quantify toxigenic *V. cholerae* using the cholera toxin (CT) gene *ctxA* have been made; while the reference El Tor strain N16961 carries a single copy of this gene, other *V. cholerae* strains carry several copies of this element and it is also occasionally found in other *Vibrio* species (Dalsgaard et al., 2001; Faruque et al., 1997; Heidelberg et al., 2000; Mekalanos et al., 1983). The *rfbO1* gene, targeting *V. cholerae* strains carrying the O1 antigens has also been used to detect pandemic *V. cholerae* (Hoshino et al., 1998; Yamasaki et al., 1996). Although it is a single copy gene, many strains unrelated to this lineage can also carry this gene (Pang et al., 2007).

To overcome the limitations of currently used molecular markers, we developed a multiplex qPCR assay for simultaneous detection and quantification of *V. cholerae* O1 (*rfbO1*), toxigenic *V. cholerae* (*ctxA*), total *V. cholerae* (*viuB*) and *V. metoecus* (*mcp).* This optimized qPCR technique allows the quantitative study of *V. cholerae* populations directly from DNA extracted from aquatic biomass, without the need for cultivation. This overcomes the problem that many *V. cholerae* cells in water are viable but not culturable (VBNC) (Colwell et al., 1985), which has so far made it difficult to the study this organism in the environment difficult. Quantifying the presence of *ctxA* and *rfbO1* simultaneously resolves drawbacks specific to the separate use of these two gene markers. Overestimation of the number of toxigenic cells because of the presence of multiple copies of *ctxA* in single cells is detected as discrepancies with *rfbO1* abundance. Likewise,

overestimation of toxigenic O1 cells due to the presence of non-toxigenic O1 strains is identified through discrepancies with *ctxA* counts.

Furthermore, by measuring the relative abundance of toxigenic and O1 serogroup strains in the total *V. cholerae* population as well as *V. metoecus* abundance, this assay provides information about intraspecies and interspecies population dynamics, which could yield insights into variations in the natural abundance of toxigenic strains. This assay has both a low detection limit (three copies per reaction) and higher specificity than comparable assays. Besides being the first assay to allow molecular quantification of *V. metoecus*, it is also the first one to have been extensively tested environmental samples for *V. cholerae*, by measuring the absolute abundance of the species and its O1 serogroup and toxigenic subpopulations in areas endemic for cholera and where the bacterium is undetectable by culture-based techniques over the course of several months.

2.4. MATERIALS AND METHODS

2.4.1. Bacterial cultures and DNA template preparation

Different isolates of *Vibrio* spp that were used to validate primer specificity and assay sensitivity in this study are listed in TABLE 2.1. and TABLE B.1. All *Vibrio* strains were grown in tryptic soy broth (TSB) (Becton Dickinson) with 1.0% NaCl (BDH), incubated at 30 °C and 200 rpm. For non-*Vibrio* strains (TABLE B.1.) TSB without 1.0% NaCl was used under the same growth conditions. Genomic DNA was extracted from overnight cultures using the DNeasy Blood and Tissue Kit (QIAGEN) and quantified using

the Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes) with a Synergy H1 microplate reader (BioTek).

2.4.2. DNA extraction from biomass and isolation of organisms from environmental water samples

Water samples were collected from Bangladesh (a cholera-endemic region) and the USA East Coast (a cholera-free region) (FIG 2.1.) at different time points. Environmental water samples from Oyster Pond (Falmouth, MA, USA) were collected during the months of June to September, 2008 and 2009, as previously described (Kirchberger et al., 2016). Briefly, triplicate samples were obtained at a 0.5 m depth and a distance of 5 m from each other. Samples from 2009 were size fractionated, where ten litres of water were first filtered through a 63 µm nylon mesh net to capture large particles such as zooplankton. Large particles were crushed in a 50 ml tissue grinder after transfer using 20 ml of sterile filtered local water. Two milliliters of crushed material (equivalent to one litre of water) was resuspended in 48 ml of sterile filtered local water and pushed through a 4.5 cm Millipore Durapore filter (0.22 µm pore size) using a polypropylene syringe.

TABLE 2.1. Bacterial strains used to validate specificity of the primers and probes designed to detect *V. cholerae* and its toxigenic serogroup O1 and *V. metoecus*

Species	No. of strains	Target genes			
		viuB	*ctxA*	*rfbO1*	*mcp*
Vibrio cholerae non O1	20	+	-	-	-
Vibrio cholerae O1	8	+	+	+	-
Vibrio parahaemolyticus	1	-	-	-	-
Vibrio vulnificus	3	-	-	-	-
Vibrio metoecus	10	-	-	-	+
Vibrio mimicus	3	-	-	-	-
Escherichia coli	3	-	-	-	-
Pseudomonas aeruginosa	3	-	-	-	-

+, indicates all strains (100%) were positive by qPCR
-, indicates no amplification

Similarly, one litre of water passed through the mesh net was pushed through a series of in-line 4.5 cm Millipore Durapore filters (sizes 5 µm, 1 µm, and 0.22 µm) using a peristaltic pump. All disposable equipment was sterile, and all filter casing and tubing was sterilized before sampling. DNA extraction from the filters using a QIAGEN DNeasy Blood and Tissue Kit was performed as follows: 0.25 g of sterile zirconium beads were added to cut-up filter pieces in a 1.5 ml screw cap tube with 360 µl Cell Lysis Buffer ATL, and bead beating performed for 30 sec at maximum speed. Proteinase K (40 µl) was added, and the tubes were vortexed for several seconds. Further steps followed the instructions of the manufacturer. Environmental strains of *V. cholerae* and *V. metoecus* were isolated from the August and September 2009 filters, as previously described (Kirchberger et al., 2016).

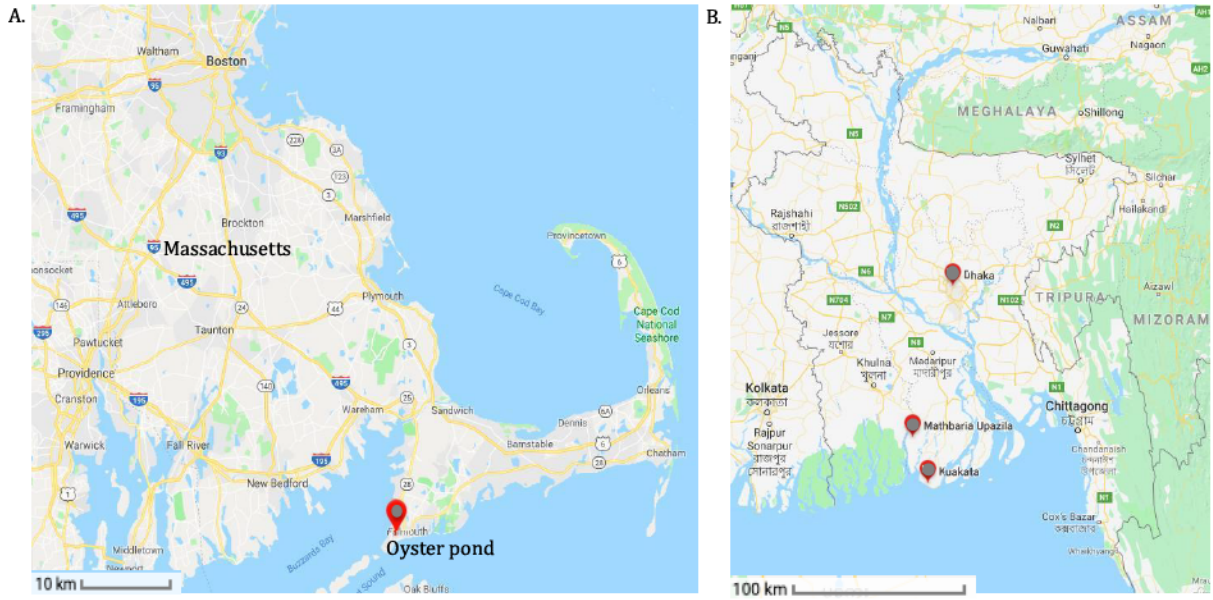

FIG 2.1. Sampling sites for environmental water samples collected to evaluate the qPCR assay developed in this study. A) Oyster Pond (Falmouth, Massachusetts, USA) a non-endemic site for cholera. B) Map of Bangladesh, identifying the two coastal regions (Kuakata and Mathbaria) and inland region (Dhaka) where samples were collected.

Three different regions in Bangladesh were selected to collect environmental water samples: two coastal regions (Kuakata and Mathbaria) and an inland region (Dhaka). Collection of water samples and extraction of DNA from filters from coastal Bangladesh sites (Kuakata and Mathbaria) were done at a single time point (May 2014) using the same protocol as the Oyster Pond sampling (Kirchberger et al., 2016). The Dhaka samples were collected from Rampura, Dhaka, Bangladesh (FIG 2.1.) bi-weekly from October 2015 to March 2016. Water samples (50 ml) were collected using 60 ml sterile polypropylene syringe and filtered through 0.22 µm Sterivex filters (Millipore). Total DNA extraction from the biomass on the filters was done by following four consecutive steps: cell lysis and digestion, DNA extraction and DNA concentrating, and washing according to the protocol described by Wright *et al* (Wright et al., 2009).

Environmental strains of *V. cholerae* were isolated from the water samples collected in Dhaka by following the protocol previously described (Huq et al., 1990).

To reduce impurities that can act as inhibitors during PCR amplification, all extracted DNA samples were further treated with One step PCR inhibitory removal kit (The Epigenetics Company, ZYMO Research), following the protocol in the user manual. Treated samples were kept at -20° C for further analysis.

2.4.3. Design and evaluation of primers and fluorogenic probes for real–time qPCR

To design a primer set suitable for the amplification of products that are unique to *V. cholerae* strains, protein-coding genes from a dataset of 77 *Vibrio* genomes were analyzed. Initial screening for genes which are not shared between *V. cholerae* and *V. metoecus* as well as their close relatives was performed on a dataset of consisting of the genomes of 42 *V. cholerae*, 10 *V. metoecus* and 25 other *Vibrio* strains. OrthoMCL (Li et al., 2003) was used to cluster all protein-coding genes into gene families based on 30% amino acid identity (Rost, 1999), resulting in sets of genes found exclusively in *V. cholerae* or *V. metoecus* and not shared with other species, respectively. After removal of multi-copy genes, remaining genes were aligned using CLUSTALW 2.0 (Larkin et al., 2007) and manually inspected for sites appropriate for the design of qPCR probes. Optimal sites for non-degenerate primer/probe design were found in the *viuB* gene encoding the vibriobactin utilization protein B (NP_23184.1) for *V. cholerae* (Butterton and Calderwood, 1994; Wyckoff et al., 2007). Primers (forward and reverse) and probe (TABLE 2.2.) for a 77-bp product were designed using the software tool PrimerQuest from integrated DNA technologies (IDT, Iowa, USA) according to supplied guidelines.

To design primers specific for *V. metoecus*, Intella (https://www.vound-software.com/) was used to analyze the unique gene contents of *V. metoecus*. Alignments of the sequence of alleles from single copy gene families present in all *V. metoecus* in the dataset were performed by using an in-house script (available upon request). The gene encoding a methyl-accepting chemotaxis protein (MCP) was selected for the presence of ideal primer and probe sites (Integrated DNA technologies), as designed using the software PrimerQuest tool from Integrated DNA technologies (IDT, Iowa, USA). This gene was targeted using the probe 5'-/5Cy5/TTG TCC GTT TCG ACA CTG AAA TCA/3IAbRQSp/-3', and forward and reverse primers, 5'-GCA GTC TCT TAC CGA AAC ACT A-3' and 5'-ATG AAC AGC TTA TCT TGC CAT TC-3', respectively, yielding an 81-bp product. The designed primers were tested for specificity by end point PCR with spiked water samples.

For estimation of toxigenic *V. cholerae* abundance, 106 bp of the *ctxA* gene (TABLE 2.2.) was targeted, as part of the genetic element encoding the major virulence factor cholera toxin and thus one of the signature genes for toxigenic potential in *V. cholerae* (Mekalanos et al., 1983). In the case of the *V. cholerae* O1 serogroup, the target was a 113 bp product of the *rfbO1* gene (TABLE 2.2.) as it detects explicitly *V. cholerae* belonging to the O1 serogroup, which includes the vast majority of strains responsible for past and ongoing cholera pandemics. Primers and probes for both of these genes were designed in this study to ensure compatibility of the assay.

TABLE 2.2. Target genes and sequences of primers and probes used in this study

Target gene	Primer and probe	Sequence (5'-3')	Amplicon size (bp)
viuB	Probe	56-FAM/TCATTTGGC/ZEN/CAGAGCATAAACCGGT/3IABkFQ	77
	Forward primer	TCGGTATTGTCTAACGGTAT	
	Reverse Primer	CGATTCGTGAGGGTGATA	
ctxA	Probe	5Cy5/AGGACAGAGTGAGTACTTTGACCGAGG/3IAbRQSp	106
	Forward primer	CAGGTGGTCTTATGCCAAG	
	Reverse primer	CTAACAAATCCCGTCTGAGTT	
rfbO1	Probe	5HEX/AGAAGTGTG/ZEN/TGGGCCAGGTAAAGT/3IABkFQ	113
	Forward primer	GTAAAGCAGGATGGAAACATATTC	
	Reverse primer	TGGGCTTACAAACTCAAGTAAG	
mcp	Probe	5Cy5/TTGTCCGTTTCGACACTGAAAATCA/3IAbRQSp	81
	Forward primer	GCAGTCTCTTACCGAAACACTA	
	Reverse primer	ATGAACAGCTTATCTTGCCATTC	

FAM, 6-carboxyfluorescein; Cy5, Cyanine 5 dye and HEX, Hexacholo-fluorescein dye were used as reporter dye. ZEN-Iowa Black FQ and Iowa Black RQ were used as quencher.

2.4.4. Real-time qPCR amplification

Dynamite qPCR Mastermix used in this study is a proprietary mix, developed and distributed by the Molecular Biology Service Unit at the University of Alberta, Edmonton, AB, Canada. It contains Tris (pH 8.3), KCl, $MgCl_2$, glycerol, Tween 20, DMSO, dNTPs, ROX as a normalizing dye, and antibody inhibited *Taq* polymerase. The volume of the PCR reaction was 10 µl containing 5 µl of 2× Dynamite qPCR master mix, 1 µl of each of 500 nM primer-250 nM probe mix, 1 µl of molecular grade water and 2 µl of DNA template. Real-time quantitative PCR was performed under the following conditions: initial primer activation at 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min in the Illumina Eco Real-Time PCR system. The assay includes standards of known copy number and negative control with no template added to assess the potential presence of contamination (FIG 2.2.).

2.4.5. Generation of standard curves and calculation of qPCR efficiency

Standard curves were prepared by amplifying gene sequences from corresponding reference strains for each target. For the preparation of standard curves, the *viuB*, *ctxA* and *rfbO1* genes of the pandemic *V. cholerae* El Tor O1 N16961 reference strain were used for total *V. cholerae* count, toxigenic *V. cholerae* and *V. cholerae* O1, respectively. To make the standard curve for the *V. metoecus* specific gene (*mcp*), the *V. metoecus* RC341 was used. Both strains were grown on LB agar (BD Difco, USA) with 0.5% NaCl at 30 °C for overnight and DNA extraction was done by DNeasy Blood and Tissue Kit (QIAGEN). Specific forward and reverse primers targeting each gene of interest were used for PCR amplification (TABLE 2.2.). A standard PCR protocol was followed for this amplification: 1 µl each of 10 pmol forward and reverse primer, 0.4 µl of 10 mM dNTP-Mix (ThermoFisher), 0.4 µl Phire Hot Start II DNA Polymerase (ThermoFisher), 4 µl of 5× Phire Buffer, 12.2 µl of molecular biology grade water and 2 µl of template DNA. The PCR reaction was performed as follows: initial denaturation at 98 °C for 30 sec, followed by annealing at 55 °C for 5 sec and extension 72 °C for 1 min for 35 cycles and a final extension of 72 °C for 1 min. PCR products were purified using the Wizard SV Gel and PCR Clean-up System (Promega). The concentrations of amplified PCR products were measured using the Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes) and the Synergy H1 microplate reader (BioTek).

Calculations mentioned in the Applied Biosystems Guideline (Applied Biosystems) for creating qPCR standard curves were used for determining the mass of amplified gene templates that correspond to copy numbers of target nucleic acid sequences. A series of standards were prepared in which a gene of interest is present at

3×10^5 copies, 3×10^4 copies, 3×10^3 copies, 3×10^2 copies 30 and 3 copies per 2 µl of the template. Once prepared, the standards were stored in 100 µl aliquots at -80° C. The standard curve was generated by plotting the log value of calculated gene copies per reaction over the quantitative cycle value (Cq) (FIG 2.2.). The Cq is described as the cycle at which the fluorescence from amplification exceeds the background fluorescence in the MIQE guideline (Bustin et al., 2009).

If a sample contains more targets, the fluorescence will be detected in earlier cycles; low Cq values represent higher initial starting copies of the target gene. The qPCR efficiency of the assay was calculated (FIG 2.2.) using the following formula: Efficiency = $10^{[-1/\text{Slope}]}$ (http://efficiency.gene-quantification.info/) by Illumina Eco Real-Time PCR system software.

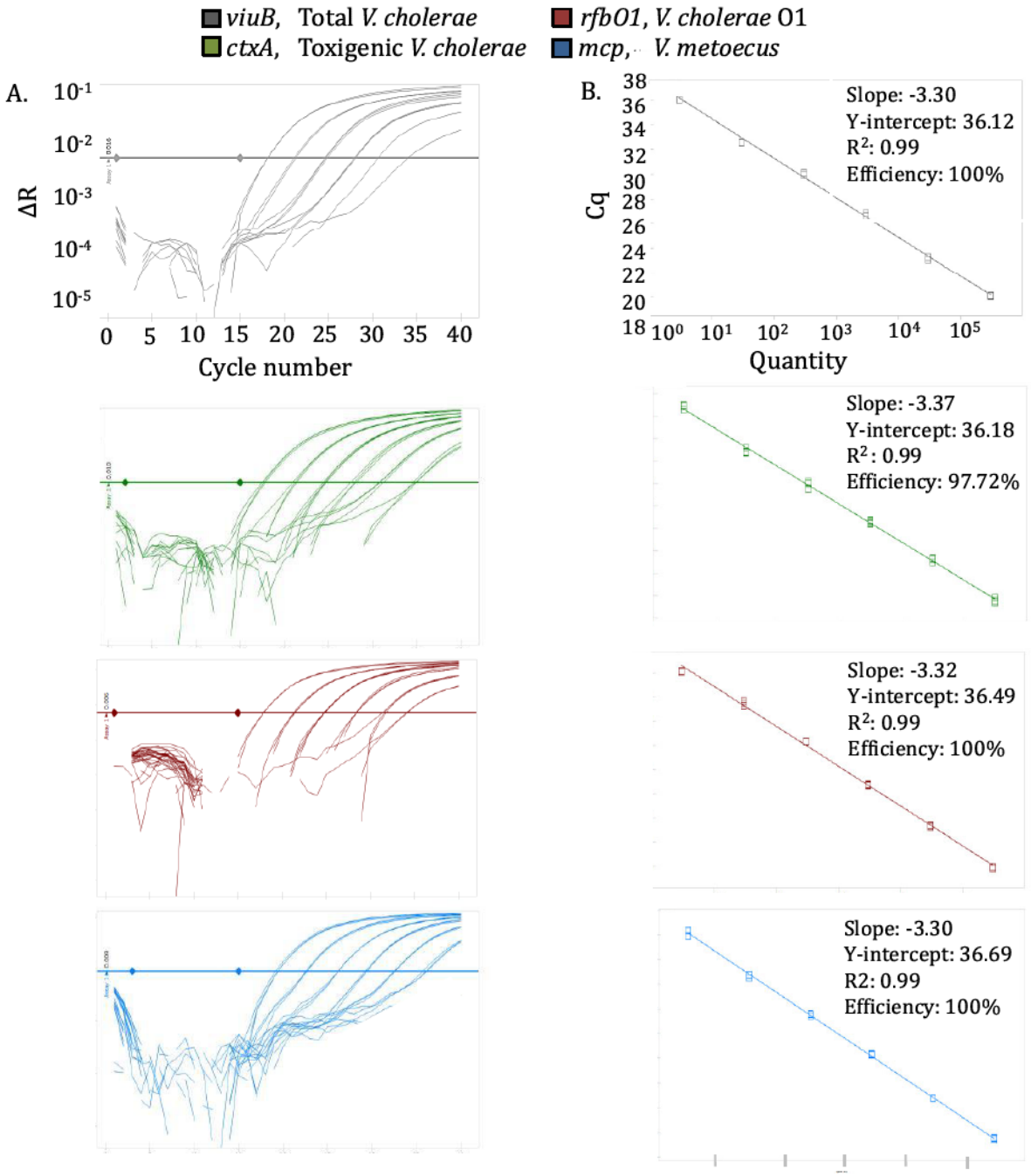

FIG 2. Multiplex real-time qPCR for simultaneous detection and quantification of *V. cholerae* and *V. metoecus*. Four gene markers with fluorogenic probes were used: *viuB* (*V. cholerae* specific), *ctxA* (toxigenic *V. cholerae* specific), *rfbO1* (*V. cholerae* O1 specific) and *mcp* (*V. metoecus* specific). Template DNA was purified from reference cultures (*V. cholerae* N16961 and *V. metoecus* RC341) and was serially diluted in 10-fold increments to yield concentration ranging from 3×10^6 to 3 copies per reaction (from left to right).

Fluorescence was measured in relative units. The panel (A) illustrates amplification curves and the panel (B) shows their corresponding standard curves. Each reaction was done in triplicate.

2.4.6. Limit of detection (LOD) and impact of inhibition testing

The LOD of the assay was determined for each of the marker genes based on the standard curve of amplified genes from reference strains (*V. cholerae* N16961 and *V. metoecus* RC341) (FIG 2.2.). The LOD of sample (per liter of water) before filtration was calculated from the LOD of the qPCR assay. We did not evaluate the quantifiable lowest minimum number of copies in the environmental water sample before any filtration step.

To test for qPCR inhibition, we compared the Cq values for 10× dilution of treated (with One step PCR inhibitory removal kit) extracted DNA samples from study sites and spiked positive and negative samples. The difference in Cq values between diluted samples were recorded (FIG B.2.).

2.4.7. Specificity testing

The specificity of the qPCR assay was evaluated using genomic DNA from bacterial strains listed in TABLE 2.2. Non-O1 *V. cholerae* (20) from environmental sources, *ctxA* positive *V. cholerae* O1(8) from both clinical and environmental sources, and *V. metoecus* (10) from environmental sources were tested (TABLE B.1.). Three other *Vibrio* species, *V. parahaemolyticus*, *V. vulnificus* and *V. mimicus,* were also tested, as well as two non-*Vibrio* gammaproteobacteria, *Pseudomonas aeruginosa* and *Escherichia coli*.

2.5. RESULTS AND DISCUSSION

2.5.1. A specific and efficient multiplex qPCR assay to detect *V. cholerae* and *V. metoecus*

We developed a specific and sensitive qPCR method for the quantification of total *V. cholerae* and *V. metoecus*, as well as toxigenic and O1 serogroup *V. cholerae*, in their natural aquatic environments. Primers and probes developed for all four marker genes displayed 100% specificity (TABLE 2.1.) using 51 bacterial strains, including 20 *V. cholerae* strains of various serogroups and toxigenic potential, as well as 10 *V. metoecus* and closely related *Vibrio* species. This assay was more specific than any previously published, with no cross-amplification between *V. cholerae* and other bacteria, including its closest relative, *V. metoecus*. The efficiency of each assay was 95% to 100% (R^2 value 0.99 and slope -3.3 ± 0.07) (FIG 2.2.).

The *viuB* and *mcp* markers are specific to *V. cholerae* and *V. metoecus* and present in single copies, facilitating quantification of the absolute abundance of these two species. Additionally, the *ctxA* and *rfbO1* markers are specific for detection of toxigenic and O1 serogroup strains, respectively. Previous qPCR studies often took advantage of these two genes but did not combine them, resulting in missed information required to correlate toxigenic *V. cholerae* and strains representing the PG lineage (Blackstone et al., 2007; Bliem et al., 2015). Although the region selected for *ctxA* gene amplification to indicate the presence of toxigenic *V. cholerae* is specific to this particular species (Goel et al., 2007), the presence of *ctxAB* has been reported in CTXφ phages present in aquatic environment, which can be the source of *ctxA* positive results (Faruque et al., 1998). Multiple copies of *ctxA* can also be present in certain *V. cholerae* genomes, biasing

quantification results (Faruque et al., 1994; Mekalanos, 1983). The simultaneous amplification of the single copy *rfbO1* specific for *V. cholerae* O1 strains compensates for these flaws, as the vast majority of cholera cases are caused by CTX positive strains of the O1 serogroup, and the co-occurrence of these two markers at similar levels allows accurate quantification of toxigenic O1 strains.

There are many rapid assays already being used to detect certain serotypes of *V. cholerae,* but most of them are confined to qualitative detection and do not provide any data on the abundance of this organism in the environment (Chua et al., 2011; Goel et al., 2007; Koskela et al., 2009; Singh et al., 2002). A few available assays are quantitative, but the limitations in sensitivity are >5 to 50 gene copies per reaction (Rashid et al., 2017b; Vezzulli et al., 2015), and they detect no more than two marker genes, or lack specificity (Gubala, 2006). For example, a real-time PCR assay using four different genetic markers i.e. *rtxA*, *epsM*, *ompW* and *tcpA* to detect *V. cholerae* also detected *V. mimicus* non-specifically by amplification of *ompW* (Gubala, 2006). Other genes currently used for detection and/or quantification of *V. cholerae* such as *hlyA*, *zot*, *ompU*, *toxR*, *groEl*, also suffer from a lack of species specificity (Fykse et al., 2007; Goel et al., 2007; Singh et al., 2002). Many of these genes share sequence similarity with homologs in closely related species or can be present in multiple copies in *Vibrio* genomes, and therefore can pose problems for specific detection and quantification. Moreover, there is no published assay that detects and quantifies the abundance of *V. metoecus* along with *V. cholerae.* Most assays have been evaluated by artificially spiking water samples with known concentrations of laboratory strains, and none has been directly applied to a significant number of environmental samples in a region endemic for cholera.

It is important for any environmental application to determine the sample limit of detection (SLOD). This value represents the lowest quantity of the target DNA that can be reliably detected and quantified in a certain volume of sample at a probability level of 95% (Bustin et al., 2009). In this study, we found the analytical detection limit for all four gene markers to be three copies per reaction (FIG 2.2.), with 1.5×10^6 copies/L of water as the SLOD without filtration, which is comparable to previous studies (Fykse et al., 2007). Concentrating samples by filtration of large volumes of water (0.05 L to 10 L, based on sampling at four sites in this study) made it possible to lower the SLOD to 9.0×10^2 copies/L for Oyster Pond (USA) samples after filtering 566 ml of water (FIG 2.3. and FIG 2.4.B). After filtration, SLODs of 3.0×10^3 copies/L (from 10 L of water) (FIG 2.5. and FIG 2.6.A) and 1.5×10^4 copies/L (from 50 ml of water) (FIG 2.7.) were determined for samples from the Kuakata and Dhaka (Bangladesh) sites, respectively.

The sensitivity of PCR-based assays for quantifying *V. cholerae* from environmental samples is highly dependent on efficient DNA extraction and removal of potential inhibitors. All the environmental DNA samples were treated with One step PCR inhibitory removal kit after DNA extraction to reduce residual inhibitors. Samples were assessed in ten-fold dilutions (1/10) to determine the effect of inhibitors in the assay. The 10× dilution of samples shifted the Cq values by 3.3 cycles ± 0.05 (FIG B.2.), consistent with no significant inhibition.

2.5.2. *V. cholerae* and *V. metoecus* co-occur seasonally in a temperate coastal location

With an optimized multiplex qPCR assay, it was possible for the first time to determine the abundance of *V. cholerae* and *V. metoecus* simultaneously in water reservoirs in cholera non-endemic areas (USA). *V. cholerae* is a ubiquitous member of bacterial communities in temperate and tropical aquatic environments around the world (Kaper et al., 1995) whereas *V. metoecus* is a recently described species, which has been co-isolated with *V. cholerae* from the USA East Coast aquatic environment and associated with eel fish in Spain, as well as clinical cases from around the USA (Boucher et al., 2011; Carda-Diéguez et al., 2017; Kirchberger et al., 2016; Kirchberger et al., 2014).

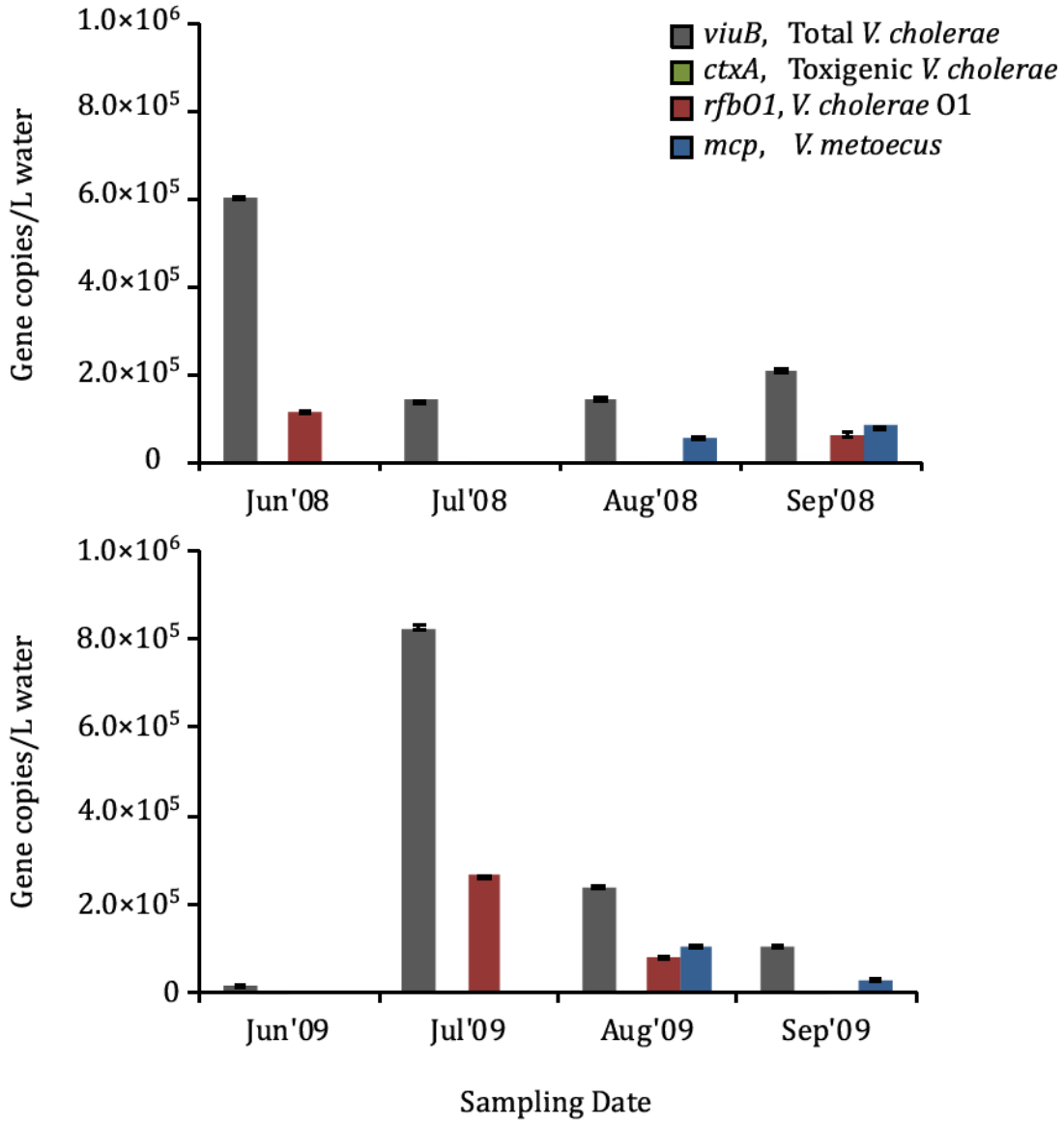

FIG 2.3. Temporal variation of abundance for *V. cholerae* along with its toxigenic and serogroup O1 subpopulations and its close relative *V. metoecus* in Oyster Pond, MA, USA. Environmental water samples collected during the months of June to August in two successive years, 2008 and 2009, were analyzed using the developed qPCR assay. The *viuB* gene was used to quantify total *V. cholerae*; *ctxA* and *rfbO1* were used to measure toxigenic *V. cholerae* and *V. cholerae* O1, respectively; the abundance of *V. metoecus* was estimated using the *mcp* gene. All four genes were tested for each sample and the absence of a bar in the graph denotes that the target gene was absent or present

below the detection limit of the assay. Each qPCR reaction was run in triplicate. Mean values are shown with error bars indicating the standard deviation between three technical replicates. *ctxA* could not be detected at any time sampled at this site.

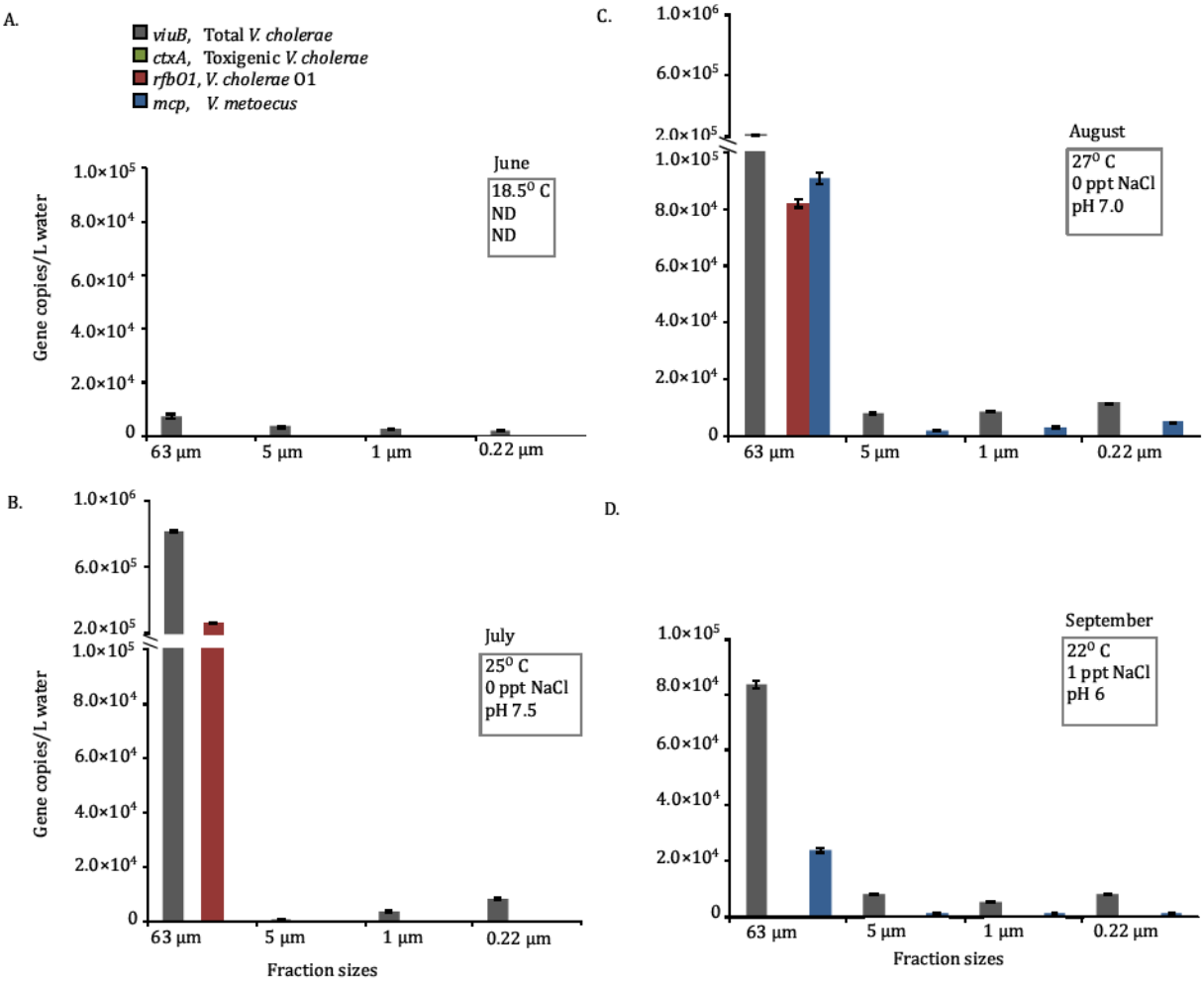

FIG 2.4. Distribution in different water fraction sizes of *V. cholerae* along with its toxigenic and serogroup O1 subpopulations and its close relative *V. metoecus* in Oyster Pond, MA, USA. Environmental water samples were collected during the months of June (A), July (B) August (C) and September (D) in 2009. These samples were fractionated by size through sequential filtration and bacteria were quantified by qPCR of marker genes on DNA extracted from the filters. The *viuB* gene was used to quantify total *V. cholerae*; *ctxA* and *rfb01* were used to measure toxigenic *V. cholerae* and *V. cholerae* O1, respectively; the abundance of *V. metoecus* was quantified using the *mcp*

gene. Each qPCR reaction was run in triplicate. Mean values are shown with error bars indicating the standard deviation between technical replicates. Temperature, pH and salinity of water collected each month are shown in boxes on the upper right corner of each graph. ND indicates not done.

To compare the qPCR assay to culture-dependent methods of detection, it was applied to samples from which *V. cholerae* and *V. metoecus* had been isolated by cultivation and samples from which no organisms had been isolated (TABLE 2.3.). These water samples were collected in summer (June to September) over two successive years (2008 and 2009) from Oyster Pond in Falmouth (MA) on the East Coast of the USA. Using our novel multiplex assay on DNA extracted from biomass of the same samples, we found the abundance of *V. cholerae* was 1.4×10^5 to 8.2×10^5 copies/L in Oyster Pond from June to September in 2008 and 2009 (FIG 2.3.). Strikingly, *V. metoecus* was only detected at the end of the season (the months of August and September) in both years, at abundances of approximately 5.6×10^4 to 1.0×10^5 copies/L (FIG 2.3.). *V. cholerae,* on the other hand, was consistently more abundant than *V. metoecus* and present throughout the summer. Using a culture-based approach, it has been suggested that *V. cholerae* is ten times more abundant than *V. metoecus* at that particular location (Kirchberger et al., 2016). Based on the qPCR approach used here, *V. cholerae* was approximately three times more abundant than *V. metoecus*, suggesting that the former is more readily culturable than the latter (FIG 2.3.). Moreover, *V. metoecus* was neither isolated by conventional culture method nor detected by qPCR in Bangladesh, suggesting a different geographical distribution than *V. cholerae* (FIG 2.5.).

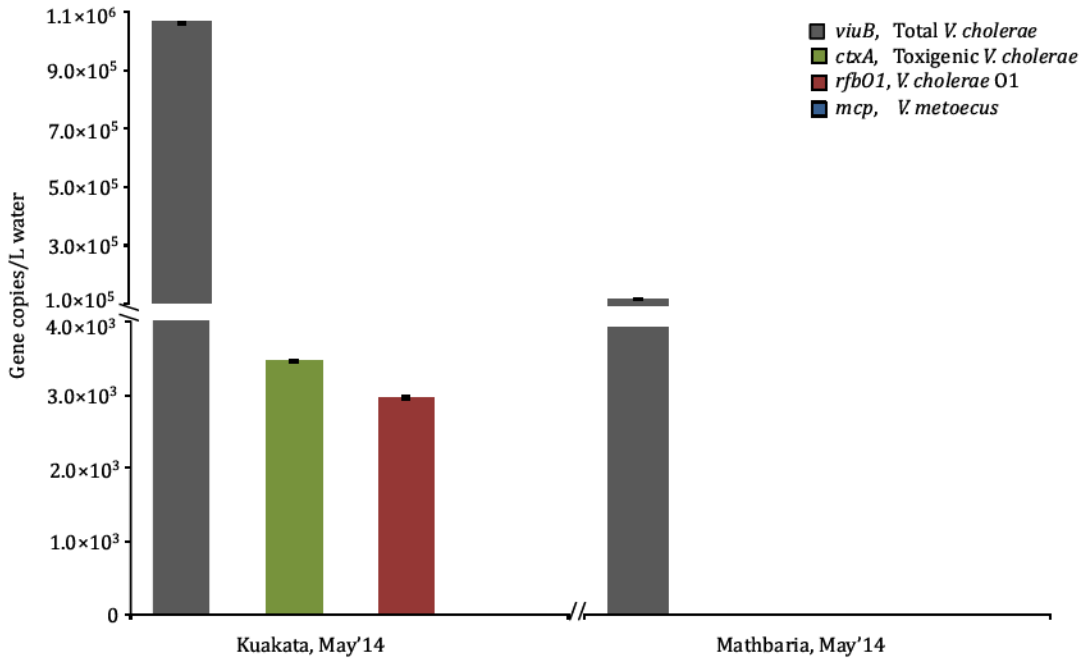

FIG 2.5. Abundance of *V. cholerae* along with its toxigenic and serogroup O1 subpopulations and its close relative *V. metoecus* in two different coastal regions in Bangladesh. Environmental water samples were collected from Kuakata and Mathbaria during the month of May in 2014 and bacteria were quantified by qPCR of marker genes. The *viuB* gene was used to quantify total *V. cholerae*; *ctxA* and *rfbO1* were used to measure toxigenic *V. cholerae* and *V. cholerae* O1, respectively; the abundance of *V. metoecus* was quantified using the *mcp* gene. Each qPCR reaction was run in triplicate. Mean values are shown with error bars indicating the standard deviation between technical replicates.

2.5.3. O1 serogroup strains are important members of a temperate coastal *V. cholerae* population

The presence/absence of toxigenic or O1 serogroup *V. cholerae* was determined using two sets of primers targeting *ctxA* and *rfbO1*. There was no amplification of *ctxA* in the Oyster Pond samples, but *rfbO1* was present sporadically: 1.1×10^5 copies/L were present in June 2008 and 6.2×10^4/L in September 2008 (FIG 2.3.), while 2.6×10^5

copies/L *rfbO1* were detected in July 2009 and 8.2×10^4 copies/L in August of 2009 (FIG 2.3.). The proportion of *rfbO1* positive *V. cholerae* was, on average, 23% of total *V. cholerae* in Oyster pond over these periods of sampling, ranging from 15% to 29% in individual samples. O1 serogroup of *V. cholerae* were consistently found during the four warmest months (June to September) of 2008 and 2009 (FIG 2.3.). Interestingly, in water samples that were fractionated by size (2009), *rfbO1* was detected only in the largest size fraction (>63 µm) (FIG 2.4.).

No O1 serogroup strains could be obtained from Oyster pond samples by conventional culture methods, despite the isolation of over 385 *V. cholerae* strains (Kirchberger et al., 2016) (TABLE 2.3.). *V. cholerae* enters into VBNC form due to environmental stress condition, where cells remain alive but cannot be revived using standard cultivation methods (Colwell, 2000). During inter-epidemic periods, toxigenic *V. cholerae* are believed to be maintained in low numbers, attaching to particles in a state of VBNC (Sultana et al., 2018).

The inability to isolate *V. cholerae* O1 in most of the samples indicates that culture-based methods could lead to an underestimation of the occurrence of *V. cholerae* O1 in environmental reservoirs due to their low abundance or VBNC state (Bliem et al., 2015). The inability to isolate *V. cholerae* O1 strains in 2009 samples collected from Oyster pond together with their detection at a relatively high abundance (TABLE 2.3. and FIG 2.3.) by qPCR likely indicates the presence of VBNC cells, questioning the assumption that *V. cholerae* O1 is rare in cholera-free regions (Islam et al., 2013).

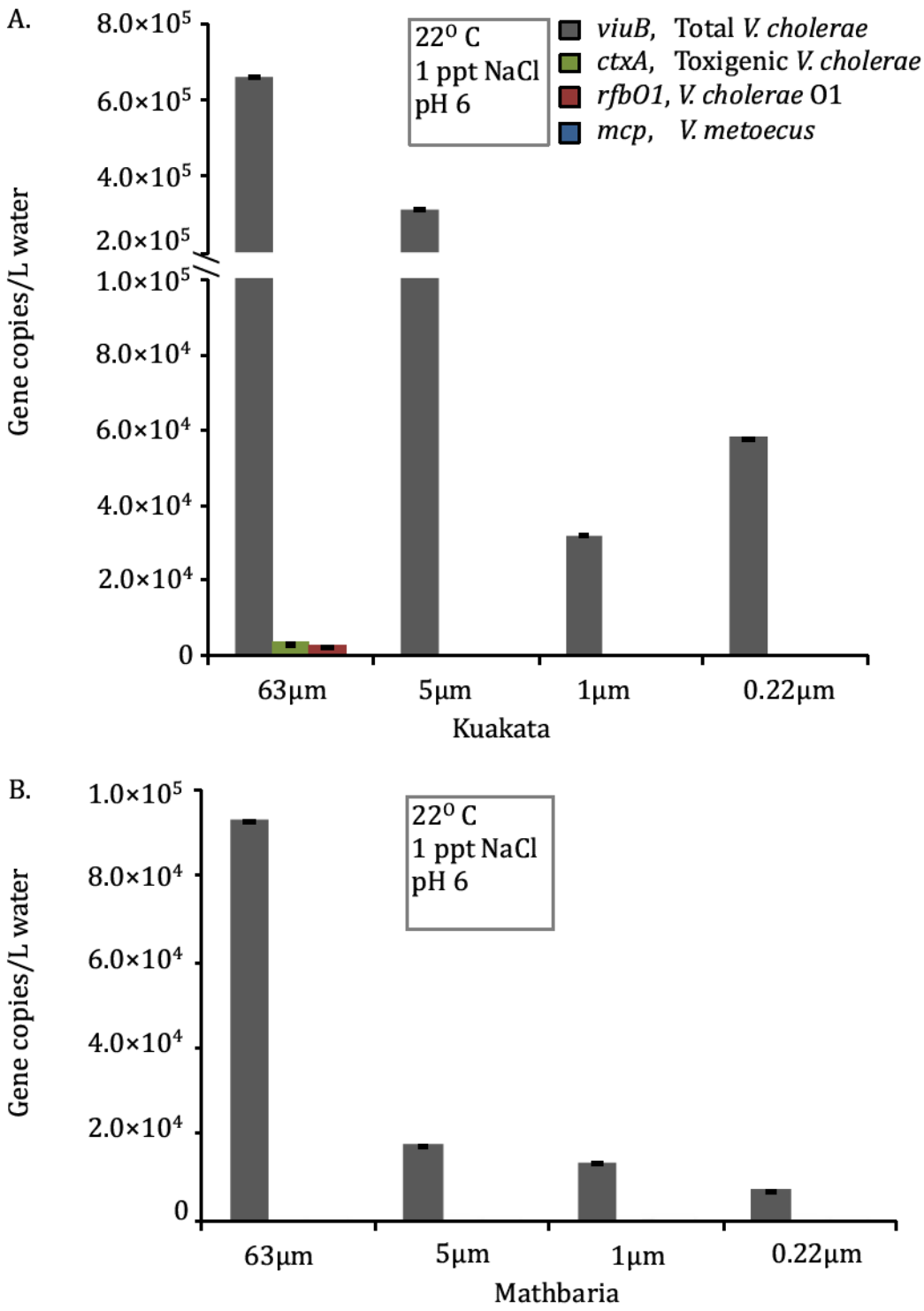

FIG 2.6. Distribution in different water fraction sizes of *V. cholerae* along with its toxigenic and serogroup O1 subpopulations and its close relative *V. metoecus* in two different coastal regions in Bangladesh. Environmental water samples were

collected from A) Kuakata and B) Mathbaria during the month of May in 2014 and bacteria were quantified by qPCR of marker genes. The *viuB* gene was used to quantify total *V. cholerae*; *ctxA* and *rfbO1* were used to measure toxigenic *V. cholerae* and *V. cholerae* O1, respectively; the abundance of *V. metoecus* was quantified using the *mcp* gene. Each qPCR reaction was run in triplicate. Mean values are shown with error bars indicating the standard deviation between technical replicates.

2.5.4. Toxigenic O1 serogroup strains are constantly present at dangerous levels in Dhaka freshwater

In Bangladesh, data on the incidence of cholera show that the disease occurs year round in the Ganges delta region of Bangladesh with seasonal peaks typically before (March to May) and after (September to December) monsoon (Alam et al., 2006; Faruque et al., 2005b). However, using culture-based methods, direct isolation of toxigenic *V. cholerae* O1 was not readily possible even during the peak season in aquatic reservoirs unless further enrichment in an alkaline peptone water medium (Huq et al., 1990). To determine if culture-based monitoring is misleading, bi-weekly sampling was done over six consecutive months in the Rampura area of Dhaka city in Bangladesh.

TABLE 2.3. Comparative result of conventional culture method and developed qPCR technique used in this study

Site	Time	Cultivation			qPCR		
		V. cholerae	*V. cholerae* O1	*V. metoecus*	*V. cholerae*	*V. cholerae* O1	*V. metoecus*
Kuakata	May'14	+	-	-	+	+	-
Mathbaria	May'14	+	-	-	+	-	-
Dhaka	Oct'15	+	+	-	+	+	-
	Nov'15	+	-	-	+	+	-
	Dec'15	+	-	-	+	+	-
	Jan'16	+	-	-	+	+	-
	Feb'16	+	-	-	+	+	-
	Mar'16	+	-	-	+	+	-
Oyster Pond	Jun'08	ND	ND	ND	+	-	-
	Jul'08	ND	ND	ND	+	+	-
	Aug'08	ND	ND	ND	+	+	+
	Sep'08	ND	ND	ND	+	-	+
Oyster Pond	Jun'09	ND	ND	ND	+	-	-
	Jul'09	ND	ND	ND	+	+	-
	Aug'09	+	-	+	+	+	+
	Sep'09	+	-	+	+	-	+

+, indicates samples were positive for corresponding organism
-, indicates there was no isolation and/or negative by qPCR
ND, indicates not done

Biomass was collected from water samples by filtration on 0.22 µm membranes with no size fractionation. The abundance of the *viuB* marker gene, which corresponds to the total *V. cholerae* population, ranged from 1.3×10^5 to 4.0×10^5 copies/L from October 2015 to March 2016 (FIG 2.7.). The *ctxA* gene used to track toxigenic *V. cholerae* was found at 3.6×10^4 copies/L to 3.2×10^5 copies/L and the *rfbO1* gene used to track *V. cholerae* O1 was detected at similar levels, ranging from 1.5×10^4 copies/L to 2.9×10^5 copies/L, corresponding to 50-60% of the total *V. cholerae* population (FIG 2.7.). There was consistent occurrence of *V. cholerae* and its toxigenic and serogroup O1 subpopulations at similar abundances throughout the six months of sampling, suggesting

that both genes were found in the same cells, which is typical of 7 th pandemic *V. cholerae* El tor strains currently responsible for most cholera cases in Bangladesh (Chun et al., 2009). Conventional culture methods only recovered *V. cholerae* O1 from October 2015, and *V. cholerae* non-O1 was isolated from each of these six months (TABLE 2.3.).

Unexpectedly, analysis of water samples from the Rampura area (Dhaka, Bangladesh) (FIG 2.1.) revealed persistent toxigenic *V. cholerae* O1 at a low abundance (1.3×10^5 to 4.0×10^5 copies/L), but a high proportion of the total *V. cholerae* population (up to 84%) (FIG 2.7.). The infectious dose of *V. cholerae* in humans being in the order of 10^3 to 10^8 bacterial cells (Schmid-Hempel and Frank, 2007); the water in this region is a permanent reservoir of toxigenic *V. cholerae* that can readily cause potential outbreaks when ingested with contaminated water or food. Water bodies around Dhaka city are surrounded by a dense population which extensively interact with them, potentially resulting in the circulation of pathogenic *V. cholerae* in that particular environment, even outside of periods in which cholera cases are frequent. Usually, this megacity experiences two seasonal outbreaks of cholera before the monsoon in March to May and just after the monsoon in September to November (Alam et al., 2011). In this study we also observed higher counts of toxigenic *V. cholerae* in early October (2.9×10^5 copies/L) and higher counts of total *V. cholerae* ($> 4.0\times10^5$ copies/L) in March but we missed the time period during April/May as the samples analyzed here were taken from October 2015 to March 2016. Another interesting observation at the Dhaka site was that the number of *ctxA* gene copies detected was always slightly higher than *rfb*O1 (~20%). It is known from previous research that El Tor strain N16961 carries a single copy of the cholera toxin prophage whereas there could be variation in copy number in other El Tor

V. cholerae strains arising from selective pressure (Chun et al., 2009; Davis et al., 1999; Mekalanos et al., 1983; Trucksis et al., 1998). Also, the presence of CTX phages in the aquatic environment may significantly impact the abundance of *ctxA* positive cells in the environment.

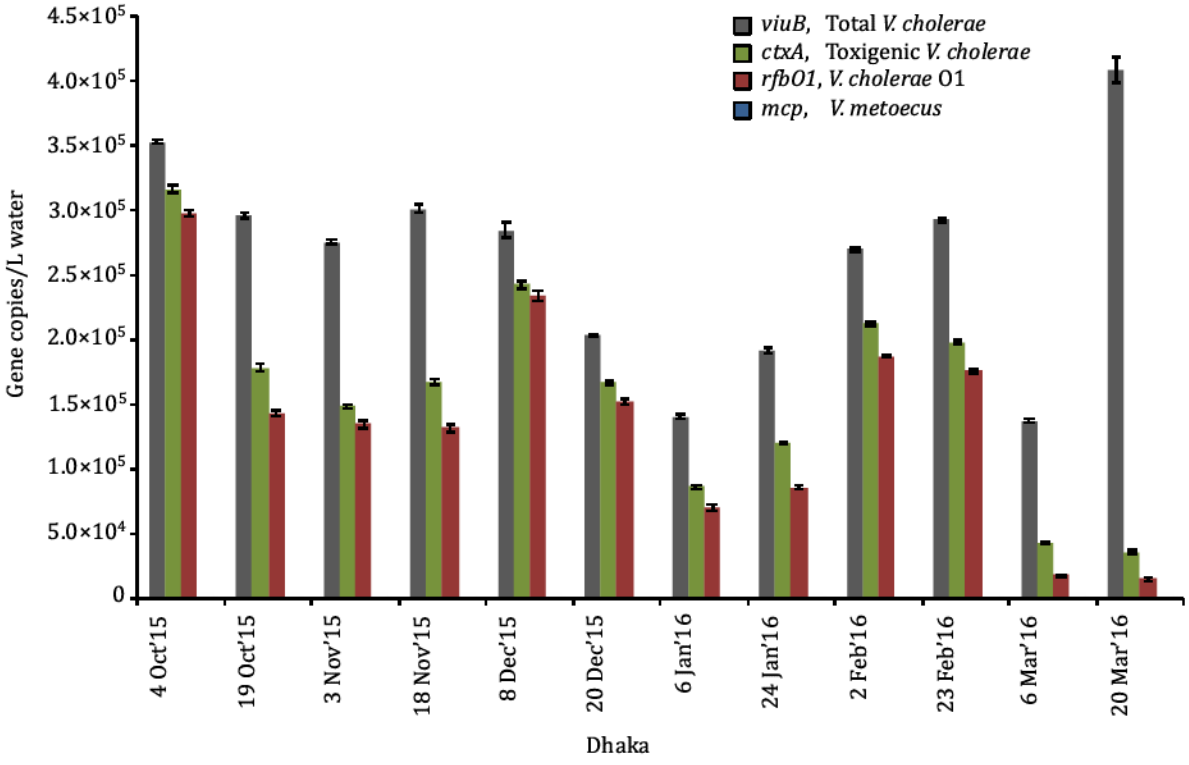

FIG 2.7. Temporal variation of abundance for *V. cholerae* along with its toxigenic and serogroup O1 subpopulations and its close relative *V. metoecus* in a central region of Bangladesh (Dhaka). Environmental water samples were collected bi-weekly from the months of October 2015 to March 2016 and bacteria were quantified by qPCR of marker genes. The *viuB* gene was used to quantify total *V. cholerae*; *ctxA* and *rfbO1* were used to measure toxigenic *V. cholerae* and *V. cholerae* O1, respectively; the abundance of *V. metoecus* was quantified using the *mcp* gene. Each qPCR reaction was run in triplicate. Mean values are shown with error bars indicating the standard deviation between technical replicates.

In contrast to the inland Dhaka site, the O1 serogroup was only detected at low abundance or was undetectable at the two coastal sites sampled in Bangladesh. *V. cholerae* O1 was found at very low abundance in Kuakata (May 2014) and absent from Mathbaria during the single month sampled (FIG 2.5. and FIG 2.6.), whereas in a previous study, detection of toxigenic *V. cholerae* O1 by DFA (direct fluorescent antibody) in water samples collected bi-weekly from March to December 2004 from six ponds in Mathbaria fluctuated from < 10 to 3.4×10^7 CFU/L (Alam et al., 2006). It is noteworthy that during the period of this latter study, cholera cases recorded in Mathbaria were due to *V. cholerae* O1. Moreover, in the same study, no strains belonging to the O1 serogroup were found among the six hundred strains isolated from coastal areas in Bangladesh, confirming our observations in an area non-endemic for cholera that *V. cholerae* O1 is challenging to isolate (TABLE 2.3.). The constant presence of *V. cholerae* O1 in Dhaka as opposed to a more stochastic appearance in coastal locations suggests that the level of human interaction with water bodies influences its population dynamic.

2.5.5. Higher abundance of *V. cholerae* and *V. metoecus* in larger size fractions indicates preference for particle association

Amongst the samples collected from endemic (Bangladesh) and non-endemic (Oyster Pond, USA) regions, overall counts of *V. cholerae* ranged from 1×10^4 copies/L to 1×10^6 copies/L for each sample (FIG 2.3., FIG 2.5. and FIG 2.7.). In Oyster pond, most of *V. cholerae* (~93%), including ~18% of the O1 serogroup *V. cholerae*, were detected in the largest fraction size (>63 µm). Similarly, the majority of *V. cholerae* cells from coastal areas of Bangladesh (~62%) were detected in the largest fraction size (> 63 µm) (FIG

2.5. and FIG 2.6.). The toxigenic *V. cholerae ctxA* marker gene and O1 serogroup strains *rfbO1* gene were found only in the >63 µm size fraction and in very low concentrations in Kuakata (3.4×10^3 and 2.9×10^3 copies/L, respectively) (FIG 2.6.).

This skewed distribution of *V. cholerae* toward the largest fraction size (FIG 2.4. and FIG 2.6.) suggests that most cells are associated with large particles, zooplankton, or phytoplankton hosts in environmental reservoirs (Ali et al., 2012; Chun et al., 2009; Faruque et al., 1998; Meibom et al., 2004). This explains why in rural areas of Bangladesh, filtering environmental water through folded cloth facilitates the reduction of particles and debris and significantly lowers the risk of cholera (Rosenberg, 2009).

2.6. CONCLUSION

This research describes for the first time a sensitive multitarget real-time qPCR application for the specific detection and quantification of *V. cholerae* and *V. metoecus* from environmental water samples. The *viuB* and *mcp* markers are specific to *V. cholerae* and *V. metoecus* and made it possible to quantify the absolute abundance of members of these two species in DNA extracted from environmental biomass. On the other hand, the *ctxA* and *rfbO1* markers are specific for detection of toxigenic and O1 serogroup strains, allowing for the first-time, determination of the proportion of the total *V. cholerae* population represented by these strains. This is also a fundamental study on quantification of *V. cholerae* on a significant scale in a cholera-endemic area. Although cholera has two seasonal peaks in this region, we showed that toxigenic *V. cholerae* O1 was persistent in the inland water reservoir at levels that pose a risk to human health. *V. metoecus* was not detected in this area, indicating a different geographical distribution

than that of its closest relative. The sporadic presence of *V. cholerae* O1 at a substantial proportion of the local *V. cholerae* total population in a region not endemic for cholera also highlights the wide distribution of this lineage displaying potential for the emergence of novel virulent variants.

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CHAPTER 3

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Nasreen T., Alam M., Yanow S.K. and Boucher Y. **"Assay for evaluating the abundance of *Vibrio cholerae* and its O1 serogroup subpopulation directly from water without DNA extraction"**

Author contribution: Y.B., T.N. and S.K.Y. designed the project. Y.B. and T.N. wrote the manuscript. M.A helped in sample collection during field trips in Dhaka, Bangladesh. T.N. performed all the experimental procedures. S.K.Y. helped in trouble shooting of any experimental problem. M.A. and S.K.Y. reviewed the manuscript. S.K.Y. and Y.B. supervised the project.

CHAPTER 3

Assay for evaluating the abundance of *Vibrio cholerae* and its O1 serogroup subpopulation directly from water without DNA extraction

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3.1. ABSTRACT

Cholera is a severe diarrheal disease caused by *Vibrio cholerae*, a natural inhabitant of brackish water. It is one of the major water related health concerns after natural disasters and during armed conflicts, because of its rapid spread through untreated drinking water sources. This communicable disease is mainly transmitted through the fecal-oral route due to compromised water management systems, sanitation and personal hygiene. Effective control of cholera outbreaks depends on prompt detection of the pathogen from clinical specimens and tracking of the source pathogen in the environment. Although cholera epidemiology is well studied, data on *V. cholerae* abundance in environmental sources are limited and rapid detection is still a challenge. The development of culture-independent on-site detection and quantification by qPCR directly from environmental water samples would greatly facilitate the identification of pathogenic *V. cholerae* in drinking water. Here, a sensitive molecular detection and quantification assay by qPCR was developed, which can easily be used on-site in low resource settings. This newly optimized method exhibited 100% specificity for total *V. cholerae* as well as *V. cholerae* O1 and allowed detection of as few as three target genes copies per reaction. The limit of detection was determined by spiking environmental samples with a known number of cells, showing that the method could quantify cells directly in water and as few as 6×10^3 cells/L of water after concentrating biomass from the sample. As a proof of concept, analysis of environmental water samples collected

from a site endemic for cholera (Gabtoli area, Dhaka, Bangladesh) showed that the O1 serotype comprises 15% of total *V. cholera*e. The ability to perform qPCR directly on water samples, the portable features of the equipment, the stability of the reagents at 4 °C and user-friendly online software facilitate fast quantitative analysis of this organism. These characteristics make this assay extremely useful for field research in resource-poor settings and could support continuous monitoring at cholera endemic areas.

3.2. INTRODUCTION

Cholera is a life-threatening diarrheal disease caused by pathogenic strains of *V. cholerae*. Still today, cholera perseveres as a global threat to public health due to its high mortality and morbidity rates (Albert et al., 1993; Bakhshi et al., 2009; Chhotray et al., 2002; Huang et al., 2009; Jesudason et al., 1994). There were 1,227,391 cholera cases and 5654 deaths reported in 2017 (WHO, 2018c). Inaccessibility to safe water and inadequate management of sanitary systems in resource-poor countries make this disease a major public health problem. Current data on the global disease burden of cholera identify 69 countries around the world as cholera-endemic and place 1.3 billion people at risk of cholera (Ali et al., 2015). Cholera outbreaks in the last five years in Haiti, Somalia, Cameroon, Guinea, Sudan, Nepal, Zimbabwe demonstrated the devastation that can be caused by this disease (WHO, 2018b). The epidemic currently taking place in Yemen is reported as the world's fastest-growing outbreak where 10,000 suspected cases are being reported per week with a death toll of over two thousand since September, 2018 and more than 1.2 million people infected so far (Reuters, 2018).

V. cholerae is naturally found worldwide, especially in brackish riverine, coastal and estuarine environments (Bliem et al., 2015). Not all *V. cholerae* present in nature are pathogenic, as only a subset of strains is known to be pathogenic to human. Amongst the 200 serogroups of this species, only O1 and O139 are associated with cholera cases and are responsible for epidemic and pandemic cholera outbreaks (Blackstone et al., 2007; Islam et al., 1994; Ramamurthy et al., 1993).

Estimation of *V. cholerae* abundance, along with that of its pandemic generating serogroups (Islam et al., 2017) in aquatic ecosystems is difficult, because of the high spatio-temporal variability exhibited by its natural populations (Bliem et al., 2018). The existence of a considerable proportion of most populations as viable but non culturable cells (VBNC), which are not revived upon culture on microbiological media also makes accurate quantification impossible through culture-based methods (Colwell et al., 1985). Epidemiological studies and analysis of cholera outbreaks revealed that the disease occurs in a regular seasonal pattern in cholera-endemic areas (Faruque et al., 1998; Glass et al., 1982; Kaper and Morris, 1995) and causes outbreaks only under certain conditions, which may be attributed to environmental and climatic factors, for example, heavy rainfall followed by blooms of phytoplankton and zooplankton (Colwell, 2000; Epstein, 1993; Huq et al., 1995).

Although cholera has been endemic to the Ganges Delta for centuries, it is an imported disease in most other locales, where it can vanish after a single outbreak or linger for decades before disappearing. For example, Haiti had no recorded cholera for centuries (Lee, 2001; Piarroux et al., 2011; Zuckerman et al., 2007), but the disease rapidly spread after the 2010 earthquake which devastated infrastructure in the country

and with the arrival of UN troops carrying the bacteria from Nepal (Guillaume et al., 2018). The country has since faced a decade of the cholera epidemic. Because of these epidemiological dynamics, cholera has been categorized as an emerging and reemerging infection (Satcher, 1995).

Surveillance of cholera outbreaks through clinical diagnosis provides an estimation of the associated disease burden but is unable to provide quantitative information on the pathogen or its abundance in source environments. To achieve a better understanding of the cholera prevalence in order to direct appropriate control measures and treatment, it is necessary to identify and promptly quantify its causative agent in its reservoir. However, the major obstacle is that the number of *V. cholerae* (toxigenic and non-toxigenic) is often below the limit of detection of current field analytical methods, even during outbreaks (Almagro-Moreno and Taylor, 2013).

Conventional culture method remains the gold standard for laboratory diagnosis of cholera and often requires pre-enrichment of the sample (Hasan et al., 1994). Moreover, two to three days are required to conduct testing with even modern laboratory infrastructure. With these methods, isolation and identification are possible, but the total abundance is an underestimate, as cells that are VBNC *V. cholerae* cannot be detected (Colwell, 1993; Hasan et al., 1994). In areas with limited or no laboratory facilities, simple darkfield microscopy is used to detect characteristic movement of *V. cholerae* in stool samples but the method is not useable on water sources, in which the bacterium is much more dilute (Benenson et al., 1964). The Crystal® VC Rapid Diagnostic Test (RDT) is also used for point-of-care detection to predict potential cholera outbreaks. This diagnostic test for cholera is mainly based on detection of either cholera toxin (Alam

et al., 2010; Almeida et al., 1990) or a lipopolysaccharide antigen (Alam et al., 2010; Harris et al., 2009; Moyenuddin et al., 1987) and is useful to detect serogroups O1 and O139. However, due to the poor sensitivity and specificity of this method, additional tests are required for confirming the presence of toxigenic *V. cholerae* in the stool (Alam et al., 2010). A direct fluorescent antibody (DFA) technique using polyclonal anti-O1 serum is also used to detect *V. cholerae* O1 in smears prepared from samples. This procedure has been used for both clinical and pre-enriched environmental samples (Hasan et al., 1994; Xu et al., 1984). Despite these methods facilitating cholera diagnosis, most of them are qualitative, only detect *V. cholerae* of the O1 serogroup, and have been optimized for clinical specimens.

To improve cholera surveillance, it is essential to know the abundance of the total and disease-causing serogroups of *V. cholerae* in its reservoir. To achieve this goal, I have designed a quantitative, portable on-site real-time quantitative PCR (qPCR) using two species-specific primers and probes to precisely detect and quantify total *V. cholerae* and *V. cholerae* O1 directly from environmental water sample without DNA extraction. This method determines the absolute abundance of this bacterium and its toxigenic serogroups across hundreds of samples in a spatiotemporal gradient, making it po ssible to pinpoint the source of cholera outbreaks and warn of potential outbreaks.

3.3. MATERIALS AND METHODS

3.3.1. Preparation of bacterial cultures

Different strains of *Vibrio* spp. and other gammaproteobacteria were used to validate analytical sensitivity and specificity of the assay in this study (TABLE 3.1.).

Twenty non-O1 *V. cholerae* from environmental sources, eight *V. cholerae* O1 from both clinical and environmental sources, and ten *V. metoecus* from environmental sources were tested (TABLE 3.1.). Single strains from three other *Vibrio* species (*V. parahaemolyticus*, *V. vulnificus* and *V. mimicus*) were tested along with *Pseudomonas aeruginosa* and *Escherichia coli*. The *Vibrio* strains were grown in tryptic soy broth (TSB) (Becton Dickinson) with 1.0% NaCl (BDH) at 30 °C and 200 rpm for overnight in a shaking incubator.

3.3.2. Collection and processing of environmental samples

Environmental water samples were collected from the river basin of the Turag river in the Gabtoli area (latitude and longitude coordinates are 23.783726, 90.344246) (LatLong.net) of Dhaka, Bangladesh (FIG 3.1.) during August 2018. Three locations, approximately 5 metres apart from each other, were chosen at this site and water samples were collected in triplicate from each of those locations (nine samples in total). Fifty ml of surface water sample was collected directly in Thermo Scientific Nunc 50-ml conical centrifuge tubes (Thermo Fisher Scientific, Korea). Collected water samples were concentrated using Amicon Ultra-0.5 ml centrifugal filter device (Merk KGaA, Darmstadt, Germany) (FIG 3.2.) according to the user's manual. Ten Amicon tubes, each containing 500 µl of water sample, were centrifuged at 14000 × g for 30 mins in a mini spin plus centrifuge (Eppendorf, Germany). All the concentrates were pooled. Altogether, 5 ml of water from each of the three replicates from each of three locations were concentrated to 100 µl.

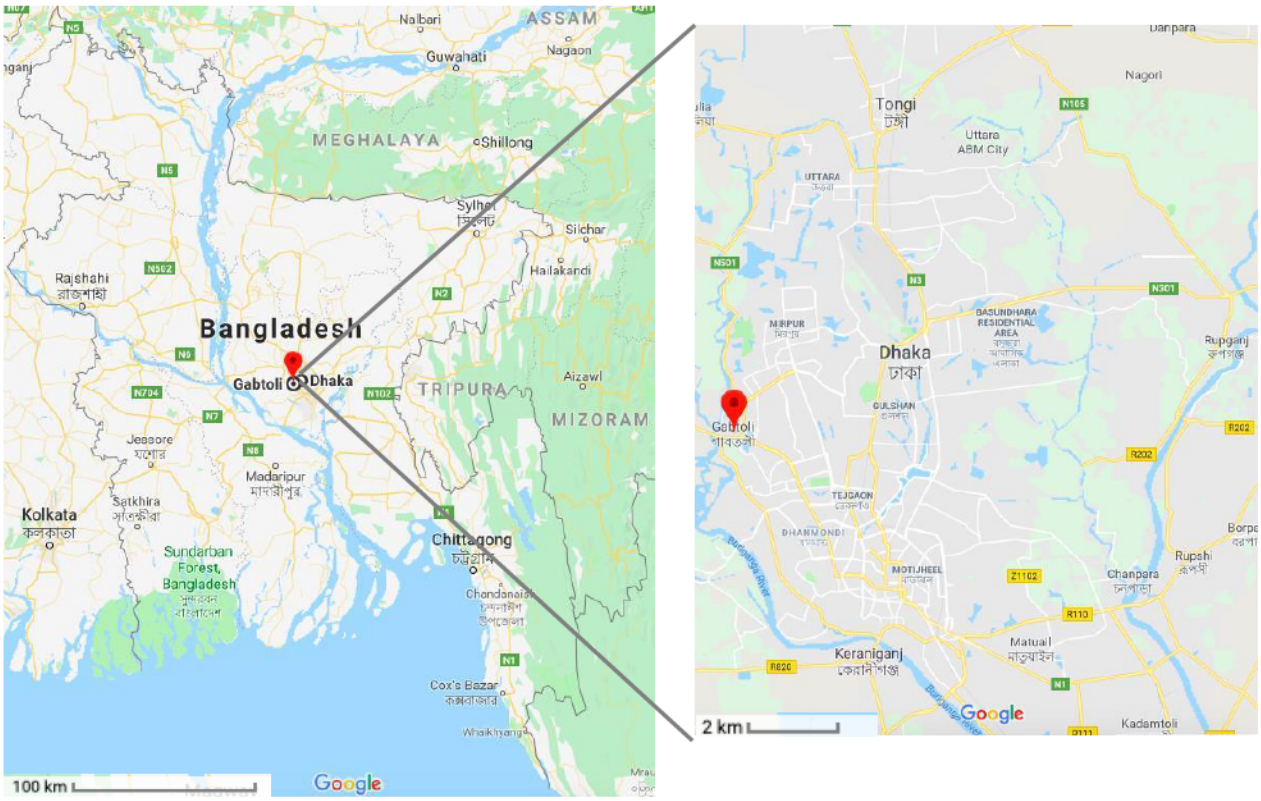


FIG 3.1. Environmental water sampling sites in Bangladesh. An endemic site for *V. cholerae* is shown on the map of Bangladesh. Environmental water samples were collected in triplicate from the river basin of Turag river from three locations that were 5 metres apart in Gabtoli area in Dhaka city that is an inland region of Bangladesh.

Table 3.1. Bacterial strains used in analytical validation of this assay

Species	No. of strains	Strain	Target genes		Source	Reference
			viuB	*rfb01*		
V. cholerae						
V. cholerae non O1	20	OYP1G01	+	–	Environmental	This study
		OYP2A12	+	–	Environmental	This study
		OYP2E01	+	–	Environmental	This Study
		OYP3B05	+	–	Environmental	Kirchberger et al (2016)
		OYP3F10	+	–	Environmental	This study
		OYP4B01	+	–	Environmental	This study
		OYP4C07	+	–	Environmental	Kirchberger et al (2016)
		OYP4G08	+	–	Environmental	This study
		OYP4H06	+	–	Environmental	This study
		OYP4H11	+	–	Environmental	This study
		OYP6D06	+	–	Environmental	This study
		OYP6E07	+	–	Environmental	This study
		OYP6F08	+	–	Environmental	This study
		OYP6F10	+	–	Environmental	This study
		OYP7C09	+	–	Environmental	This study
		OYP8C06	+	–	Environmental	This study
		OYP8F12	+	–	Environmental	This study
				–		
V. cholerae O1	8	N16961	+	+	Clinical	Heidelberg et al (2000)
		V52	+	+	Clinical	Chun et al (2009)
		EDC-728	+	+	Environmental	This study
		EDC-753	+	+	Environmental	This study
		EDC-754	+	+	Environmental	This study
		EDC-755	+	+	Environmental	This study
		EDC-772	+	+	Environmental	This study
		EDC-805	+	+	Environmental	This study

(continued in the next page)

Species	No. of strains	Strain	Target genes		Source	Reference
Other Vibrio species						
V. parahaemolyticus	1	ATCC 17802	–	–	Clinical	Yang et al (2015)
V. vulnificus	3	ATCC 27562	–	–	Clinical	Li et al (2012)
		CVD7	–	–	Clinical	Stine et al (2000)
		A9	–	–	Clinical	Fouz et al (2007)
V. metoecus	10	RC341	–	–	Environmental	Haley et al (2010)
		OP3H	–	–	Environmental	Kirchberger et al (2014)
		OYP4D01	–	–	Environmental	Orata et al (2015)
		OYP4E03	–	–	Environmental	This study
		OYP5B04	–	–	Environmental	Orata et al (2015)
		OYP5B06	–	–	Environmental	Orata et al (2015)
		OYP5H08	–	–	Environmental	This study
		OYP8G05	–	–	Environmental	This study
		OYP8G09	–	–	Environmental	This study
		OYP8G12	–	–	Environmental	This study
V. mimicus	3	ATCC 33653	–	–	Clinical	Davis et al (1981)
		ATCC 33654	–	–	Environmental	Davis et al (1981)
		ATCC 33655	–	–	Clinical	Davis et al (1981)
Other bacterial species						
Escherichia coli	2	CU1	–	–	Clinical	Taniguchi et al (1998)
		CU2	–	–	Clinical	McGilvrary et al (1974)
Pseudomonas aeruginosa	2	PA103	–	–	Clinical	Ohman et al (1980)
		PA14	–	–	Clinical	Kukavica-Ibrulj (2008)

3.3.3. Amplification using the Chai open qPCR platform

Species-specific primers designed in the previous study (Chapter 2) were used to detect and quantify *V. cholerae* in this assay. To quantify total *V. cholerae* (all serogroups), we selected the *viuB* gene encoding vibriobactin utilization protein B, as it is a single copy gene present in all *V. cholerae* with only distant homologs present in other species. To detect and quantify *V. cholerae* from the O1 serogroup, we used specific primers and probe to amplify the *rfbO1* gene essential for the synthesis of this antigen (TABLE 3.2.) (Chapter 2). For this study, the reporter dye 56-FAM (Fluorescein) was used for both probe sets as this qPCR assay was optimized in the Chai Open qPCR thermocycler (CHAI, USA) which had a single channel to detect wavelengths of 513-555 nm.

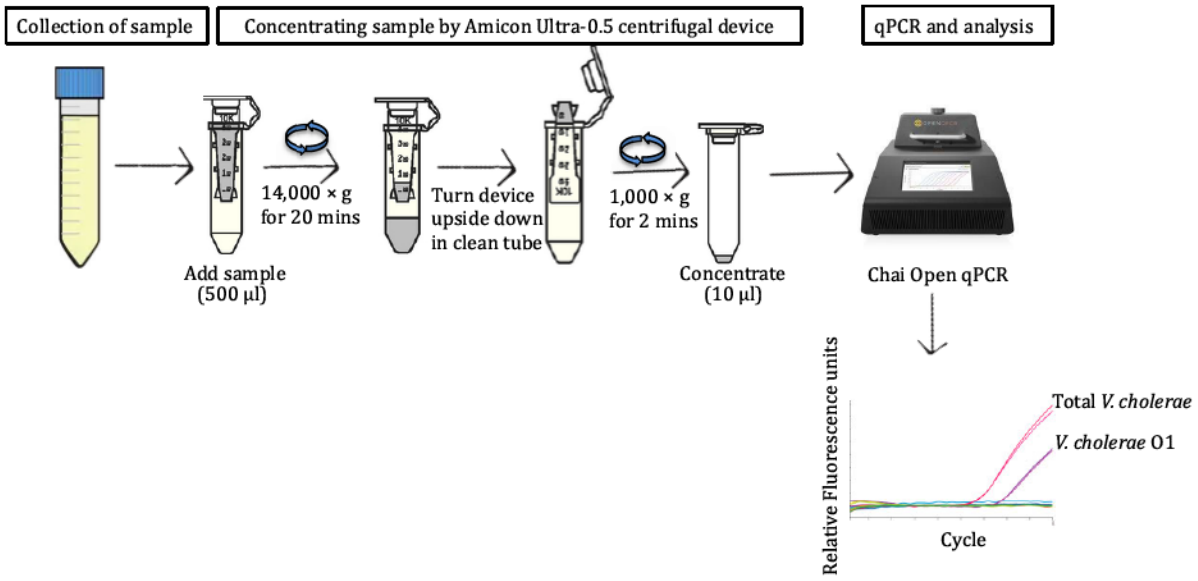


FIG 3.2. Method for processing of environmental samples for qPCR. A. Sample collection. B. Concentrating sample by Amicon Ultra-0.5 centrifugal device. C. qPCR in the Chai Open qPCR thermocycler (https://www.chaibio.com/openqpcr).
5mL of water was collected from each location. Ten Amicon tubes, each had 500 µl of water sample were centrifuged at 14000 × g for 30 mins in a mini spin plus centrifuge,

and all the concentrates were pulled together. Thus, the total volume of concentrate was 100 µl. There was no amplification directly from the water without concentration.

Dynamite qPCR Master mix used in this study is a proprietary mix, developed and distributed by the Molecular Biology Service Unit (University of Alberta, Canada). It contains Tris (pH 8.3), KCL, $MgCl_2$, glycerol, Tween 20, DMSO, dNTPs, ROX as a normalizing dye and antibody inhibited Taq polymerase. The volume of each PCR reaction was 50 µl and contained 25 µl of 2× Dynamite qPCR master mix, 5 µl of 500 nM primer-250 nM probe mix, 10 µl of molecular grade water and 10 µl of the concentrated environmental water sample. The recommended long-term storage temperature for this master mix is 4 °C. Conditions for the real-time qPCR are as follows: initial activation of the enzyme at 95 °C for 2 min followed by 45 cycles of 95 °C for 30 s, 60 °C for 1 min in the Chai Open qPCR system. In this qPCR method, the detection and quantification of the *viuB* and *rfbO1* gene markers were carried out in parallel. Standards of known copy number of cells and no template control were included in every assay.

TABLE 3.2. Target genes and sequences of primers and probes used in this study

Target gene	Primer and probe	Sequence(5'-3')	Amplicon size (bp)	References
viuB	Probe	56-FAM/TCATTTGGC/ZEN/CAGAGCATAAACCGGT/3IABkFQ	77	Chapter 2
	Forward primer	TCGGTATTGTCTAACGGTAT		
	Reverse Primer	CGATTCGTGAGGGTGATA		
rfbO1	Probe	56-FAM/AGAAGTGTG/ZEN/TGGGCCAGGTAAAGT/3IABkFQ	113	Chapter 2
	Forward primer	GTAAAGCAGGATGGAAACATATTC		
	Reverse primer	TGGGCTTACAAACTCAAGTAAG		

3.3.4. Standard curve for the qPCR assay

Standard curves were generated using the *V. cholerae El Tor* O1 N16961 reference strain. Pure bacterial culture was grown on LB agar (Becton Dickinson) at 30 °C overnight. Bacteria were then diluted in sterile water prepared by filtering 50 ml of water from the study location pushed through a 0.2 µm PES filter media (Whatman, GE Healthcare, UK) using a 50 ml syringe (BD, USA). A series of standards were prepared in which bacterial cells were present at 3×10^5 copies, 3×10^4 copies, 3×10^3 copies, 3×10^2 copies 30 and 3 copies per 10 µl of filter sterilized water. Inoculum concentration were quantified using a standard drop plate method (Herigstad et al., 2001). Standards were run in three independent experiments, with three replicates per dilution and repeated on three different days. The average of each experiment was assessed to define intra- and inter-assay variation (TABLE 3.3). A standard curve was generated by plotting the log value of the calculated colony forming unit (CFU) per reaction against the quantitative cycle value (Cq). The Cq value is the cycle at which the fluorescence from amplification exceeds the background fluorescence in the Minimum Information for Publication of Quantitative qPCR Experiments (MIQE) guidelines (Bustin et al., 2009).

3.3.5. Determination of the limit of detection (LOD)

The LOD of the assay was determined from the standard curve constructed from serially diluted standards of *V. cholerae* N16961 as mentioned above (FIG 3). To determine the LOD of samples which were concentrated, known numbers of *V. cholerae* N16961 were spiked in filter sterilized environmental water samples collected from the same location (Gabtoli, Dhaka) and subjected to the same concentration protocol as

environmental samples using the Amicon ultra-0.5 ml centrifugal filter device. 1ml of spiked environmental water sample was concentrated to 20 µl after 30 min spinning. The qPCR assay was carried out on these concentrated samples containing known bacterial cell numbers in the Chai Open qPCR system (TABLE C.1.). Cq values were used to define LOD of the assay. The LOD typically is assumed to be the highest Cq value observed for the lowest concentration that can be determined based on the dilution where all replicates were positive across ten repeated experiments.

3.3.6. The calculation for qPCR efficiency

The qPCR efficiency of the assay was calculated using the following formula: Efficiency = $10^{[-1/\text{Slope}]}$ (Blackstone et al., 2007; Pfaffl, 2004) in Excel.

3.4. RESULTS

3.4.1. Analytical validation

The qPCR assay was validated by using a blind panel of filter-sterilized environmental water samples collected from the Gabtoli area (Dhaka, Bangladesh) and spiked with *V. cholerae* reference strains. Forty-six bacterial isolates of known concentration (3×10^4 CFU/ml) were tested including non-O1 *V. cholerae*, *V. cholerae* O1, *V. metoecus* and the other *Vibrio* species *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *P. aeruginosa* and *E. coli* (TABLE 3.1.). All 20 non-O1 *V. cholerae* and 8 *V. cholerae* O1 were positive for the *viuB* gene. Only samples spiked with *V. cholerae* O1 strains were positive for the *rfbO1* gene. The other *Vibrio* spp. and non-*Vibrio* bacterial species were negative for these two gene targets. Thus, the analytical specificity of this method was 100%,

referring to the ability of an assay to detect and/or measure a specific organism in a sample (TABLE 3.1.). Analytical sensitivity was also found to be 100% based on detection of 3 CFU/reaction (TABLE 3.3.).

TABLE 3.3. Reproducibility and repeatability of qPCR assays

	Assay for Total *V. cholerae* (*viuB*)				Assay for *V. cholerae* O1 (*rfbO1*)			
No of CFU/Reaction	Intra-assay Mean	%CV	Inter-assay Mean(Cq)	%CV	Intra-assay Mean	%CV	Inter-assay Mean (Cq)	%CV
300000	19.45	0.03	19.47	0.07	19.55	0.03	19.50	0.21
30000	22.75	0.05	22.78	0.20	22.53	0.01	22.56	0.11
3000	25.94	0.02	25.92	0.07	25.94	0.01	25.93	0.07
300	29.12	0.01	29.08	0.19	29.23	0.02	29.28	0.13
30	32.57	0.02	32.56	0.08	32.63	0.01	32.62	0.03
3	35.83	0.01	35.85	0.07	35.83	0.01	35.85	0.03

3.4.2. Limit of Detection (LOD)

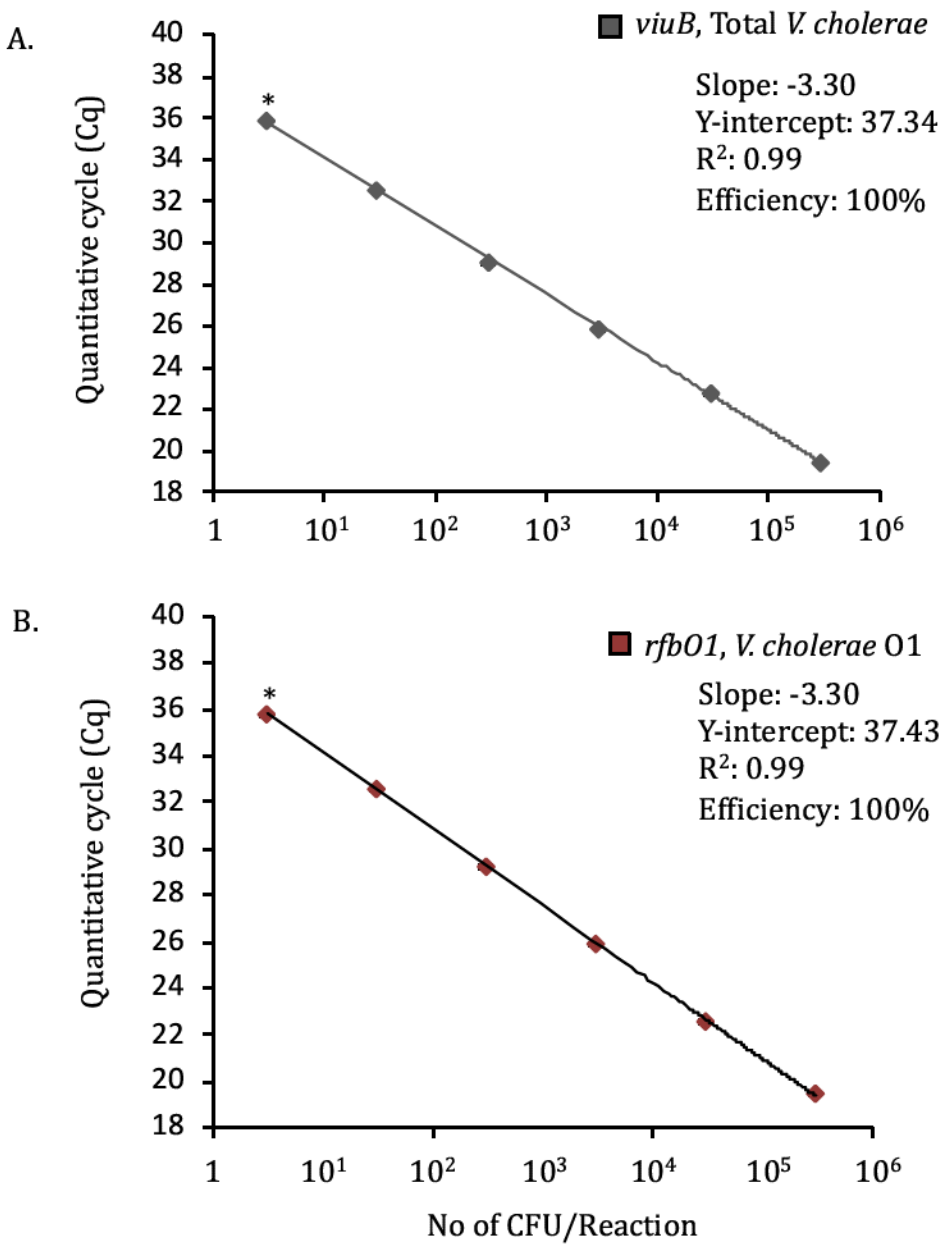
LOD of the assay was determined to be as low as 3 copies per reaction from the standard curve constructed using serially diluted standards of the *V. cholerae* El Tor O1 N16961 cells (FIG 3.3.). We also determined the SLOD (Sample limit of detection) of 6×10^3 CFU/L for the spiked filter-sterilized environmental water samples with a known number of *V. cholerae* N16961 concentrated with Amicon ultra-0.5 centrifugal filter device (TABLE C.1.).

3.4.3. Assay precision and efficiency

Following the MIQE guidelines, intra-assay variation (variation between replicates in the same experiment) and inter-assay variation (variation between replicates from different experiments) were evaluated to determine the repeatability and reproducibility of the assay for detecting and quantifying total *V. cholerae* and its O1 serogroup subpopulation. Precision analysis to test random variation of repeated measurement was

done for this assay by calculating the coefficient of variation (%CV) of multiple replicates of standards run in the same experiment and experiments on different days. Intra-assay %CV ranged from 0.01 to 0.05% for the *viuB* assay and 0.01 to 0.03% for the *rfbO1* assay. Inter-assay %CV ranged from 0.07 to 0.2% for *viuB* and 0.03 to 0.2% for *rfbO1* (TABLE 3.3.). The efficiency of both assays was 100% based on the standard curve generated from a serial dilution of *V. cholerae* N16961 (FIG 3.3.) with $R^2 = 0.99$ and slope of -3.3.

To test for qPCR inhibition, we compared the Cq values for different dilutions of filter-sterilized environmental water samples spiked with the reference *V. cholerae* strains. The 10× dilution of samples shifted the Cq values by 3.3 cycles $\pm$ 0.07 (TABLE C.1.), indicating no inhibition.



* indicates LOD of the assay

FIG 3.3. Standard curves for simultaneous detection and quantification of total *V. cholerae* and *V. cholerae* O1 by qPCR. Two gene markers with fluorogenic probes, A) *viuB* (*V. cholerae* specific) and B) *rfbO1* (*V. cholerae* O1 specific) were used. Cells of reference culture (*V. cholerae* N16961 El Tor O1) were serially diluted in 10-fold increments to yield concentration ranging from 3×10^6 to 3 copies per reaction (from left to right). Fluorescence was measured in relative units.

3.4.4. Analysis of environmental water samples

With this field-ready method, it was possible to quantify the abundance of both total *V. cholerae* and *V. cholerae* O1 in the same experiment (FIG 3.4.). Five ml of environmental water samples were concentrated to 100 µl, from which 10 µl was used in the qPCR amplification. Each reaction was run in triplicate. The abundance of *V. cholerae* in the water samples collected from the Gabtoli area, Dhaka, Bangladesh was 3.7×10^4 CFU/L to 3.9×10^4 CFU/L (FIG 3.5.). *V. cholerae* O1 was found at 4.7×10^3 to 5.4×10^3 CFU/L, representing 13% of the total *V. cholerae* population (FIG 3.5.).

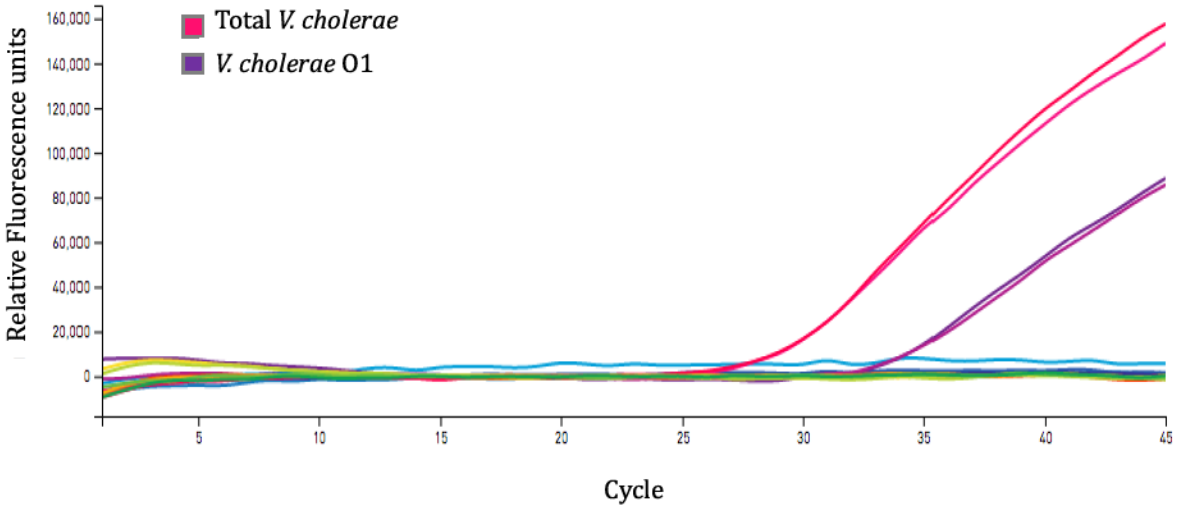


FIG 3.4. Simultaneous detection and quantification of total *V. cholerae* and *V. cholerae* O1 in the same qPCR experiment. Filter sterilized environmental water samples collected from Gabtoli site were inoculated with known of cells of reference strain *V. cholerae* N16961 El Tor O1. Quantification was performed with both *viuB* and *rfbO1* primers in the same PCR cycle to detect total *V. cholerae* and O1 serogroup *V. cholerae*, respectively.

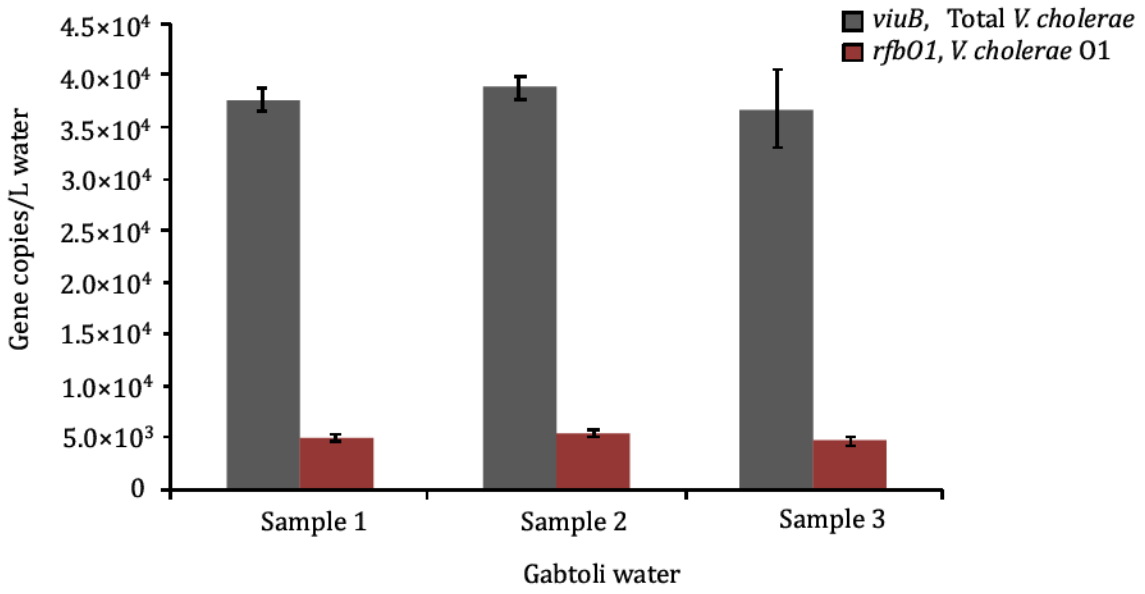


FIG 3.5. Abundance of *V. cholerae* along with its toxigenic serogroup O1 subpopulation in water from an inland urban region (Gabtoli, Dhaka) of Bangladesh. Water samples were collected in August 2018 and analyzed using the developed qPCR assay. The *viuB* gene marker was used to determine for total *V. cholerae*, and *rfbO1* was used to measure *V. cholerae* O1. Each qPCR reaction was done in triplicate and evaluated with corresponding standards. Error bars represent the standard deviation of means from biological replicates.

3.5. DISCUSSION

Since cholera is a waterborne infectious disease and the primary mode of transmission is via the fecal-oral route, environmental water bodies serve as an inevitable reservoir for pathogenic *V. cholerae*. This bacterium is associated with plankton mainly in brackish waters and is ubiquitous in temperate and tropical estuarine microbial communities (Takemura et al., 2014). As a survival strategy, *V. cholerae* goes into a VBNC state under unfavorable environmental conditions, in which it assumes a coccoid shape and cannot be revived using traditional culture methods (Colwell, 2000;

Colwell and Huq, 1994). Non-culturable *V. cholerae* in biofilm were reported in the environmental water samples from Mathbaria (Bangladesh), and were able to resume active growth after passage through the gastrointestinal tract of rabbits (in animal passage of non-culturable *V. cholerae* O1) following a period of more than a year in a microcosm (Alam et al., 2007). Therefore, passing through a human host could be a means of revival from the VBNC state and contribute to the amplification of *V. cholerae* prior to an outbreak (Colwell et al., 1996; Faruque et al., 2005a) and subsequent transmission through the fecal-oral route due to poor management of drinking water and hygiene. This survival state frequently found in the environment for *V. cholerae* means that waters bodies can serve as a long-term reservoir for this pathogen, leading to the consistent and persistent pattern of cholera epidemics historically documented on the coast of Bangladesh.

The ability of *V. cholerae* to exist in a VBNC state has hindered our ability to quantify it in environmental reservoirs using traditional methods. Limited resources and lack of infrastructure in countries where cholera is endemic has also been problematic in monitoring its causative agent. The qPCR method developed here allows the detection and quantification of *V. cholerae* and the O1 serogroup strains responsible for most outbreaks. Furthermore, the portable and low-cost instrument used (Chai Open qPCR), as well as our streamlined protocol, allow processing of water samples on site, without the need for DNA extraction or any pre-enrichment procedure. The main challenge for direct quantification is that the number of bacteria in environmental water is usually below the LOD of existing qPCR methods (Huq et al., 1990). To overcome this problem, the water sample was concentrated 50-fold using simple size-exclusion centrifugation.

The targeted *viuB* gene sequence is only present in *V. cholerae* and can be amplified from both O1 and non-O1 strains (TABLE 3.1.). Moreover, *V. cholerae* O1 could be detected with primers targeting the *rfbO1* gene, thus allowing the determination of the abundance of both populations with 100% specificity. The assay sensitivity was as low as three copies per reaction, which is the norm for a well-designed sensitive assay, as described in the MIQE guidelines (Bustin et al., 2009). The limit of detection for concentrated water samples was 6×10^3 CFU/L, which is promising as the infectious dose of *V. cholerae* was reported to be 10^3 to 10^8 cells (Schmid-Hempel and Frank, 2007). More specifically, the infectious dose for toxigenic *V. cholerae* is around 10^3 cells, whereas the infective dose for non-O1 strains is around 10^6 (Kothary and Babu, 2001). Without the concentration procedure, the limit of detection of *V. cholerae* in environmental water samples would be 3×10^7 CFU/L, which is relatively high. This assay determines the absolute abundance of both *V. cholerae* O1 and non-O1 in parallel qPCR reactions and thus is very useful to calculate the proportion of pandemic *V. cholerae* in a particular geographical location. Therefore, this assay is useful to track cholera in endemic areas like Bangladesh, where all detected *V. cholerae* O1 strains were found to be toxigenic (Alam et al., 2006; Alam et al., 2007).

The possibility of storing the qPCR master mix on ice (4 °C) for extended periods of time and the portable feature of the Chai thermocycler (small footprint of 28 cm × 24 cm, weight of 4 kg) facilitate the use of this method in field-level surveillance and also avoid transportation problems leading to deterioration of the samples. The convenient set up of the whole procedure makes this assay workable in most resource-limited

settings. Moreover, quick processing of the sample reduces the chance of cross-contamination.

As proof of concept, we analyzed water samples collected from the river basin of the Turag river in a region endemic for cholera, the Gabtoli area of Dhaka, Bangladesh. Approximately 25,000 people live in this area, which is surrounded by brickfields with high traffic (Ahmed et al., 2017). This site was chosen as previous studies indicated the presence of *V. cholerae* O1 and cholera infections are often detected in people living in the surrounding area. The number of total *V. cholerae* detected was about 3.9×10^4 CFU/L, 13% of which was *V. cholerae* O1 (5.0×10^3 CFU/L), which is of concern as it is within the range of the infectious dose for cholera if someone were to ingest a few hundred millilitres (>10^3 cells) (Schmid-Hempel and Frank, 2007). Previous studies based on the Crystal VC® dipstick test after sample enrichment for 18h in alkaline peptone water showed that in urban Dhaka, Bangladesh, 30% of water sources used by households of cholera patients were contaminated with *V. cholerae* (George et al., 2016; Rashid et al., 2017a). From our observation during sampling, and also reported by other studies, the water bodies around Dhaka city serve as drinking water sources and are frequently used for domestic purposes, such as washing utensils and bathing (Aktar and Moonajilin, 2017). It is therefore likely that local rivers and ponds serve as an important reservoir of the cholera pathogen in Dhaka city. There has never been a direct link between a rise in *V. cholerae* numbers and the start of a seasonal epidemic, despite the timing of these being well known from the tracking of cholerae cases in hospitals.

Many qPCR assays for the quantification of *V. cholerae* have been previously developed, but all of them require modern laboratory facilities and are not amenable to

extensive field studies. Furthermore, there is not a single study of the environmental tracking of *V. cholerae* in a cholera-endemic setting of significant size. With the currently available techniques, either spiked environmental samples were evaluated after pre-enrichment in alkaline peptone water or DNA extraction after filtration (Blackstone et al., 2007; Vezzulli et al., 2015). Amongst these methods, culture-based techniques are still the most frequently used and only reveal a small proportion of naturally occurring bacterial populations in water samples. These methods do not capture coccoid non-culturable cells within clusters of biofilms in estuarine environments present in waters bodies are Bangladesh during the period between outbreaks when reported cases of cholera are low (Sultana et al., 2018).

Another widely used method is dipstick, which is valuable to detect *V. cholerae* O1 and O139 in both water and stool samples. This is a rapid and inexpensive method, but has a limit of detection of 10^7 CFU of enriched culture that is higher than the usual infectious dose of cholera (Schmid-Hempel and Frank, 2007). This method only provides a qualitative output and requires six hours of enrichment before testing. Also, because of compromised sensitivity, it was recommended for use in combination with a traditional bacterial culture method (Rashid et al., 2017a).

The developed assay is both specific and sensitive as well as convenient for field studies to make it possible to overcome the limitations of current "rapid" techniques. In this study, a single channel Chai thermocycler was used, which only permitted FAM to be used as a fluorescein dye. A dual channel Chai system also exists, where HEX or VIC fluorescein dyes can also be used with FAM, making a multiplex qPCR assay is possible.

Thus, it is possible to optimize a multiplex assay where O1 and O139 can be rapidly identified and quantified simultaneously in a single reaction along with total *V. cholerae*.

During the environmental sample analysis, we did not find significant inhibition to PCR amplification in our assay. However, for testing water from more contaminated sources, as determined by multivariate analysis of environmental factors where the industrial waste disposal and high population density matters (Pia et al., 2018; Staff Correspondent, 2016), further treatment of the sample to remove inhibitors could be required. A recent study on water quality assessment of the roadside surface of Savar, Dhaka explained the impact of vehicle emission, atmospheric deposition from brick field, industrial pollution and massive urbanization on the water reservoirs as total suspended solids (>25 mg/l), total dissolved solids (>840mg/l), biological oxygen demands (0.758 mg/l), dissolved oxygen (4.5 mg/l) were high or even higher than the standards (Ahmed et al., 2017).

In conclusion, we developed a species-specific qPCR method, which can be used at the sampling site without any need for pre-enrichment or DNA extraction. This test can support a continuous monitoring program for *V. cholerae* O1 in water reservoirs used by residents, which in coordination with local authorities could limit risks of contracting cholera and allow the identification of sources of contamination.

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CHAPTER 4

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Nasreen T., Islam M.T., Johura F.T., Alam M. and Boucher Y. **"Intraspecies interactions and changes in intrinsic environmental factors influence the prevalence of pandemic *Vibrio cholerae* in an urban region endemic for cholera"**

Author contribution: Y.B., T.N. and M.A. designed the project. Y.B. and T.N. wrote the manuscript. M.T.I, F.T.J. and M.A. helped in sample collection and sample processing during field trips in Dhaka, Bangladesh. T.N. performed the qPCR and M.T.I did the amplicon sequencing. T.N. and M.T.I. did the analysis. K.L. helped in bioinformatics analysis. M.T.I., K.L., F.T.J. and M.A. reviewed the manuscript. M.A. and Y.B. supervised the project.

CHAPTER 4

Intraspecies interactions and changes in intrinsic environmental factors influence the prevalence of pandemic *Vibrio cholerae* in an urban region endemic for cholera

4.1. ABSTRACT

Cholera has been endemic to the Ganges delta for centuries. Although its causative agent, *Vibrio cholerae,* is a coastal bacterium mostly found in brackish water, cases of cholera occur continually in Dhaka, the inland capital city of Bangladesh, which is surrounded by freshwater. Despite the persistence of this problem, little is known about the environmental abundance and distribution of *V. cholerae* serogroup O1 from the pandemic generating (PG) lineage responsible for most cholera cases. To understand spatial and temporal dynamics of PG and other lineages belonging to the *V. cholerae* species in Dhaka's water reservoirs, we performed high-throughput sequencing of a species-specific, highly diverse protein-coding gene (vibriobactin utilization protein, *viuB*) amplified from aquatic biomass DNA. Coupled with real-time qPCR, this method provided subspecies level resolution of the abundance and lineage composition of *V. cholerae* populations. Seven different sites across Dhaka were investigated for six consecutive months, and the total abundance of *V. cholerae* was found to be relatively stable, varying between 2 to 4 × 10^5 cells/L at six sites and around 5 × 10^5 cells/L at the site in the most densely populated part of Dhaka city. Moreover, cells from the PG lineage displaying the O1 antigen composed between 24% to 92% of the *V. cholerae* population, only showing occasional but sudden reductions in abundance. In these rare instances in which PG O1 lost its dominance, other lineages underwent a rapid expansion while the

size of the total *V. cholerae* population remained unchanged. This suggests that intraspecies competition might be responsible for PG O1 population crashes. Amongst the environmental parameters measured, salinity and total dissolved solids (TDS) correlated with *V. cholerae* lineage richness positively. A negative correlation was also observed between PG O1 and salinity, even though changes in this parameter were minor (0-0.8 ppt). This suggests that even at the subspecies level, population composition can be influenced by interactions among the close relatives as well as by fluctuations in environmental factors. Pandemic *V. cholerae* strains might be especially susceptible to changes in salinity, which is one of the environmental changes that could trigger their loss of dominance in their competition with other lineages within the species.

4.2. INTRODUCTION

The Gram-negative, comma-shaped facultative anaerobic bacterium *V. cholerae* is a natural inhabitant of estuarine water systems (Centers for Disease and Prevention, 1994; Xu et al., 1982). Pathogenic strains of *V. cholerae* are responsible for cholera, an acute life-threatening diarrheal disease which is a major public health concern because of its high morbidity and mortality with an estimation of 1.3 to 4 million cholera cases and 21, 000 to 143, 000 deaths worldwide each year (Ali et al., 2012). The first pandemic of cholera struck in 1817 and was followed by six others in the next two hundred years, leaving a devastating human death toll. More than 200 serogroups of *V. cholerae* have been identified based on their surface polysaccharide O antigen (Chatterjee and Chaudhuri, 2003). However, only O1 serogroup dominates the pandemic generating (PG) lineage (Islam et al., 2017) and other serogroups mostly represent *V. cholerae*

environmental strains which are generally non-pathogenic (Chun et al., 2009; Faruque et al., 1998). Serogroup O1 is further classified into two biotypes, Classical biotype that was evident to cause the fifth and sixth pandemic and believed to be associated with the earlier pandemics, and El Tor biotype which has been the causative agent for the seventh pandemic of cholera (Hu et al., 2016).

Cholera never went away and remained as an emerging and reemerging disease (Satcher, 1995). The seventh pandemic of cholera started in 1961 and is still ongoing, known as the world's longest persistent pandemic (Ali et al., 2012; WHO, 2018a). The world's worst epidemics can be traced back to Ganges delta that serves as a reservoir for the disease (Boucher et al., 2015; Mutreja et al., 2011). High population density and improper sewage disposal along with uncontrolled industrialization constantly pollute the water sources in this region (Martinez, 2018). Dhaka, the capital city of Bangladesh, is considered as hyper-endemic for cholera, the disease continually occurs at a low incidence but exhibits biannual seasonal outbreaks (Longini Jr et al., 2002).

Recent advancement on cholera surveillance intended to increase prevention, preparedness and awareness of the disease has been chiefly established based on clinical cases and analysis of clinical samples. Such surveys help to understand epidemiological patterns, trends of the disease over time and predict potential outbreaks. However, they are most useful in endemic settings where outbreaks are regular and associated with climate and socio-economic conditions (Bwire et al., 2017; Haque et al., 2013; Olago et al., 2007). They also only yield limited insight on how outbreaks start, and which water reservoirs are responsible for the spread of the disease. As *V. cholerae* is an autochthonous member of aquatic environments, understanding its life cycle and

ecology requires a knowledge of the temporal and spatial variations in abundance of various genotypes in its natural habitat.

There are few substantial environmental studies of *V. cholerae*. Culture-based studies underestimate *V. cholerae* abundance and diversity due to the viable but non-culturable (VBNC) state posed by this bacterium in response to unfavorable conditions (Alam et al., 2006; Colwell, 2000). Real-time qPCR analyses of environmental DNA targeting *V. cholerae* or O1 serogroup-specific genes are quantitative but do not provide information on the subspecies composition of the populations analyzed. Additionally, culture-independent studies, including 16S rRNA sequencing, can be helpful for species identification but do not provide resolution at the subspecies level (Chun et al., 1999). The fluorescent antibody staining method used to enumerate viable *V. cholerae* cells, but only target the O1 or O139 serogroups (Brayton and Colwell, 1987; Chowdhury et al., 1995) and cannot distinguish between O1 strains belonging to the pandemic-generating lineage and strains from other lineages bearing that antigen.

To overcome these limitations, we performed high-throughput sequencing of a species-specific, highly variable protein-coding gene (vibriobactin utilization protein, *viuB*) from aquatic biomass coupled with qPCR. This technique allowed us to understand spatio-temporal variations in abundance of the pandemic-generating (PG) and other lineages belonging to the *V. cholerae* species in Dhaka's water reservoirs over six consecutive months. It revealed the continual presence of the PG lineage and co-occurrence dynamics of *V. cholerae* at subspecies level along with the influence of environmental factors in this cholera endemic urban region.

4.3. MATERIALS AND METHODS

4.3.1. Study area

Water samples were collected from seven different locations (site 1 to site 7) in Dhaka, Bangladesh (FIG 4.1.) biweekly for six consecutive months from October 2015 to March 2016. Dhaka (23.8103° N, 90.4125° E) is the capital city of Bangladesh surrounded by the river system of four rivers: Turag, Buriganga, Shitalakshya and Balu. A population of >18 million was recorded for this area in 2016, with a density of 23,234 people/ km^2 within a total area of 300 km^2 (FIG 4.1.) (http://worldpopulationreview.com/world-cities/dhaka-population/). The physical distance between site 1 and site 7 is shorter (9.9 km) than the distance between site 1 and site 7 to the other five sites (approximately 21 to 25 km) (Google maps). Based on the epidemiological records, Dhaka experiences two seasonal cholera outbreaks each year and is considered hyper-endemic for the disease (Longini et al., 2002). The climate of Dhaka is categorized as tropical wet and dry with a distinct monsoon season. The average temperature recorded is 26.1 °C (19.1 °C in Jan and 29.1 °C in June)(Weatherbase). The visual inspection of the study areas indicated that local markets surrounded these sites, and the human intrusion such as bathing, swimming, washing dishes as well as bathing domestic animals were massive.

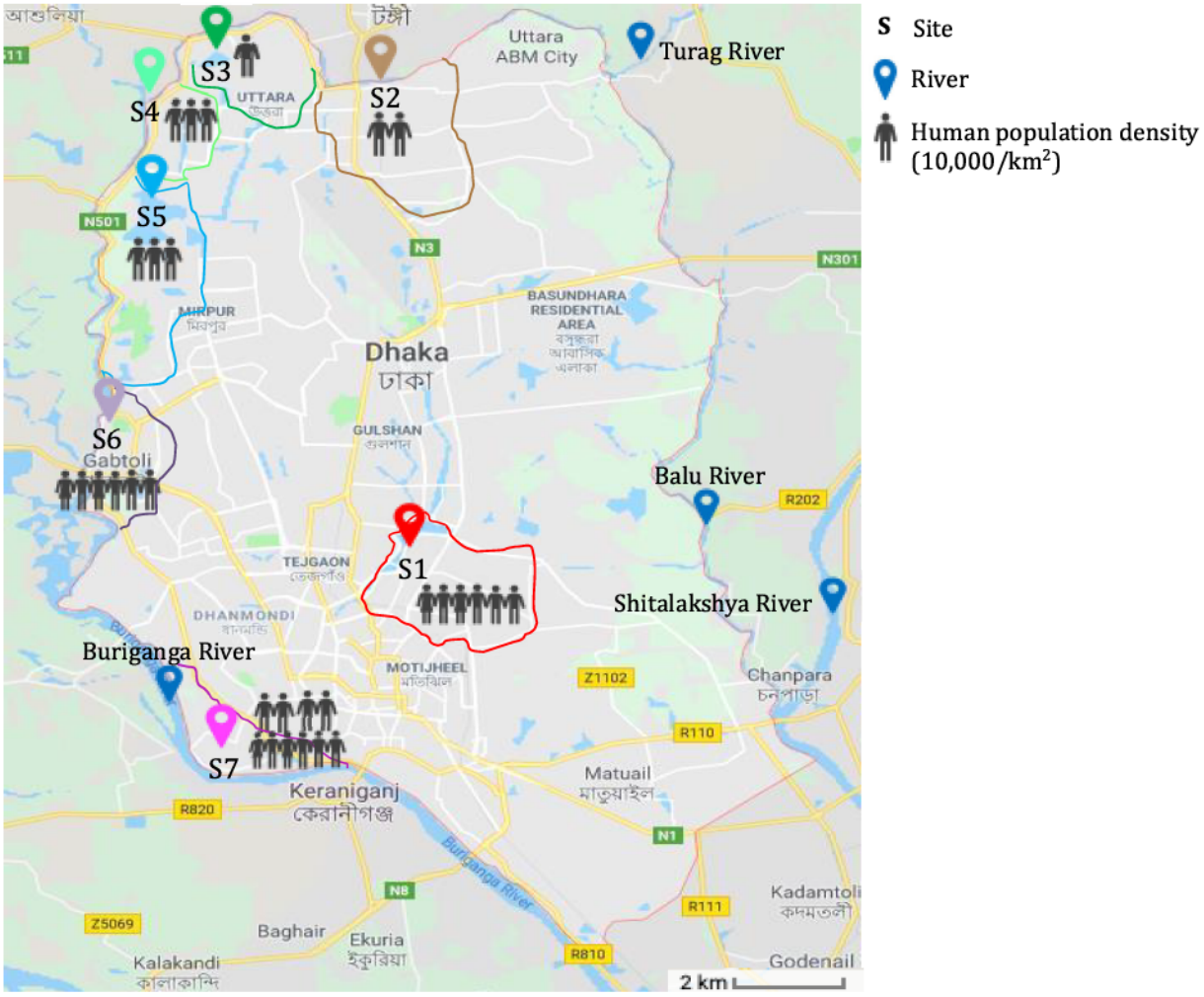

FIG 4.1. Sampling sites in Dhaka, Bangladesh. Dhaka, the capital city of Bangladesh, is surrounded by the river system of four rivers including Turag river, Buriganga river, Shitalakshya river and Balu river, as indicated on the map. Seven different sampling sites located in the inland capital city of Bangladesh are indicated with 'S' (from S1 to S7) along with the human population density corresponding to each site.
[Information on the human population density in this figure was adapted from (Khatun et al., 2017)]

4.3.2. Sample collection and processing

Water samples (50 ml) were collected using 50 ml sterile polypropylene syringes and filtered through 0.22 µm Sterivex filters (Millipore). Total DNA extraction from the

biomass on the filters was done through the following three consecutive steps: cell lysis and digestion, DNA extraction, and DNA concentrating and washing according to the protocol developed by Wright et al. (Wright et al., 2009).

To reduce impurities that can act as PCR inhibitors during amplification, all extracted DNA samples were further treated with One step PCR inhibitory removal kit (The Epigenetics Company, ZYMO Research) by following the user manual instructions with 90-180 µl of yield achieved from 100-200 µl of the eluted extracted DNA sample. Treated samples were kept at -20°C for further analysis.

4.3.3. Physicochemical parameters

Surface water quality was measured in water reservoirs at the seven sampling sites. EXO2 multiparameter sonde (YSI, Xylam Brand, USA) allowed for simultaneous measurement of pH, dissolved oxygen (DO), conductivity, total dissolved solids (TDS), salinity and water temperature. Properly calibrated sensors attached to the instrument were placed in each site while sampling and data were recorded for further analysis.

4.3.4. PCR amplification and Illumina sequencing

A touchdown PCR was performed to amplify a 293bp region of the *viuB* gene from DNA extracted from biomass. Master mix for PCR contained 5 µl of 5× Phire Buffer (ThermoFisher), 0.4 µl of 10 mM dNTP mix (ThermoFisher), 0.4 of µl Phire Hot Start II DNA Polymerase (ThermoFisher), 0.5 µl of Molecular Biology Grade Bovine Serum Albumin (20 mg/mL, New England Biolabs), 0.5 µl each of 10 pmol forward and reverse primers (for *viuB*: *viuB*2f 5'-CCGTTAGACAATACCGAGCAC-3' and *viuB*5r 5'-

TTAGGATCGCGCACTAACCAC-3') and 2 µl of template DNA. The PCR reaction was performed as follows: initial denaturation at 98 °C for 4 min, followed by 10 cycles of denaturation at 98 °C for 10 sec, annealing at 60 °C for 6 sec (reduced by 1°C per cycle), and extension 72 °C for 1 sec; followed by 23 cycles of denaturation at 98 °C for 10 sec, annealing at 50 °C for 6 sec (reduced by 1°C per cycle), and extension at 72 °C for 1 sec; and a final extension at 72 °C for 1 min.

Dual-indexed sequences using indices developed by Kozich et al. (Kozich et al., 2013) were used to prepare amplified *viuB* products for sequencing. 2 µl of amplified *viuB* products were used as template for a second PCR reaction with the same reagents as above with a set of forward and reverse primers that contained appropriate Illumina-adapters, a sample-specific 8 nucleotide index sequence, a 10 nucleotide pad, 2 nucleotide linker, and the gene specific sequence described above, for a total of 70 and 65 bp (Oksanen, 2014). This tagging PCR reaction was performed as follows: initial denaturation at 98 °C for 30 sec; followed by two cycles of denaturation at 98 °C for 10 sec, annealing at 55 °C for 6 sec, and extension at 72 °C for 1 sec; and final extension at 72 °C for 1 min. Use of gene-specific primers during amplification and subsequent tagging to create dual-indexed PCR products facilitated improved yield of amplicons and prevented biased amplification due to unexpected interaction of non-primer sequences with the template.

Additionally, eight tagging reactions were done for each sample to obtain an adequate concentration of amplicons. All eight reactions of the same sample were pooled together and run on a 2% agarose gel in 1× Tris-Acetate-EDTA buffer where two bands of very similar size, a smaller band (around 360 bp) representing only half-tagged PCR

products, and a slightly bigger band (428 bp) of the fully tagged product were visualized. The larger bands were cut out of the gel. PCR products were then purified using Wizard SV Gel and PCR Clean-Up System (Promega) according to the instructions by the manufacturer. The concentration of cleaned PCR products was then measured using a Qubit Fluorometer (ThermoFisher) with a Qubit dsDNA HS Assay Kit (ThermoFisher) and pooled together in equal concentrations (> 10 ng/µl). The pooled samples were then concentrated using a Wizard SV Gel and PCR Clean-Up System (Promega) according to the instructions by the manufacturer. Quality control of the pooled and the concentrated sample was performed using an Agilent 2100 Bioanalyzer. Sequencing was performed using Illumina MiSeq technology with a v3 (600 cycles) reagent kit. The nucleic acid concentration was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes) and the Synergy H1 microplate reader (BioTek).

4.3.5. Sequence Analysis

Processing of amplicon sequence reads was performed following the procedure described by Kirchberger (2017). Briefly, de-multiplexed raw reads were processed in R (RC, 2013) using the DADA2 pipeline (Callahan et al., 2016). Forward and reverse reads were trimmed due to a drop-off in read quality in the first 10 bp as well as after 240 bp and 160 bp for forward and reverse reads, respectively. Assembled overlapping forward and reverse reads were therefore 272 bp in length, 11 bp shorter than the completely sequenced region. Reads with a maximum expected error rate > 1% were also discarded. After this procedure, 1072 unique sequences remained in the dataset. Chimera detection implemented in DADA2 was then performed on pooled samples, leaving a total of 460

unique reads. Sequence reads were assigned to *viuB* alleles, 25 of which were composed of more than 1,000 reads (with an average of 100,000 reads per sample) and considered for further analysis.

4.3.6. Real-time qPCR amplification

A real-time qPCR assay was performed to determine the abundance of total *V. cholerae* and toxigenic *V. cholerae* O1. For total *V. cholerae*, the *viuB* gene encoding vibriobactin utilization protein B was targeted using the probe, 5'-/56-FAM/TCA TTT GGC/ZEN/CAG AGC ATA AAC CGG T/3IABkFQ/-3', and forward and reverse primers, 5'-TCG GTA TTG TCT AAC GGT AT-3' and 5'-CGA TTC GTG AGG GTG ATA-3', respectively, for a 77-bp product and *V. cholerae* O1 serogroup, the target was 113bp product of O1 specific *rfbO1* gene.

Probe, 5'-/ 5HEX/AGAAGTGTG/ZEN/TGGGCCAGGTAAAGT/3IABkFQ/-3', forward 5'-GTAAAGCAGGATGGAAACATATTC-3' and reverse, 5'- TGGGCTTACAAACTCAAGTAAG-3' primers were used.

Dynamite qPCR Mastermix (Molecular Biology Service Unit, University of Alberta, Edmonton, AB, Canada) was used in this study is a proprietary mix, developed and distributed by the Molecular Biology Service Unit (MBSU). It contains Tris (pH 8.3), KCL, $MgCl_2$, glycerol, Tween 20, DMSO, dNTPs, ROX as a normalizing dye and antibody inhibited Taq polymerase. The volume of the PCR reaction was 10 µl containing 5 µl of 2× Dynamite qPCR master mix, 1 µl of each of 500 nM primer-250 nM probe mix, 1 µl of molecular grade water and 2 µl of DNA template. Real-time quantitative PCR was performed under conditions: initial primer activation at 95 °C for 2 min followed by 40

cycles of 95 °C for 15 s, 60 °C for 1 min in Illumina Eco Real-Time PCR system. The assay includes standards of known copy number and a no template control for each PCR reaction to ensure the reaction is contamination-free. The limit of detection was determined to be 3 copies per reaction (Chapter 2). Briefly, standard curves were prepared using amplified gene sequences from corresponding reference strains as templates for each target. *viuB* and *rfb01* genes were used for total *V. cholerae* count, toxigenic *V. cholerae* O1, respectively. *V. cholerae* N16961 strain was used as a reference strain for these two genes. A series of standards were prepared in which a gene of interest was present at 3×10^5 copies, 3×10^4 copies, 3×10^3 copies, 3×10^2 copies 30 and 3 copies. Once prepared, the standards were aliquoted (100 µl) and stored at -80° C. The standard curve was generated by plotting the log value of calculated gene copies per reaction over the quantitative cycle value (Cq). The Cq is described as the cycle at which the fluorescence from amplification exceeds the background fluorescence in MIQE guideline (Bustin et al., 2009) and therefore lower Cq values represent a higher initial copy number of the target gene. The qPCR efficiency of the assay was calculated using the formula, Efficiency = $10^{[-1/Slope]}$ (http://efficiency.gene-quantification.info/).

4.3.7. Statistical and multivariate analyses

Two-dimensional visualizations of the overall *V. cholerae* community structure were performed using NMDS (Non-metric multidimensional scaling) with Bray-Curtis distance at OTU levels based on *viuB* alleles. For NMDS analysis in R program, we used the scripts developed in the bioenv project (Torondel et al., 2016) which finds a non-

parametric monotonic relationship between the dissimilarities in the samples matrix and plots the location of each site in a low-dimensional space.

Abundance data together with environmental variables data were analyzed in R. The script used for analysis was an extension of vegan library's bioenv function that finds the best set of environmental variables with maximum (rank) correlation with *V. cholerae* community dissimilarities and then plots them as vectors along with the best subset of *viuB* alleles on the NMDS plot. Multiple subplots were also generated for all the environmental variables against *viuB* allele richness in a single plot, and a correlation test is performed between them to determine any significant relationships (Torondel et al., 2016). Hierarchical clustering analysis was done with the R function hclust to identify clusters (*viuB* alleles) with the closest distance based on the abundance data (https://www.datanovia.com/en/lessons/assessing-clustering-tendency/).

4.3.8. Phylogenetic analysis and determination of genome similarity

At most two representative strains (TABLE S1) were chosen for each major *viuB* allele. Whole genome analysis was done using mugsy v1.2.3 with default parameters (Angiuoli and Salzberg, 2011). Mugsy outputs were analyzed using galaxy (https://usegalaxy.org), and the phylogenetic tree was constructed using Raxml v8.2.11 under the GTRGAMMA model with 100 bootstrap replicates (Stamatakis, 2014). Defaults were chosen for all other parameters. The phylogenetic tree was visualized using iTOL (Letunic and Bork, 2007).

Two metrics were used to determine genomic similarities. The first metric used was *in-silico* DDH, which is a proxy for traditional DDH values, and it retained the same

species-level cut-off of 70% (Auch et al., 2010). All pairwise comparisons were calculated using GGDC with default parameters (Meier-Kolthoff et al., 2013). Allelic differences were the second method used to determine genomic similarities. To determine allelic differences each genome was first annotated using RAST (Aziz et al., 2008). A set of 2443 genes common in most *V. cholerae* strains were then identified using Usearch (RC, 2010). Allele designations and identification were subsequently performed using automated scripts made available by BIGSdb (Jolley and Maiden, 2010). Finally, pairwise allelic differences for all isolates were calculated using an in-house script and only loci present in both isolates of a pair are considered. Sequences for all gene alleles are available on https://pubmlst.org/vcholerae under the cgMLST scheme.

4.4. RESULTS AND DISCUSSION

4.4.1. The pandemic generating lineage is the most abundant *Vibrio cholerae* genotype in Dhaka's water reservoirs

Dhaka, one of the most densely populated cities in the world (>18 million as of 2016), is located within the Ganges delta. It has reported previously that 7th pandemic of cholera has originated and spread out from this region (Mutreja et al., 2011). Although Dhaka is an inland city, its water system primarily dominated by rivers and canals of fresh water. In this city, cholera exists at a hyperendemic level and shows biannual peaks in reported cases during the spring and fall, i.e. before and after the monsoon (Alam et al., 2011), but little is known about the abundance and the distribution of the causative agent in natural waterbodies and how it correlates with environmental and human factors. To determine variation in abundance and lineage composition of *V. cholerae*

populations, a 272 bp hypervariable stretch of the *viuB* marker gene, which is present in a single copy in *V. cholerae* (Kirchberger, 2017) was amplified and sequenced from fortnightly samples taken in seven different water reservoirs inside Dhaka city from October 2015 to Mach 2016 (FIG 4.1.). Twenty-five *viuB* alleles were found, differing from each other by two or more single nucleotide polymorphisms. Amongst them, 12 *viuB* alleles (> 20,000 sequence reads) were included for further analysis of *V. cholerae* community composition, while the other 13 alleles representing < 1% of the total population were excluded (FIG 4.2.).

The *viuB*-73 allele corresponding to the PG lineage (Kirchberger, 2017) was present in all seven sites throughout the six months of sampling (FIG 2A and FIG 2B), having a relative abundance of 24% to 92%. That this allele dominates *viuB* profiles in the water bodies of Dhaka is not surprising given the likely influence of human intrusion as a major criterion to set dynamics in *V. cholerae* community in Dhaka which could have impact on regular cholera episodes that occurs year-round with two seasonal peaks (Alam et al., 2011). In contrast, data from cholera non-endemic regions revealed that *V. cholerae* population is dominated by other *viuB* alleles and *viuB*-73 was found sporadically at lower relative abundance (~18% of the total *V. cholerae* population) (Kirchberger, 2017).

Three other predominant *viuB* alleles were *viuB*-05, *viuB*-06 and *viuB*-07 (5×10^4 to 1.5×10^5 copies/L) (FIG 4.2.A), which are typical of strains representing a basal long branch clade in whole-genome phylogeny of known *V. cholerae* from around the world (Dorman et al., 2019; Islam et al., 2018). The representative isolates of this clade found to be indistinguishable from *V. cholerae* based on conventional phenotypic tests, but they

were divergent from *V. cholerae* based on phylogenetic and genotypic analysis (https://www.cdc.gov/vibrio/surveillance.html)(Islam et al., 2018). These alleles were especially dominant at site 7 (53.94% of total *V. cholerae*), where *viuB*-73 has the lowest abundance among all sites. This suggests that some form of competition could be taking place between different *V. cholerae* lineages.

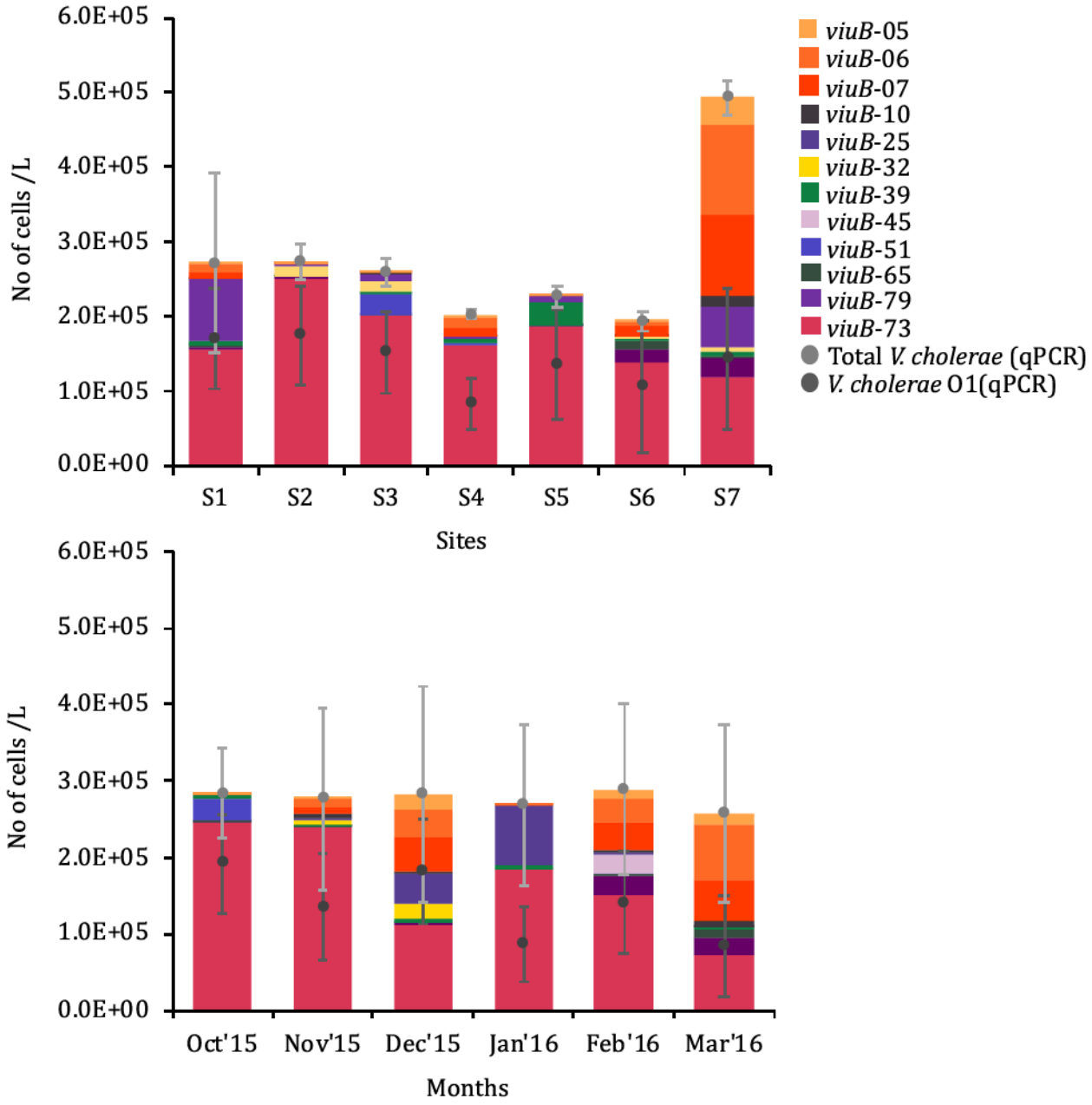

FIG 4.2. Spatial and temporal distribution of *viuB* alleles found in a cholera endemic urban region. Aquatic biomass was extracted from water sample collected from seven different sites around Dhaka, Bangladesh in six consecutive months (Oct 2015 to Mar 2016). Total *V. cholerae* abundance was determined by qPCR amplification of the *viuB* gene marker and used to normalize the number of *viuB* alleles sequences. Light grey dots denote the average count of total *V. cholerae* in each site enumerated by qPCR. Proportions of different *viuB* alleles are denoted with colors specific to each allele. The relative abundance of *viuB*-73, a proxy for PG *V. cholerae* (mostly composed by O1

serogroup strains), was confirmed by qPCR with the amplification of *rfbO1* gene (specific for the O1 serogroup), which is represented by dark grey dots. Error bars represent the standard deviation between abundance in each site. A) Spatial variation of *viuB* alleles. Error bars represent standard deviation between the abundance in each site; B) Temporal variation of *viuB* alleles. Error bars represent standard deviation between the monthly abundance across all sites.

4.4.2. Intraspecies competition could reduce the relative abundance of PG *V. cholerae* O1

Samples analyzed from the seven sampling sites over six consecutive months (Oct' 15 to Mar' 16) indicated the total abundance of *V. cholerae* was relatively stable, varying between 2 to 4 × 10^5 cells/L at six sites (site 1 to site 6) (FIG 4.2.A and FIG D.1.) and around 5 × 10^5 cells/L at the site in the most densely populated part of Dhaka (site 7) (FIG 4.1.., FIG 4.2.A and FIG 4.3.). Little temporal variation was observed, irrespective of sites and total abundance of *V. cholerae* (all sites combined) ranged between 2.5 × 10^5 cells/L to 2.8 × 10^5 cells/L (FIG 4.2.B). Cells belonging to the O1 serogroup were quantified separately and composed between 24 to 92% of the *V. cholerae* population their abundance ranged from 1.2 × 10^5 cells/L to 2.5 × 10^5 cells/L (FIG 4.2.A). They subsisted at every site throughout the six months sampled (FIG 4.4), only showing occasional but sudden reductions in abundance (FIG 4.3). Whenever *viuB*-73 (corresponding to PG O1) lost its dominance, another lineage corresponding to *viuB*-05, *viuB*-06, *viuB*-07, *viuB*-25, *viuB*-45, *viuB*-51 or *viuB*-79 underwent a rapid expansion while the total *V. cholerae* population size remained almost unchanged (FIG 4.3). For example, *viuB*-25 surged when the *viuB*-73 abundance was reduced at sites 1 and 7.

Notably, a new *viuB* allele identified for the first time in this study, *viuB*-79 (genome sequence is not available for the representative strain of this allele because no cultured isolates were found), also appeared when *viuB*-73 abundance was reduced at sites 6 and 7 (FIG 4.4.). All of these alleles showed a negative correlation with *viuB*-73, indicating possible antagonistic interactions between these genotypes (FIG 4.5.).

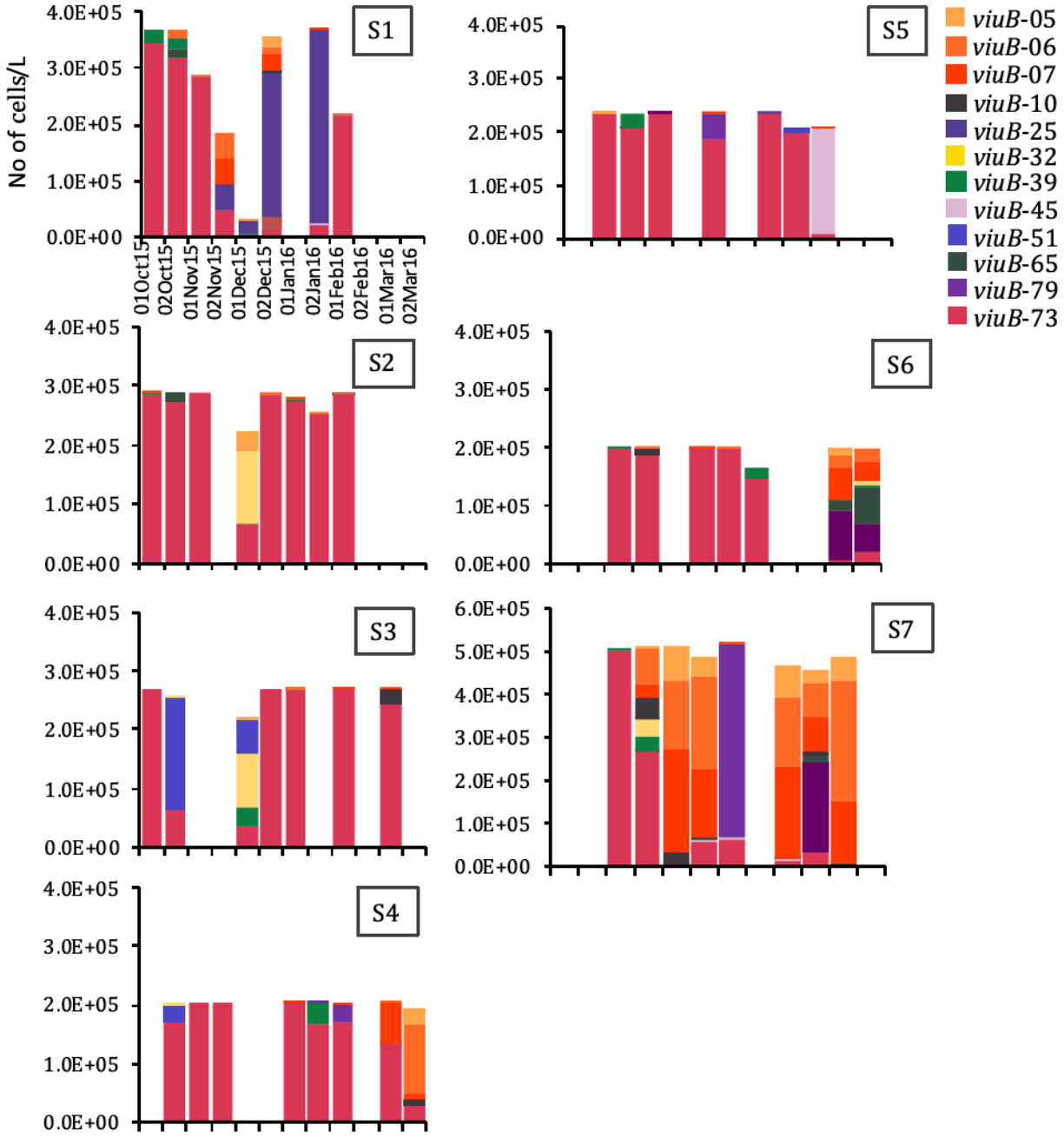

FIG 4.3. Abundance of *V. cholerae* along with the proportion of different *viuB* alleles present in a cholera endemic urban region (Dhaka, Bangladesh). Water samples were collected from seven different sites in six consecutive months (Oct 2015 to Mar 2016). The partial *V. cholerae* specific *viuB* gene was amplified and sequenced from DNA extracted from the biomass of these samples. Relative abundance of *viuB* alleles representing various lineages is presented on a scale of absolute abundance of *V.*

cholerae determined by qPCR. Blank space indicates loss of extracted DNA sample during shipment.

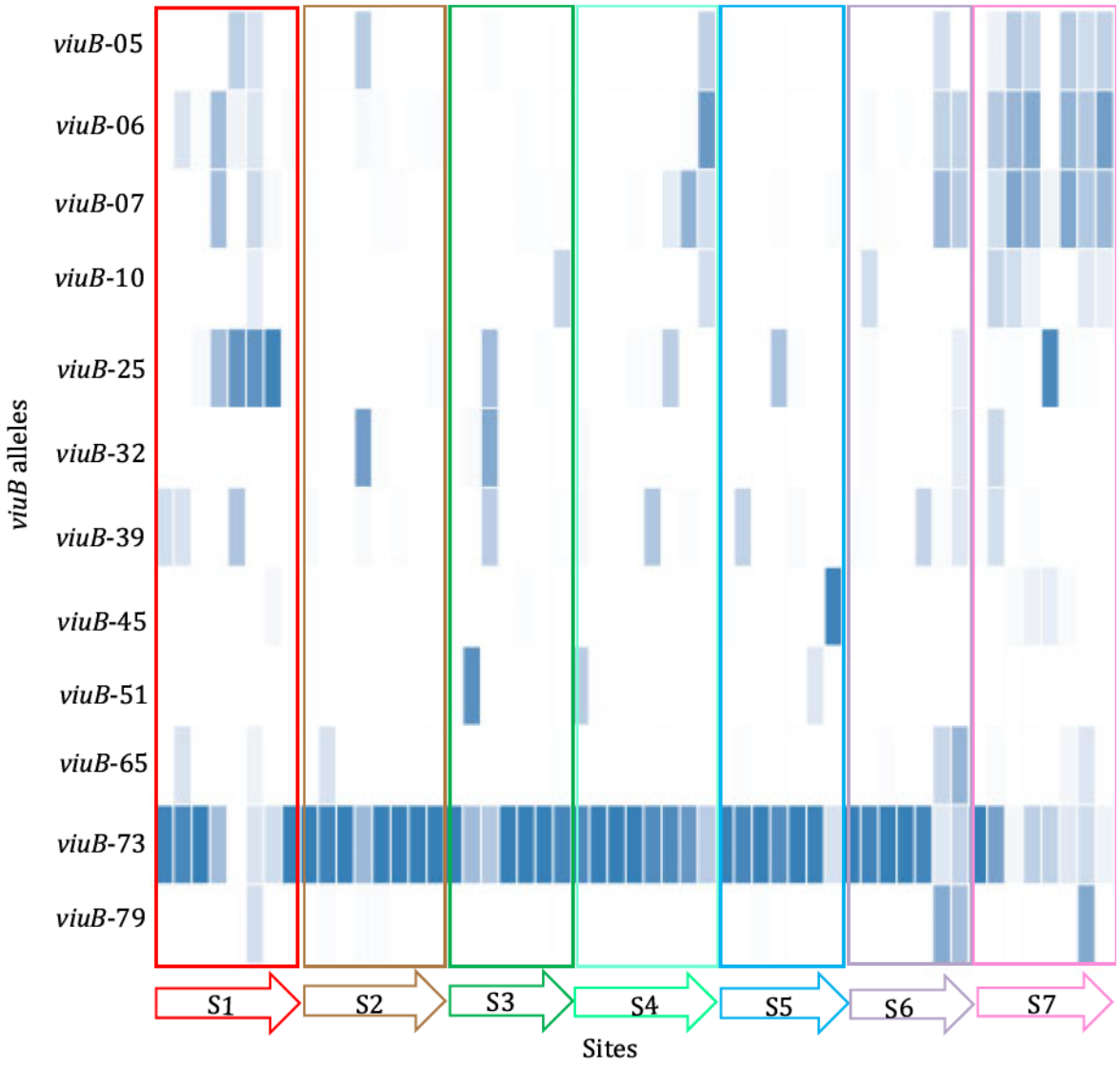

FIG 4.4. Temporal variation of the most abundant *viuB* alleles at seven different sites around Dhaka city. The heatmap illustrates the abundance of the 12 most abundant *viuB* allele at seven different sites (S1 to S7) in Dhaka city from October 2015 to March 2016. Gradients of color (dark to light blue) represent the abundance (high to low).

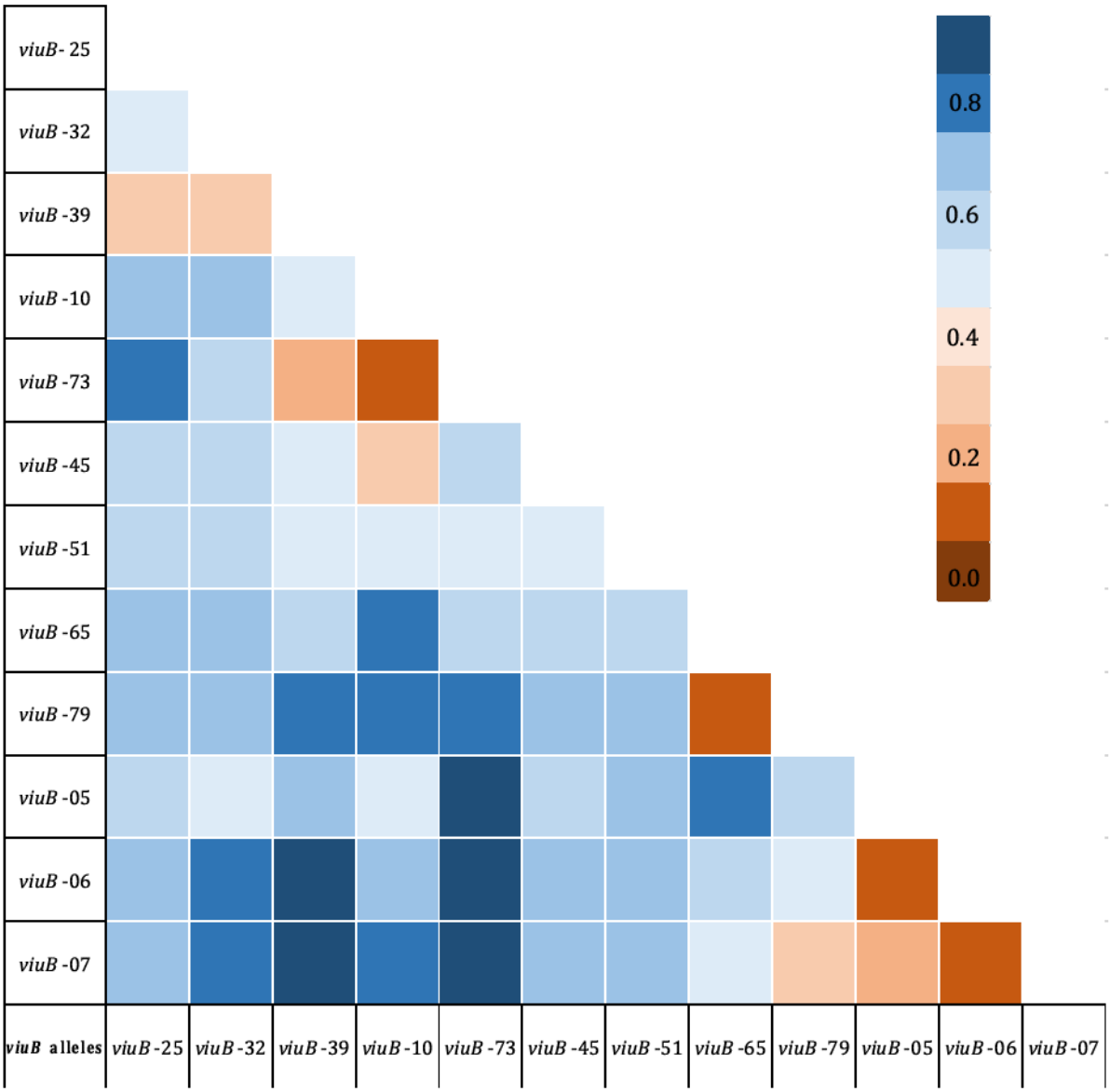

FIG 4.5. Co-occurrence of *viuB* alleles in same sample. The Hopkins statistics (H) was used to assess the clustering tendency of the *viuB* amplicon sequencing dataset. The threshold value was 0.5 which means if $H < 0.5$, data has statistically significant clusters. Visualization of analyzed *viuB* amplicon sequencing data is presented with blue and brown color gradients ($H < 0.5$, indicating that the data is highly clusterable which is shown with brown color gradient and $H > 0.5$, indicating that the data is not clusterable which is described by blue color gradient). Significant clustering shown between *viuB* alleles 05, 06 and 07.

The temporally stable abundance for the species suggests that changes in population composition could be caused by intraspecies competition. Such competition could be direct (mediated by antagonistic mechanisms such as the Type VI secretion system or T6SS, ubiquitous in *Vibrios*)(López-Pérez et al., 2019; MacIntyre et al., 2010) or indirect (due to a differential response in changes in the environment or microbial predation). In aquatic environments, the abundance of *V. cholerae* is often influenced by grazing from heterotrophic protists (Lutz et al., 2013). To overcome the grazing pressure *V. cholerae* executes different strategies such as morphological shift, i.e. from smooth to rugose resulting in the production of VPS (Vibrio polysaccharide) that helps to encase themselves in biofilm and resist predation (Matz and Kjelleberg, 2005). It has been shown in previous studies that *V. cholerae* can survive intracellularly in a range of amoeba which serve as environmental reservoirs and as a vehicle for dispersal in the aquatic environment (Abd et al., 2004, 2005; Thom et al., 1992). Surviving predation from *Dictyostelium discoideum* has been attributed to T6SS, which is used by *V. cholerae* to kill both competing bacteria and eukaryotes (MacIntyre et al., 2010; Pukatzki et al., 2006). The T6SS, ubiquitously found in *V. cholera*, has been hypothesized to play a role in competing with normal gut flora of infected individuals (Fu et al., 2013). This system encodes a syringe-like structure that can pierce cellular envelopes of other bacteria and some eukaryotes, injecting effector proteins that can kill the recipient (Miyata et al., 2013; Pukatzki, 2013; Unterweger et al., 2014). This same phenomenon could influence the population composition of *V. cholerae*, with different lineages in direct competition. Typing of T6SS in *V. cholerae* genomes representative of the *viuB* alleles found in Dhaka indicate that in most cases, genotypes are incompatible (FIG D.2.). This is also

statistically shown in the Hierarchical clustering analysis of amplicon sequencing data (FIG 4.5.) [The Hopkins statistics (*H*)] was used to assess the clustering tendency of the *viuB* amplicon sequencing dataset and the threshold value was 0.5 which means if $H < 0.5$, data has statistically significant clusters, where significant clustering only occurred between *viuB* alleles representing strains from the basal divergent clade (*viuB*-05, *viuB*-06 and *viuB*-07) ($H < 0.5$) and *viuB*-73 showed significant negative correlation with most other *viuB* alleles ($H > 0.5$) (Lawson and Jurs, 1990). The only *viuB* alleles positively correlated with *viuB*-73 was *viuB*-10, which cannot kill strains carrying *viuB*-73 using T6SS, and *viuB*-39 (FIG 4.5. and FIG D.2.), which is a ubiquitous allele present at low abundance across sampling sites (FIG 4.4.). This incompatibility in sub species level likely plays a role in the diversity and dynamic of the *V. cholerae* populations in Dhaka.

As crashes in the PG *V. cholerae* O1 populations appear stochastic, and our sampling took place during a period of low cholera incidence (between the two peaks in the frequency of clinical cholera cases which occur in March to May and September to November), it does not seem likely that changes in fecal input in the water reservoirs would cause these fluctuations. We do not know if PG *V. cholerae* O1 (and other genotypes) are maintained in Dhaka waters through continual fecal contamination by infected individuals or whether it has established itself in this ecosystem. There is some indication, however, that the amount of fecal contamination could increase nutrient loads thus can be impactful on *V. cholerae* abundance in its' habitat (Santo Domingo and Edge, 2010).

4.4.3. The site with the highest surrounding human population density displays the most pronounced changes in *V. cholerae* population density and composition

Although total *V. cholerae* abundance remained relatively stable throughout the six months sampling period within any given site, there was substantial variation among some of the sites. Population composition, for its part, changed temporally within each site and spatially among sites. The most striking differences appeared at site 7, where total *V. cholerae* abundance was close to double than that of other sites, specifically 28% to 43% higher than any other site (FIG 4.2.). Furthermore, unlike sites 1 to 6, which were mainly dominated by *viuB*-73, (FIG 4.3. and FIG 4.4.), three *viuB* alleles (*viuB*-05, *viuB*-06 and *viuB*-07) were relatively more abundant than *viuB*-73 at site 7, especially from Dec'2015 to Mar'2016 (FIG 4.3.). They were only present at low abundance in site 1 and 6 and have not been found in environmental surveys outside of Dhaka. Genomic similarities have been deduced by in-silico DDH and analysis of allelic difference for representative strains of each *viuB* allele. Allelic difference and *in-silico* DDH of whole genome sequences show that organisms represented by these three alleles (*viuB*-05, *viuB*-06 and *viuB*-07) are not more closely related to each other on average than most pairs of *V. cholerae* strains from the main clade would be (FIG D.3.). Despite this lack of genetic similarity, their presence is strongly correlated, as they are usually all absent or present at the same time (FIG 4.2.A and FIG 4.2.B).

Based on the demographic records of Dhaka city, the area surrounding site 7 is the most densely populated (100,000/km^2) among the seven sites studied. Population density at the other six sites ranged from 10,000/km^2 to 60,000/km^2 (Khatun et al., 2017) (FIG 4.1.). Human interference in the water reservoir in this area is expected to be

much higher than other sites, with a possible higher level of fecal contamination and industrial waste mostly from tannery industrial units (Chakraborty et al., 2013). This area is mostly occupied by clusters of slums, inhabited by the low-income population where people use shared facilities, for example showers and toilets. Open defecation has been reported from poorly maintained shared facilities.

Additionally, overpopulation in this area frequently causes an overload of septic tanks, which results in the overflow of untreated effluent to the local environment (Mansour et al., 2017) and due to the open drainage system, mixing between sewage and fresh water is common, increasing the possibility of *V. cholerae* transmission between the environment and human population. the observation of coexistence and high abundance of *viuB*-05, *viuB*-06 and *viuB*-07 at site 7, we hypothesize that bacteria carrying these alleles are associated to some degree with the human gut. Water levels in this area vary seasonally [2.71 to 3.06 m recorded in 2003 -2007 (Thiele-Eich et al., 2015)], the water quality of the reservoir deteriorates during December to April (Chakraborty et al., 2013), coinciding with the rise in the abundance of *viuB*-05, *viuB*-06 and *viuB*-07.

4.4.4. Subspecies level diversity can be influenced by variation in environmental parameters

Studies conducted on the population dynamics of *V. cholerae* in natural habitats have shown that different environmental factors influence their abundance (Takemura et al., 2014). The abundance of *V. cholerae* in aquatic environments was previously found to be significantly influenced by temperature and salinity. *V. cholerae* is found at a wide

range of temperatures (10 to 30 °C), and its abundance decreases with increasing salinity. The highest abundance is observed at >20 °C temperature and between 0-10 ppt of salinity (Takemura et al., 2014). In this study, several environmental variables were measured during sampling (pH, dissolved oxygen, conductivity, total dissolved solids, salinity and water temperature) along with the abundance of the different *V. cholerae* *viuB* alleles found in Dhaka water reservoirs. Dhaka's water is considered freshwater, and the salinity is very low and shows little variability (0-0.8 ppt) (FIG 4.6.). Correlation of environmental parameters with *V. cholerae* population composition were analyzed by envfit function of vegan program in R. The abundance of *viuB*-07 was positively correlated with increasing salinity, whereas *viuB*-73 showed a negative correlation with this parameter (stress 0.13 and $P < 0.01$) (FIG 4.6.). Salinity and conductivity (100 to 1600 µs/cm) were also positively correlated with richness (Pearson correlation coefficient 0.44 and 0.41 respectively; $P < 0.01$), indicating that *V. cholerae* lineage diversity increased with these parameters. Total dissolved solids (TDS) also had a significant impact (Pearson correlation coefficient 0.40; $P < 0.01$) on the richness of different *viuB* alleles representing strains of *V. cholerae* in different sites. In contrast, water temperature and pH did not differ much and did not have a significant effect (FIG 4.7.).

Adaptation to a wide range of salinity levels facilitates *V. cholerae*'s survival in various aquatic environments (from coastal to inland waters) (Brenner, 2005). It was recently found that *viuB*-73 alleles are rarely found in the ocean (Kirchberger, 2017), suggesting that high salinity possibly represents a barrier to the dissemination of cholera. The negative correlation between *viuB*-73 abundance and salinity levels also

demonstrated that PG *V. cholerae* might have a competitive advantage at low salinity. The PG lineage represented by *viuB*-73 may have higher tolerance of rapid variations at low salinities, as it was consistently predominating in all inland sites of Dhaka where salinity levels constantly fluctuated between 0-0.8 ppt.

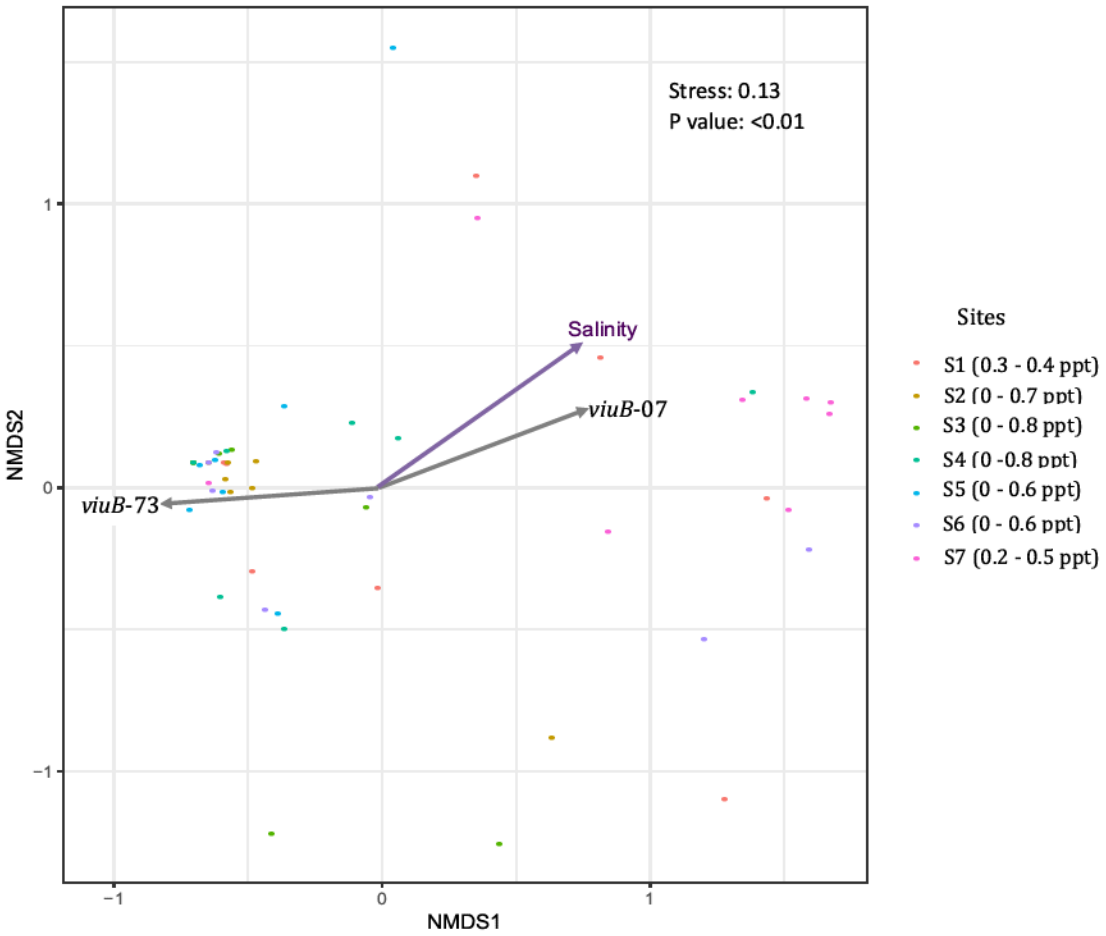

FIG 4.6. Correlation of environmental variables with *V. cholerae* population composition. *viuB* amplicons sequenced from aquatic biomass over six consecutive months collected from seven different sites in Dhaka, Bangladesh were categorized in to twelve lineages. The envfit function of vegan program in R (stress= 0.13 and p<0.01) was used to calculate the dissimilarity of *V. cholerae* populations based on their lineage composition and their correlation with recorded physicochemical parameters. Sites are indicated in different colors.

The only site at which *viuB*-73 was not dominant was site 7 at which salinity was more stable and only varied from 0.4 ppt to 0.5 ppt, never dropping below 0.4 ppt. This suggests that even at the subspecies level, small environmental fluctuations have an influence on population composition. In the low lying Ganges delta, where Dhaka is located, salinity intrusion is considered a major threat due to gradual climate change, resulting in reduced upstream discharge, sea level rise and other catastrophic events such as cyclones (Akter et al., 2019). A salinity increase of about 26% was recorded in coastal regions in Bangladesh over the last 35 years (Mahmuduzzaman et al., 2014). Such a shift in salinity could be affecting the composition of *V. cholerae* populations, possibly changing the distribution and abundance of various lineages, some of which could become a threat to human health.

However, as site 7 also differs from other sites by having a consistently high amount of total dissolved solids (TDS)than other sites, it is difficult to know which variable(s) causes the shift in population composition. The total concentration of dissolved matter in water is defined as total dissolved solids (TDS), that comprise both inorganic salts and a small portion of organic substances. Typically, natural bodies of water have a certain amount of dissolved solids due to the dissolution and weathering of rocks and soil. However, human activities also influence the concentration of TDS in water reservoirs, especially considering Dhaka's inland water bodies where TDS may vary extensively because of urban runoff as well as wastewater discharge. According to WHO guidelines for drinking water quality (WHO, 2004), TDS above 1000 mg/L could be indicative of a pond that has an existing water quality problem. Amongst the seven different sites studied, most of them had TDS concentration on an average of < 300mg/L

except sites 1 and 7, where TDS varied from 308 to 472 mg/L and from 476 to 575 mg/L, respectively. These two sites display the most consistently high levels of TDS. Increase in TDS in a system could have an impact on the growth of particle-associated bacteria, of which *V. cholerae* is a clear example, as over 90% have been found to be particle-associated (Kirchberger, 2017). The increase in available nutrients at higher TDS concentrations may have an impact on *V. cholerae* richness which describes the allelic diversity (FIG4.7.). As Dhaka sites are predominately enriched with waste disposal, we can hypothesize that this could potentially affect consistent retention and circulation of PG lineage representatives as well as other lineages.

As particle/host attachment of *V. cholerae* is dependent on temperature, increases in temperature above 22 °C can be impactful and contribute to changes in the lineage composition of a *V. cholerae* population when a seasonal change occurs (Kirchberger, 2017). However, we did not observe any significant changes in *viuB* allele diversity associated with temperature fluctuations (Pearson correlation coefficient 0.1) (FIG 4.7.). Water temperatures remained high (27.4 °C to 30.8 °C) throughout the sampling period, a pattern that differs dramatically from seasonally different regions where shifts in temperature are noticeable during summer and winter (Jiang and Fu, 2001). It is likely that at consistently high temperatures such as those found in a tropical region like Bangladesh, *V. cholerae* is not overly responsive to this parameter, either in terms of its growth rate or particle attachment behavior.

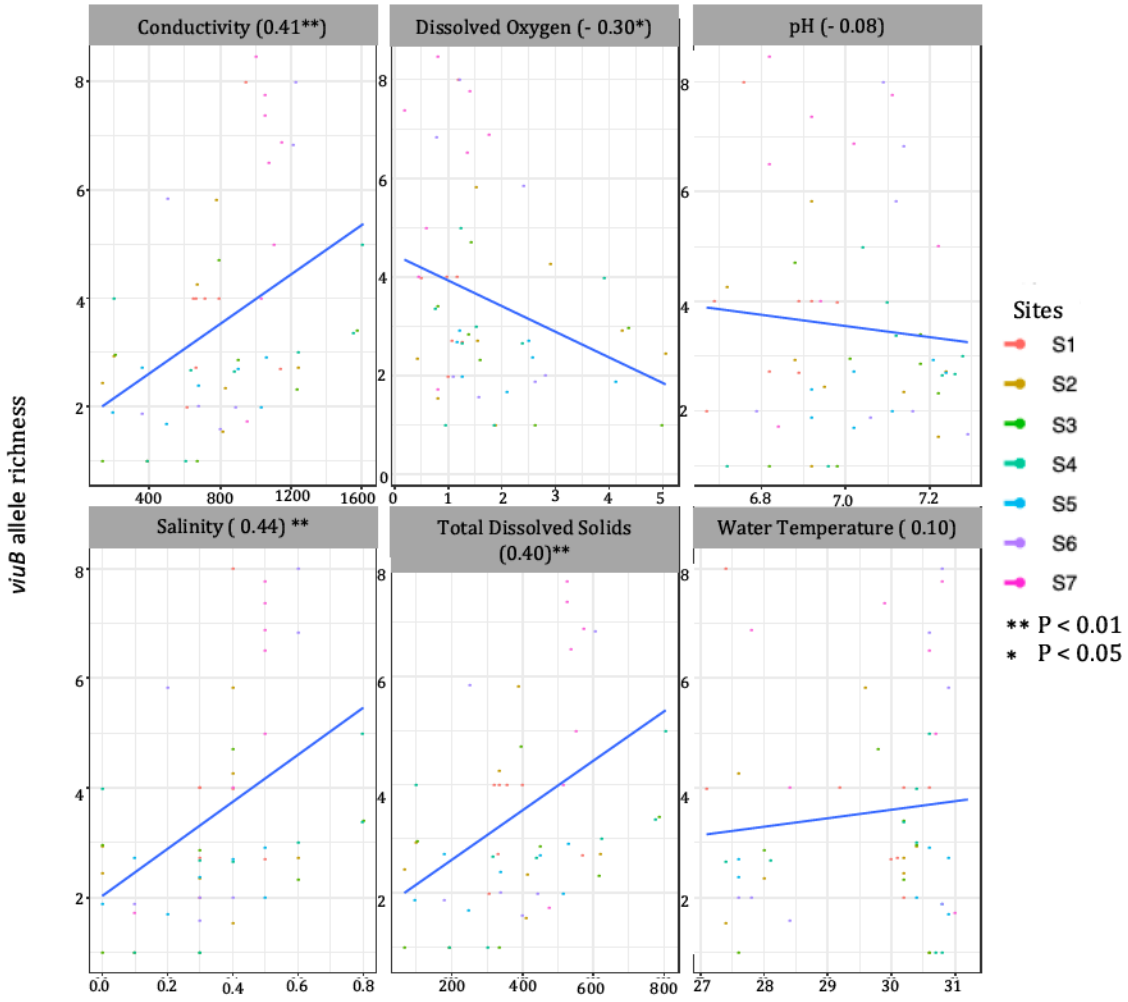

FIG 4.7. Impact of environmental variables on *viuB* allele richness (based on abundance of *viuB* alleles). Correlation test was performed between *viuB* allele richness and each environmental variable in R. Significance of each test was calculated and mentioned on top of each panel. Each scatter plot shows a different environmental variable (x axis) versus rarefied allele number (y axis). Pearson's correlations are given, and significance indicated (*P<0.05 and **P<0.01). Sites are indicated in different colors.

Dissolved oxygen (DO), a measure of free non-compound oxygen present in an aquatic system, was negatively correlated (Pearson correlation coefficient -0.30, P< 0.05) with *V. cholerae* diversity based on richness estimates at all seven sites of Dhaka city (DO range 0.26 – 5.05 mg/L). A study in Hood Canal, Washington, USA, demonstrated that

there was a strong negative correlation between bacterial richness and DO (Spietz et al., 2015). So far, there are no studies describing the correlation between *V. cholerae* diversity and DO, but it was demonstrated earlier that *V. cholerae* is most abundant in low DO environments (Blackwell and Oliver, 2008; León Robles et al., 2013). As *V. cholerae* is known to be a facultative anaerobe, and it is possible that some strains live better than others at lower oxygen concentrations, leading to reduced diversity at higher concentration of DO. Additionally, DO concentration is also inversely correlated with TDS, which could also explain the reduced diversity at high DO.

4.5. CONCLUSION

The Ganges delta has been a reservoir for pandemic and non-pandemic *V. cholerae* lineages for centuries. Cholera endemicity is usual in this area, and Dhaka is one of the most densely populated inland megacities in the world, with cholera epidemics occurring biannually. Environmental water bodies in seven different sites around Dhaka city were analyzed biweekly for six consecutive months. In this study, the *V. cholerae* PG lineage was consistently found in Dhaka's water bodies at a considerably higher proportion of the total *V. cholerae* population, suggesting it is established in that environment.

Moreover, subspecies level resolution revealed that other *V. cholerae* lineages were coexisting with PG *V. cholerae* O1 in Dhaka waters. Intraspecies competition with these other lineages can decrease PG *V. cholerae* O1 dominance, especially when environmental parameters, such as consistently high TDS (possibly from fecal contamination) or salinity could favor another lineage. Variability of these parameters,

even on a small scale, affected *V. cholerae* population composition and richness, with more temporal stability of TDS and salinity at one site leading to a dramatically different lineage composition. The same site also displayed the highest human population density in Dhaka, which could be the cause for the stability of these parameters, but also a possible source of contamination altering *V. cholerae* population composition. Consistent interaction of people with waterbodies and mixing up sewage with these waterbodies suggest that human gut may also serve as a potential reservoir for PG O1 and other lineages of *V. cholerae,* resulting in year-long persistence of *V. cholerae* belonging to these lineages through the transmission cycle between human and aquatic environment.

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CHAPTER 5

CHAPTER 5

Discussion

5.1. Culture-independent quantitative analysis is essential for an accurate description of *V. cholerae* populations in aquatic environments

In a viable but non-culturable (VBNC) state, bacteria have very limited metabolic activity, no cell division, and cannot be cultured by standard methods. This state is caused by unfavorable conditions such as nutrient limitation, temperature shifts, changes in osmotic pressure, oxygen, and light conditions (Baker et al., 1983; Fakruddin et al., 2013; Li et al., 2014; Oliver, 2005). In the VBNC state, the cells are morphologically smaller and can remain in this stage for over a year. Many bacteria such as *E. coli*, *Listeria monocytogenes*, *Campylobacter jejuni*, *V. vulnificus* including *V. cholerae* demonstrate this feature to escape adverse environmental conditions (Lindbäck et al., 2010; Thomas et al., 2002; Xu et al., 1982). As this state makes culture-based studies of bacterial populations unreliable, it can affect environmental monitoring, investigation and control of infectious disease burden (Thandavarayan 2014).

V. cholerae, a facultative human pathogen and common inhabitant in brackish and estuarine waters, where they are often associated with a wide range of aquatic phytoplankton and zooplankton. In many studies, it was shown that *V. cholerae* enters into a state of restrained metabolic activity when exposed to low temperature and nutrient deprivation conditions (Carroll et al., 2001; Chaiyanan et al., 2007).

In this research, the second chapter describes scenarios where *V. cholerae* O1 was not detected by the traditional culture method but detected by a molecular technique,

qPCR. With the use of species-specific primers for *V. cholerae,* it was possible to quantify their abundance in two geographical locations: Oyster Pond, Falmouth, MA, USA, a non-endemic site for cholera, and Bangladesh, a cholera endemic site. From this extensive investigation of aquatic biomass, I showed that there was substantial temporal and spatial variation in *V. cholerae* abundance, especially in the locations where significant seasonal variation occurred year-round. We found consistent presence of the O1 serogroup of *V. cholerae* determined by the *rfbO1* gene marker in Oyster Pond during the warmest month of the summer and also it was mostly detected in the largest size fraction (>63 µm). Moreover, there was not any O1 serogroup strain detected by conventional culture method. Failure of isolation by culture-based methods often causes misinterpretation of the existence of *V. cholerae* O1 in environmental reservoirs because of their VBNC state or low abundance (Bliem et al., 2015; Sultana et al., 2018). Thus, the absence of *V. cholerae* O1 isolates in 2009 samples collected from Oyster Pond which were detected by qPCR indicate the presence of VBNC cells which is also indicative that non-toxigenic *V. cholerae* O1 might not be completely absent in cholera-free region (Islam et al., 2013).

Analysis of samples collected from cholera-endemic regions of inland and coastal Bangladesh also demonstrated the consistent presence of *V. cholerae* O1 by qPCR. In the inland region, which is densely populated, I observed the persistent presence of toxigenic *V. cholerae* O1 throughout six months (October 2015 to March 2016) as a relatively high proportion of total *V. cholerae*. These data suggest that both genes (*ctx*A and *rfb*O1) were found in the same cells, which is typical of 7th pandemic *V. cholerae* El Tor strains currently responsible for most cholera cases in Bangladesh (Chun et al.,

2009). Water bodies around this area thus serve as a reservoir for toxigenic strains at a dangerous level (1.5×10^5 to 4×10^5 CFU/L), which can cause potential outbreaks when ingested with water or food. The infectious dose of *V. cholerae* is from 10^3 to 10^8 bacteria (Schmid-Hempel and Frank, 2007).

5.2. A field-ready method of quantitation of bacteria is key to outbreak prediction

Another goal of the research conducted in this thesis was to develop a method which can be applied in the field to monitor *V. cholerae* abundance in environmental reservoirs, especially in the area where cholera has a consistent and persistent pattern of epidemics.

The Crystal® VC dipstick assay is one of the most widely used field ready methods, which detects the presence of *V. cholerae* O1 and O139 antigens in both water and stool samples through an immunochromatographic method (Sinha et al., 2012). This is a rapid and inexpensive method, but the limit of detection is 10^7 CFU of enriched culture that is higher than the usual infectious dose of cholera (Schmid-Hempel and Frank, 2007). Also, this method requires 6 hours of enrichment before testing, and the output is only qualitative. Because of compromised sensitivity, it is recommended for use in combination with the bacterial culture method (Rashid et al., 2017a). Other assays, for example, the Direct fluorescent antibody (DFA) technique using polyclonal anti-O1 serum facilitate detection of *V. cholerae* O1 in smears prepared from samples. This procedure has been used for both clinical and pre-enriched environmental samples (Hasan et al., 1994; Xu et al., 1984). Although these assays are useful for cholera diagnosis, pre-enrichment of sample is often needed, and the interpreted result is

qualitative. Furthermore, these assays are optimized for the screening of clinical specimens.

The developed qPCR method discussed in Chapter 3 is a field-ready method aiming to monitor the causative agent of cholera in inadequate infrastructure and limited resource settings. The instrument used (Chai Open qPCR) was chosen for its small footprint and easy setup features, while the streamlined protocol facilitates the processing and analyzing of water samples on site without the need of pre-enrichment or DNA extraction. In this method, the water sample was concentrated 50 folds to increase the sensitivity and lower the limit of detection to overcome the limitation of direct quantification due to the lower number of bacteria present in environmental water (Huq et al., 1990). The assay was designed to obtain maximum sensitivity [as of MIQE guideline (Bustin et al., 2009)], as low as 3 copies per reaction. The limit of detection for concentrated water samples was therefore 6×10^3 CFU/L, which is below the infectious dose for toxigenic *V. cholerae,* which can be as low as 10^3 cells, and far below that for non-O1 strains, which is 10^6 cells (Kothary and Babu, 2001). It is possible to determine absolute abundance of both *V. cholerae* O1 and non-O1 simultaneously in this assay, which is useful to delineate the proportion of pandemic *V. cholerae* in a geographic location and thus helpful to predict any risk of an outbreak. As proof of concept, we used this assay to analyze water samples collected from the Turag river from an endemic cholera region, the Gabtoli area, Dhaka, Bangladesh and were able to determine the proportion of *V. cholerae* and *V. cholerae* O1 precisely. We showed that almost 13% of total *V. cholerae* ($\sim 4 \times 10^4$ CFU/L) in that particular location belonged to the O1 serogroup, which is not surprising because in a previous study based on the Crystal VC®

dipstick test, 30% of water sources used by cholera patient households were contaminated with *V. cholerae* (George et al., 2016; Rashid et al., 2017a).

The qPCR-based technique developed here could be beneficial to monitor *V. cholerae* abundance along with its toxigenic serogroup and thus provide information to the local residents to be aware of any form of contamination that could lead an outbreak of cholera.

5.3. High-throughput protein-coding gene amplicon sequencing combined with qPCR is useful to describe population dynamics at the subspecies level

I extended my research beyond simple detection and quantification and conducted a detailed study of the *V. cholerae* population structure for six consecutive months with water samples collected from surrounding waterbodies of Dhaka, a densely populated capital city (>18 million as of 2016) (https://populationof2019.com/population-of-dhaka-2019.html) of Bangladesh. I chose this location because Dhaka is known to have a hyperepidemic level of cholera distinguished by biannual peaks during the spring and fall just before and after the monsoon (Alam et al., 2011). In Chapter 4 we combined two methods to investigate diversity and abundance of *V. cholerae.* High throughput amplicon sequencing of the species-specific gene, *viuB,* provided us the opportunity to explore diversity at the subspecies level and quantification of this marker gene by qPCR added information on abundance. Here we observed that in Dhaka's water reservoir, the prevalence of pandemic generating (PG) *V. cholerae* lineage dominates characteristic *viuB* allele as *viuB* 73 corresponding to the PG lineage (Kirchberger, 2017) were found to be present in all

seven sites throughout the six months of sampling. Other predominant *viuB* alleles were *viuB*-05, *viuB*-06 and *viuB*-07 (5×10^4 to 1.5×10^5 copies/L); these alleles are typical in long branch strains in a whole genome phylogeny of known *V. cholerae* isolate collection from worldwide, most involved in clinical cases (Islam et al., 2018). Local population density and improper waste disposal may play a vital role in the circulation of PG *V. cholerae* in this particular region. This study also provided vital information on subspecies level diversity in this cholera endemic region, which was never done before.

Studies on interspecies competition strategies explained that bacterial species can coexist or compete with each other for the same resources by diverse mechanisms that result in emergence or decline of specific microbial lineages in the natural population. Intraspecies competition and cooperative behaviors are also observed in the bacterial community (Hibbing et al., 2010). Generation of different niche specialized *Pseudomonas fluorescens* variants were reported in static culture in which one of the variants overproduces extracellular polysaccharide (EPS) that enables it to float to the surface and thus gain access to oxygen (Rainey and Travisano, 1998). One classic experiment with three different variants of *E. coli* depending on production and resistant to the molecule colicin demonstrated that these variants can exist in the well-structured environment where they create their niches. In contrast, when they are in a mixture, variant which is resistant to the colicin but unable to produce it quickly dominate and out-compete the others (Kerr et al., 2002).

Interestingly, my study highlighted that intraspecies competition could limit the levels of specific *viuB* alleles as the total *V. cholerae* abundance remained the same, but the proportion of *viuB* alleles varied temporally and spatially. In several instances, *viuB*-

73, representing the pandemic lineage, lost its dominance and other lineages experienced a rapid expansion. This stochastic change of *viuB*-73 could be correlated with the intermediate period between two peaks of cholera outbreak based on clinical cases because the samples investigated in this chapter were collected mostly during the dry season when PG O1 is usually low in number as they have tendencies to go transition to the VBNC stage (Colwell, 2000).

The T6SS, which is described as a syringe-like structure consisting of effector and immunity proteins serves as a survival tool for *V. cholerae* (Miyata et al., 2013; Pukatzki, 2013; Unterweger et al., 2014) and possibly plays a role in this intraspecies competition. Further investigation through typing of T6SS that correspond to *viuB* alleles from the available database showed that their effector-immunity protein composition was different from each other. This may result in the incompatibility of most strains, which was also demonstrated statistically by Hierarchical cluster analysis to identify clusters (*viuB* alleles) with the closest distance based on the abundance data. In this study (Chapter 4), clustering only existed between *viuB* alleles representing long branch strains/isolates (Hopkins correlation coefficient, H <0.5) and *viuB* 73 showed a significant negative correlation with other *viuB* alleles (Hopkins correlation coefficient H >0.5). Thus, it can be predicted that intraspecies competition can be led by T6SS incompatibility, which ultimately influences the dynamics of *V. cholerae* population.

5.4. Rapid urbanization and change in environmental parameters could be impactful on subspecies level diversity

In this research, I noticed that surrounding human population density could affect the dynamics of *V. cholerae* populations, as I observed higher abundance in total *V. cholerae* with notable variation in subspecies level diversity at site 7 in Dhaka. This location of Dhaka city is reported as one of the most densely populated and industrialized areas. Unlike the other six sites explored in Dhaka city, this location mainly serves as a reservoir for *viuB*-05, *viuB*-06 and *viuB*-07 that correspond to representative members of a divergent clade associated with human infections (Islam et al., 2018). Variation in *V. cholerae* populations at the subspecies level has never been explored before. Variable water levels due to seasonal changes usually result in mixing of sewage and fresh water around this area as there are many open drainage systems giving the opportunity for consistent circulation of dominant *viuB* alleles in this location or facilitating transmission from the environment to humans.

Moreover, I also examined whether there was any correlation between changes in environmental parameters and population dynamics of *V. cholerae* at the subspecies level. There have been many studies that describe the impact of environmental parameters on *V. cholerae*. Temperature, salinity and pH had a substantial effect on growth and multiplication of toxigenic *V. cholerae* O1. In the same study, attachment to planktonic crustaceans such as copepods was also influenced by these environmental factors (Huq et al., 1984). Maximum growth of *V. cholerae* and attachment to copepods were demonstrated at 30 °C, pH 8.5 and salinity of 15% (Huq et al., 1984). Here we

recorded pH, dissolved oxygen (DO), conductivity, total dissolved solids (TDS), salinity and water temperature for six months during Dhaka sites study period (Chapter 4).

Interestingly, *viuB*-73 (corresponding to the PG lineage) abundance did not change due to the small fluctuations in salinity as it was found in all seven sites in consistence abundance. In contrast, little variation in salinity found at site 7 seemed favorable for the other three alleles (*viuB*-05, *viuB*-06 and *viuB*-07). As I sampled mostly in the dry season, where the temperature shift was limited (ranged from 28° C to 33.4° C), I did not find any correlation amongst temperature change and changes in abundance or diversity. However, TDS (476 to 575 mg/L) was consistently higher at site 7 and could have been advantageous for *viuB* 05, *viuB* 06 and *viuB* 07. It was demonstrated earlier that *V. cholerae* is more abundant in low DO environments (4.9 – 8.5 mg/L)(León Robles et al., 2013). Negative correlation to DO was also described in a study of North Carolina estuaries (Blackwell and Oliver, 2008). Here we also noticed a negative correlation between DO (DO range in Dhaka sites 0.26 – 5.05 mg/L) and *V. cholerae* abundance (Pearson's correlation coefficient, -0.30).

5.5. Co-occurrence of *V. cholerae* and its closely related species *V. metoecus*

An extensive molecular-based study of the samples from the historical Continuous Plankton Recorder archive revealed that gradual increase s in sea surface temperature changed the dynamics of ocean planktonic community over the last 50 years (Vezzulli et al., 2012). The abundance of *V. cholerae* increased amongst the other representative bacteria of the family Caulobacteraceae, Hypphomicrobiaceae, Bradyrhizobiaceae, Rhizobiaceae, Methylobacteraceae, Rhodobacteraceae, Rhodospirillales, Holosporaceae,

Alteromonadales, Pseudomonadaceae, Aeromonadaceae which were ubiquitous in the same environment, the North Sea, where increased incidence of bathing infection has been reported (Vezzulli et al., 2012). Narrowing it down to sea food contamination, *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* are often co-isolated from clam or other raw and undercooked seafood (Passalacqua et al., 2016; Robert-Pillot et al., 2014).

V. metoecus is a recently described species, which has been co-isolated with *V. cholerae* from the USA East Coast aquatic environment and associated with eel fish in Spain, as well as clinical cases from around the USA (Boucher et al., 2011; Carda-Diéguez et al., 2017; Kirchberger et al., 2016; Kirchberger et al., 2014). This was the first study to track the abundance of *V. metoecus* in aquatic environments (Chapter 2). In temperate and tropical aquatic environments around the world, *V. cholerae* is found to be a ubiquitous member of bacterial communities (Kaper et al., 1995). However, *V. metoecus* was neither isolated by conventional culture method nor detected by qPCR in Bangladesh (Chapter 2). We suggest that *V. metoecus* may have a different geographical distribution than *V. cholerae.* In a previous study, with a culture-based approach, *V. cholerae* was ten times more abundant than *V. metoecus* in the aquatic environment of USA East Coast (Kirchberger et al., 2016). In Chapter 2, I found that *V. cholerae* was approximately three times more abundant than *V. metoecus* based on the qPCR approach. In both studies, *V. cholerae* was more abundant than *V. metoecus* although they coexist. Interestingly *V. metoecus* was only found during the warmer months of the summer (June and July) which was similar to the previous observation (Kirchberger et al., 2014) based on culture method that described the impact of seasonal variability.

5.6. Future Research

There are several ways to extend my investigations, which could strengthen the findings described in this thesis. We have a extensive collection of isolated strains of *V. cholerae* and *V. metoecus* from the USA East Coast aquatic environment. So far, *V. metoecus* was found to be the closest species to *V. cholerae*. As they coexist in the same environment, horizontal gene transfer could occur between them (Orata, 2017). As *V. metoecus* has been isolated from clinical cases in the USA, we can speculate that in future there would be a chance of emergence of more virulent *V. metoecus.* To investigate this hypothesis, we could identify the genes responsible for *V. metoecus* pathogenicity; therefore, it will be useful to design an assay that can facilitate the screening of *V. metoecus* strains. In a previous study, *V. cholerae* was ten times higher in abundance than *V. metoecus* in the environment based on culture method (Kirchberger et al., 2014) which was again proven in the chapter 2 by quantitative analysis (qPCR) that *V. cholerae* was approximately three times higher than *V. metoecus* which is indicative that chances of acquiring genes from *V. cholerae* is higher. Also, the inclusion of *V. metoecus* screening in any cholera surveillance study would provide additional information on the geographical distribution of *V. metoecus* as this species was so far isolated only in USA and Europe (Choopun, 2004; Kirchberger et al., 2014), and not found in cholera endemic regions of Bangladesh.

In this thesis (Chapter 2 and 3) I explored environmental aquatic samples mostly from the cholera-endemic region of Dhaka, Bangladesh. In this area, cholera exists for over a century and displays biannual peaks of outbreaks that were recorded based on clinical cases occurring just before and after the monsoon season. In this study, I mostly

analyzed samples collected in between peak seasons. From the analysis, it was found that whether or not cholera outbreak was there at that period, pandemic generating *V. cholerae* O1 was always in the water reservoir around Dhaka city at a dangerous level. It would be ideal to execute a yearlong study with the methods developed here to monitor the abundance of total *V. cholerae* and toxigenic *V. cholerae* O1 at the same time, providing valuable information on the circulation and fluctuation of these two strains in the same environmental reservoir. These data would inform our understanding of the factors that drive the two annual seasonal peaks of cholera in this region as well as prove or disprove the assumption that there is a peak in abundance of toxigenic *V. cholerae* before outbreaks.

Moreover, amongst the seven sites around Dhaka city included in this study (Chapter 4), I noticed the interesting distribution of *V. cholerae* genotypes based on *viuB* allele scheme in site 7. The *viuB*-73 allele (corresponds to PG O1) was found at all seven sites but site 7 was specifically predominated by three other *viuB* alleles (*viuB*-05, *viuB*-06 and *viuB*-07). In a previous study these three *viuB* alleles corresponded to the representative strain of a divergent clade which was named as long branch strain (Domman et al., 2018; Islam et al., 2018). We have a collection of isolates from each of these sites. Genome-based study of these isolates could yield information on these three *viuB* alleles carrying isolates. I hypothesize that dense urbanization and massive industrialization could have an influence on shaping the diversity of *V. cholerae* populations. Further investigation of the local population and usage of the water reservoirs residing in the same location could be helpful to track these variants in the

human gut. It will be beneficial to know if the human population is serving as a natural carrier.

In Chapter 4, I discussed the role of T6SS on the incompatibility of coexistence of *V. cholerae* at subspecies level. In this study I analyzed the T6SS of *V. cholerae* corresponding to the *viuB* alleles (detected in Dhaka water reservoirs) from the available database and found that in most cases, the effector-immunity composition was different. We have isolated strains in our collection from this study which would be useful in future to study the outcomes of co-culture in a competition assay, generally performed to interpret coexistence or non-coexistence in the form of killing matrix.

5.7. Conclusion

In this thesis, I developed and optimized different methods that could be important in the study of cholera. In the second chapter, I optimized a multiplex qPCR system where I was able to detect and quantify *V. cholerae* and *V. metoecus*, a closely related species of *V. cholerae* from environmental biomass simultaneously. It was also possible to know toxigenic *V. cholerae* O1 abundance at the same time. In Chapter 3, I developed a field-ready qPCR method and tested water samples collected from an endemic site of cholera. Development of this method gives us an opportunity to measure the abundance of total *V. cholerae* and *V. cholerae* O1 in the same assay on site that would be immensely useful to monitor the burden of pathogenic *V. cholerae* in any water reservoir. Surveillance can inform residents of a predicted outbreak of cholera and take measures to limit the spread of cholera. Lastly, in Chapter 4, I investigated subspecies level diversity and the proportion of each genotype with a combined method of high

throughput amplicon sequencing and qPCR. This assay allows us to gain knowledge about the diversity of *V. cholerae* in the environmental aquatic reservoir with subspecies level resolution which plays an important role to understand any shift in local *V. cholerae* population due to intraspecies competition or variation of environmental factors and also impactful for finding new variant. Altogether, research performed in this thesis has added valuable information on the population of *V. cholerae* as well as *V. metoecus* in their aquatic habitats.

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APPENDICES

APPENDIX A

List of publications

Refereed Publications

1. **Nasreen T**, Case R and Boucher Y (2013). Lateral gene transfer and Microbial Diversity. In **Encyclopedia of Metagenomics,** edited by Karen E. Nelson, SpringerReference.com

Publications (in preparation)

1. **Nasreen, T.**, Hussain, N.A.S., Islam, M.T., Orata, F.D., Kirchberger, P.C., Alam, M., Yanow, S.K. and Boucher, Y. Simultaneous quantification of *Vibrio metoecus* and *Vibrio cholerae* with its O1 serogroup and toxigenic Sub-populations in environmental reservoirs (prepared for submission to Applied and Environmental Microbiology in June 2019)

Author contribution: Y.B. and T.N. designed the project and wrote the manuscript. M.T.I and M.A. helped in sample collection and sample processing during field trips in Bangladesh. T.N. performed all the experimental procedures. S.K.Y. helped in trouble shooting of any experimental problem. F.D.O performed the bioinformatics analysis for *V. metoecus*. P.C.K. helped to find out the unique gene for *V. cholerae*. N.A.S.H., M.T.I., F.D.O., P.C.K., M.A. and S.K.Y. reviewed the manuscript. S.K.Y. and Y.B. supervised the project.

2. **Nasreen, T.**, Alam, M., Yanow, S.K. and Boucher, Y. Assay for evaluating the abundance of *Vibrio cholerae* and its O1 serogroup subpopulation directly from water without DNA extraction (prepared for submission to the Journal of Microbiological Methods in July 2019)

Author contribution: Y.B., T.N. and S.K.Y. designed the project. Y.B. and T.N. wrote the manuscript. M.A helped in sample collection during field trips in Dhaka, Bangladesh. T.N. performed all the experimental procedures. S.K.Y. helped in trouble shooting of any

experimental problem. M.A. and S.K.Y. reviewed the manuscript. S.K.Y. and Y.B. supervised the project.

3. **Nasreen, T.**, Islam, M.T., Liang, K., Johura, F.T., Alam, M. and Boucher, Y. Intraspecies interactions and changes in intrinsic environmental factors influence the prevalence of pandemic *Vibrio cholerae* in an urban region endemic for cholera [in preparation for submission in Frontiers in Microbiology (Infectious Diseases) in August 2019, abstract has been accepted on February 15, 2019]

Author contribution: Y.B., T.N. and M.A. designed the project. Y.B. and T.N. wrote the manuscript. M.T.I, F.T.J. and M.A. helped in sample collection and sample processing during field trips in Dhaka, Bangladesh. T.N. performed the qPCR and M.T.I did the amplicon sequencing. T.N. and M.T.I. did the analysis. K.L. helped in bioinformatics analysis. M.T.I., K.L., F.T.J. and M.A. reviewed the manuscript. M.A. and Y.B. supervised the project.

4. Kirchberger, P.C., Orata, F.D., **Nasreen, T.**, Kauffman, K.M. Case, R.J., Polz, M.F. and Boucher, Y. Culture-independent tracking of *Vibrio cholerae* at strain level resolution reveals complex spatio-temporal dynamics in a natural population. (submitted in Environmental Microbiology in June 25, 2019)

Author contribution: M.F.P. and Y.B. designed the project. K.M.K. and Y.B. did sampling. P.C.K. developed all methods. P.C.K. and T.N. performed *viuB* sequencing. P.C.K. did *viuB* sequencing analysis. F.D.O. performed whole-genome sequencing and analysis. T.N. performed qPCR. P.C.K., R.J.C. and Y.B. wrote the manuscript. Y.B. supervised the project.

5. Orata, F.D., Liang, K., **Nasreen, T.**, Hussain, N.A.S., and Boucher, Y. Differences in abundance and seasonal patterns of *Vibrio cholerae* and *Vibrio metoecus* lead to a directional bias in interspecies horizontal gene transfer.

Author contribution: F.D.O. and Y.B. designed the project and wrote the manuscript. Y.B. did sampling. F.D.O. and T.N. did the DNA extraction and prepared samples for sequencing. F.D.O. performed whole-genome sequencing and assembly. F.D.O. and K.L. performed bioinformatic analysis. T.N. performed qPCR. F.D.O. and N.A.S.H. performed the natural transformation assay. Y.B. supervised the project.

6. Islam, M.T., **Nasreen, T.**, Kirchberger, P.C., Liang, K., Johura, F.T., Orata, F.D., Alam, M. and Boucher, Y. Intra-species diversity in geographically distinct *V. cholerae* populations.

Author contribution: Y.B. and M.A. designed the project. Y.B. and M.T.I. wrote the manuscript. M.T.I, F.T.J., T.N. and M.A. did sample collection and sample processing in Dhaka, Bangladesh. T.N. performed the qPCR. M.T.I and T.N. did the *viuB* amplicon sequencing. M.T.I. did the *viuB* amplicon sequencing analysis. M.T.I. did whole-genome sequencing. M.T.I. and K.L. performed bioinformatics analysis. M.T.I., T.N., P.C.K., F.D.O. and M.A. reviewed the manuscript. M.A. and Y.B. supervised the project.

APPENDIX B

Supplementary data for chapter 2

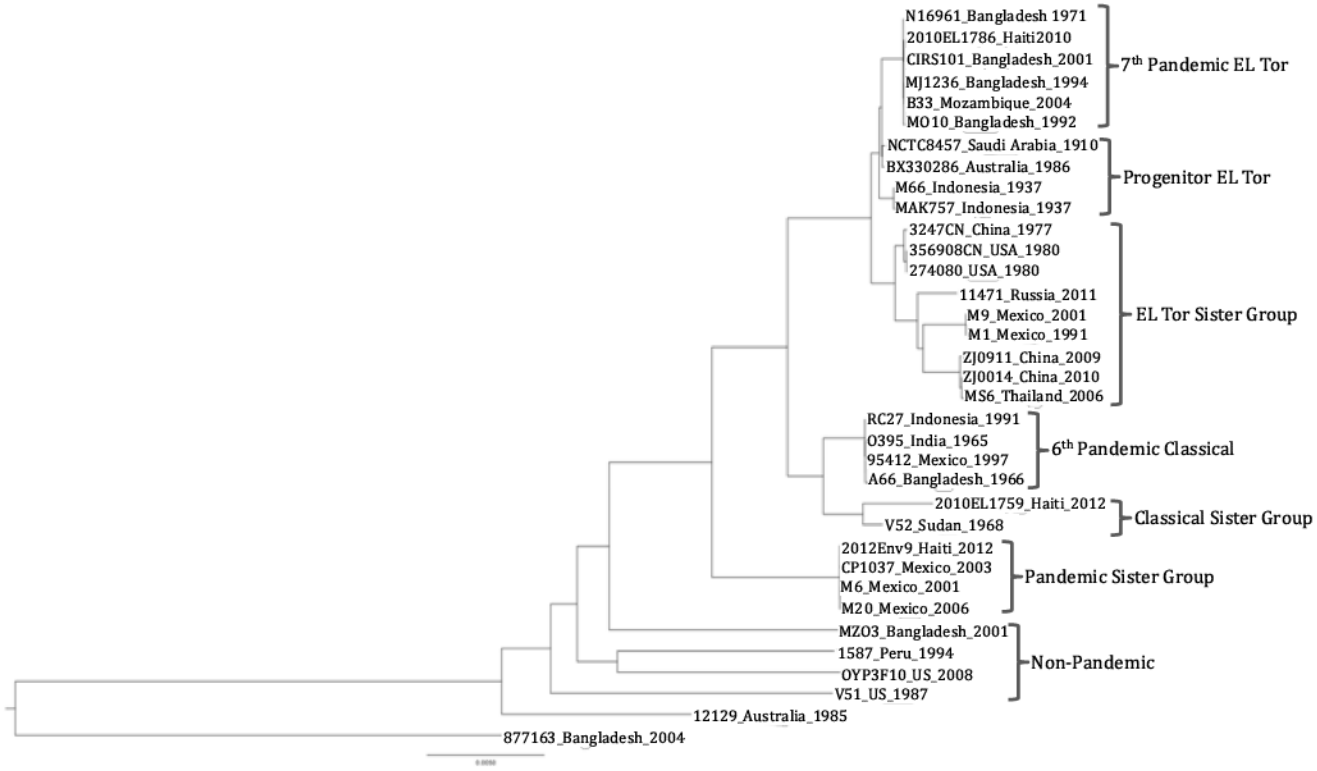

FIG B.1. Phylogeny of the pandemic generating lineage of *V. cholerae*. The maximum likelihood phylogenomic tree was constructed from the alignment of locally collinear blocks (2,367,372 bp) using GTR gamma substitution model with 100 bootstrap replicates (all nodes had >98% bootstrap support). Environmental strain *Vibrio sp.* strain 877-163 was used to root the tree. The source and isolation year of the strains used are indicated next to the strain names.

TABLE B.1. Bacterial strains used for validating primers and probes in this study

Species	No. of strains	Strain	Classification	Accession number*
V. cholerae				
V. cholerae non O1	20	OYP1G01	Environmental	NMTO00000000
		OYP2A12	Environmental	NMTN00000000
		OYP2E01	Environmental	NMTK00000000
		OYP3B05	Environmental	LBGB00000000
		OYP3F10	Environmental	NMTJ00000000
		OYP4B01	Environmental	NMTI00000000
		OYP4C07	Environmental	LBGE00000000
		OYP4G08	Environmental	NMTH00000000
		OYP4H06	Environmental	NMTG00000000
		OYP4H11	Environmental	NMTE00000000
		OYP6D06	Environmental	NMTC00000000
		OYP6E07	Environmental	NMTB00000000
		OYP6F08	Environmental	NMTA00000000
		OYP6F10	Environmental	NMSZ00000000
		OYP7C09	Environmental	NMSX00000000
		OYP8C06	Environmental	NMSV00000000
		OYP8F12	Environmental	NMSU00000000
V. cholerae O1	8	N16961	O1/O139 group, Clinical	AE003852: AE003853
		V52	non O1/non O139, Clinical	MIPN00000000
		EDC-728	O1/O139 group, Environmental	NA
		EDC-753	O1/O139 group, Environmental	NA
		EDC-754	O1/O139 group, Environmental	NA
		EDC-755	O1/O139 group, Environmental	NA
		EDC-772	O1/O139 group, Environmental	NA
		EDC-805	O1/O139 group, Environmental	NA

Species	No. of strains	Strain	Classification	Accession number*
Other *Vibrio* species				
V. parahaemolyticus	1	ATCC 17802	Type strain, Clinical	CP014046: CP014047
V. vulnificus	3	ATCC 27562	Type strain, Clinical	CP012882: CP012881
		CVD7	Environmental	NA
		A9	Environmental	NA
V. metoecus	18	RC341	Environmental	ACZT00000000
		OP3H	Environmental	JJMN00000000
		OYP4D01	Environmental	LBGO00000000
		OYP4E03	Environmental	NMST00000000
		OYP5B04	Environmental	LBGP00000000
		OYP5B06	Environmental	LBGQ00000000
		OYP5H08	Environmental	NMSR00000000
		OYP8G05	Environmental	NMSQ00000000
		OYP8G09	Environmental	NMSP00000000
		OYP8G12	Environmental	NMSO00000000
		OYP8H05	Environmental	NMSN00000000
		OYP9B03	Environmental	NMSM00000000
		OYP9B09	Environmental	NMSL00000000
		OYP9C12	Environmental	NMSK00000000
		OYP9D03	Environmental	LBGR00000000
		OYP9D09	Environmental	NMSJ00000000
		OYP9E03	Environmental	NMSI00000000
		OYP9E10	Environmental	NMSH00000000
V. mimicus	3	ATCC 33653	Type strain, Clinical	NA
		ATCC 33654	Type strain, Environmental	CP014042: CP014043
		ATCC 33655	Type strain, Clinical	AOMO00000000
Other bacterial species				
Escherichia coli	3	CU1	Clinical	NA
		CU2	Type strain	NA
		MSU1	Clinical	NA
Pseudomonas aeruginosa	3	12A1	Clinical	NA
		PA103	Type strain, Clinical	JARI00000000
		PA14	Clinical	CP000438

* Accession number obtained from Genebank

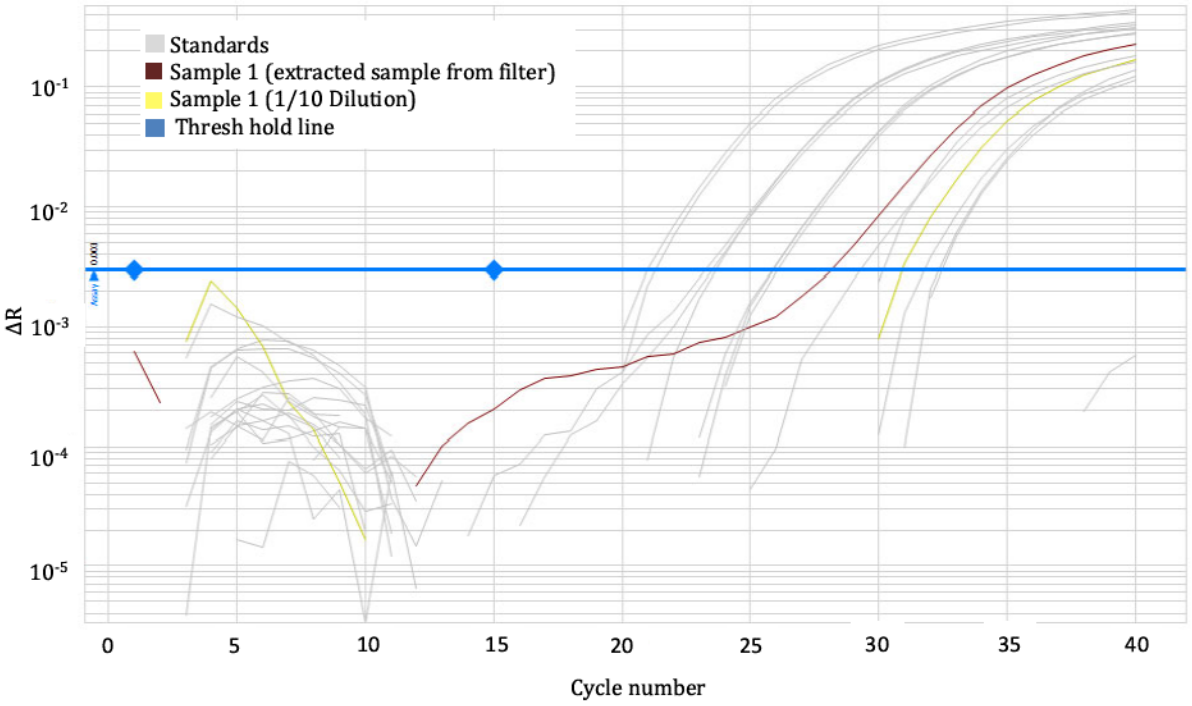

FIG B.2. Testing of inhibition in qPCR amplification. The qPCR assay was performed on an extracted DNA sample treated with One step PCR inhibitory removal kit (sample1) from Oyster Pond large fraction size (> 63 µm) and a 10× dilution. Standards (3×10^4 copies to 3 copies per reaction indicated with grey colored lines) were run in the same experiment. ΔR, normalized reporter value. The difference in Cq values of the sample and 10× dilution was 3.3 ± 0.05, indicating no inhibition.

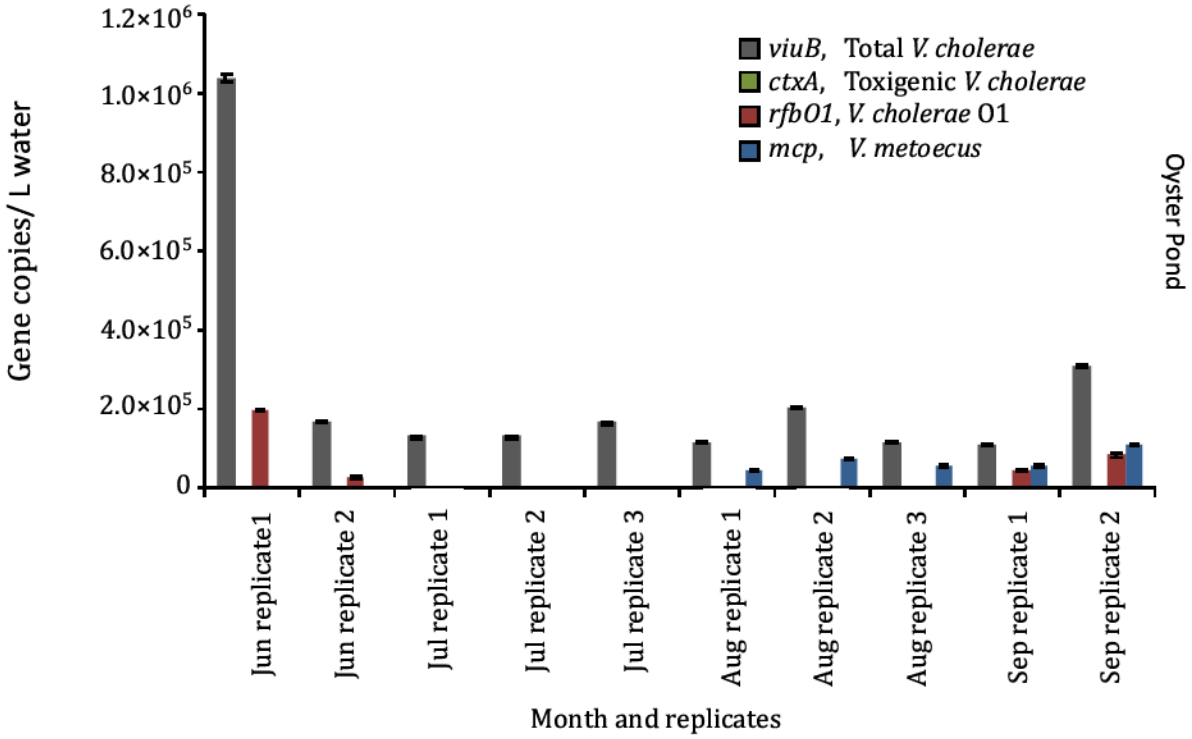

FIG B.3. Biological replicates of the quantification of *V. cholerae* along with its toxigenic and serogroup O1 subpopulations and its close relative *V. metoecus* in Oyster Pond, MA, USA. Environmental water samples were collected in two or three replicates during the months of June to September in 2008. Each replicate represents 50 ml filtered independently from the same 10 L sample as its matching replicate. Bacteria were quantified by qPCR of marker genes. The *viuB* gene was used to quantify total *V. cholerae*; *ctxA* and *rfbO1* were used to measure toxigenic *V. cholerae* and *V. cholerae* O1, respectively; the abundance of *V. metoecus* was quantified using the *mcp* gene. Each qPCR reaction was run in triplicate. Mean values are shown with error bars indicating the standard deviation between technical replicates.

APPENDIX C

Supplementary data for Chapter 3

Table C.1. Determination of limit of detection of sample after concentrating step and testing inhibition in the assay

No of copies/1mL sample	No of copies/500µL sample	No of copies/10µL concentrate	Cq values with STDEV of Replicates				
			Replicate 1	Replicate 2	Replicate 3	Mean(Cq)	STDEV
3000	1500	1500	27.01	27	27.02	27.01	0.01
300	150	150	30.2	30.04	30.2	30.15	0.08
30	15	15	33.5	33.7	33.5	33.57	0.09
3	1.5	1.5					
6	3	3	35.8	34.74	35.9	35.48	0.52
5	2.5	2.5	36.74	36.87			

'Blank' indicates no amplification

APPENDIX D

Supplementary data for Chapter 4

TABLE D.1. Genomes representative to *viuB* alleles found in Dhaka, Bangladesh used to construct the phylogenetic tree and DDH analysis

Name of the isolate	*viuB* type	Phylogeny	Country of Origin	Accession number
EDC_690	6	LB	Bangladesh	NA
Vc_229135	6	LB	Spain	SRR7062498
EDC_716	7	LB	Bangladesh	NA
EDC_717	7	LB	Bangladesh	NA
Vc_2016V_1091	5	LB	Unknown	GCA_003312065.1
Vc_PNUSAV000170	5	LB	USA	SRR6456907
Vc_236140	10	Non-PG	India	SRR7062513
Vc_229143	10	Non-PG	India	SRR7062619
Vc_YB2H11	39	Non-PG	USA	GCA_003349365.1
Vc_YB1A01	39	Non-PG	USA	GCA_001402185.1
Vc_EDC_800	45	Non-PG	Bangladesh	NA
Vc_EDC_688	45	Non-PG	Bangladesh	NA
Vc_BD21	32	Non-PG	Bangladesh	GCA_003348245.1
Vc_BD23	32	Non-PG	Bangladesh	GCA_003348215.1
Vc_L11	51	Non-PG	Sweden	GCA_001718105.1
Vc_NHCC-008D	65	Non-PG	Bangladesh	GCA_000348425.2
Vc_493492	65	Non-PG	Thailand	SRR7062586
Vc_EDC_754	73	El Tor	Bangladesh	NA
Vc_EDC_755	73	El Tor	Bangladesh	NA
Vc_BD04	25	Non-PG	Bangladesh	GCA_003348485.1
Vc_BD34	25	Non-PG	Bangladesh	GCA_003348055.1

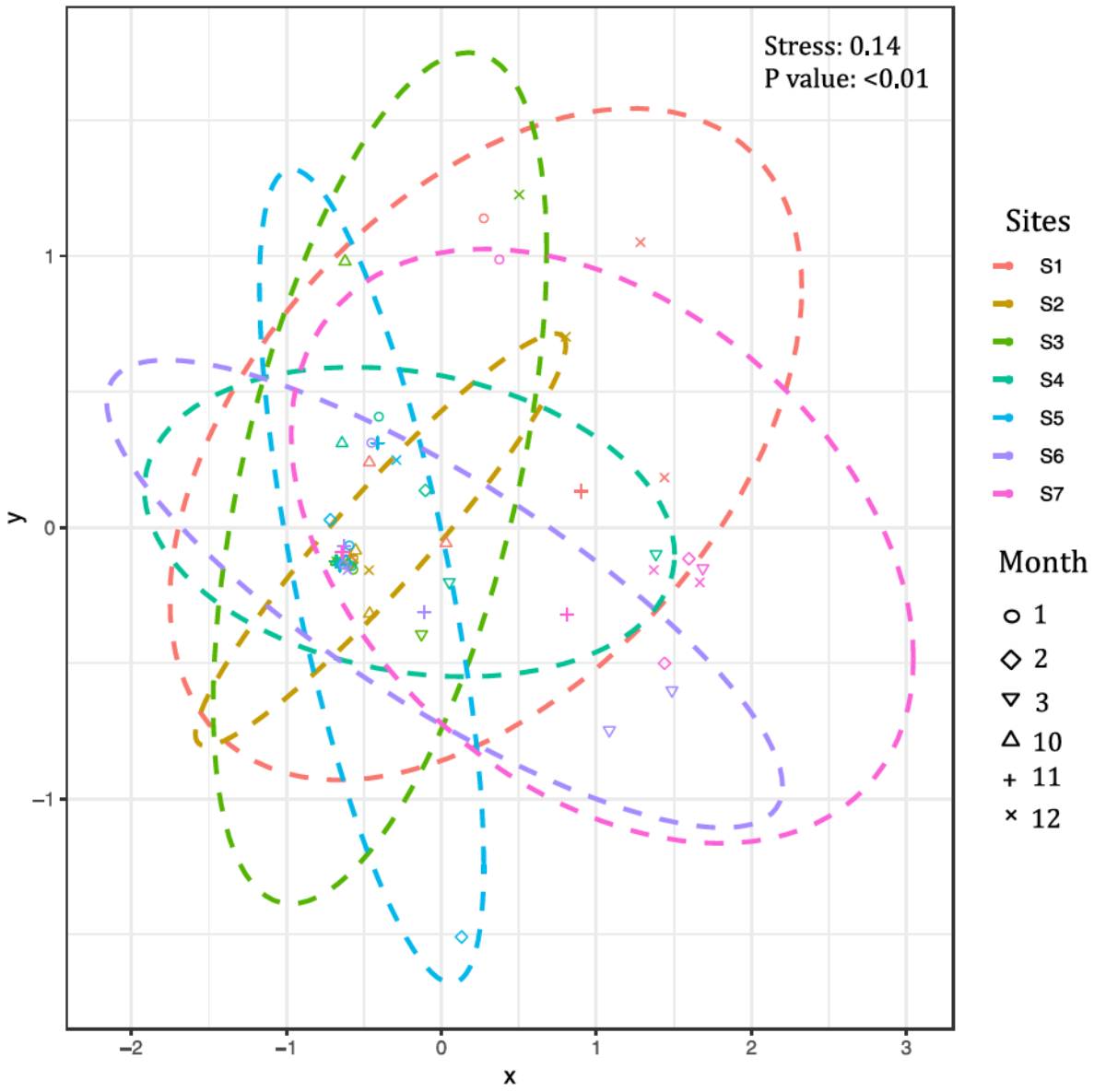

FIG D.1. The non-parametric monotopic relationship between dissimilarities in the sample matrix based on abundance of different *viuB* alleles found in seven sites in six consecutive months plotted in low dimensional space (stress= 0.13 and p< 0.01).

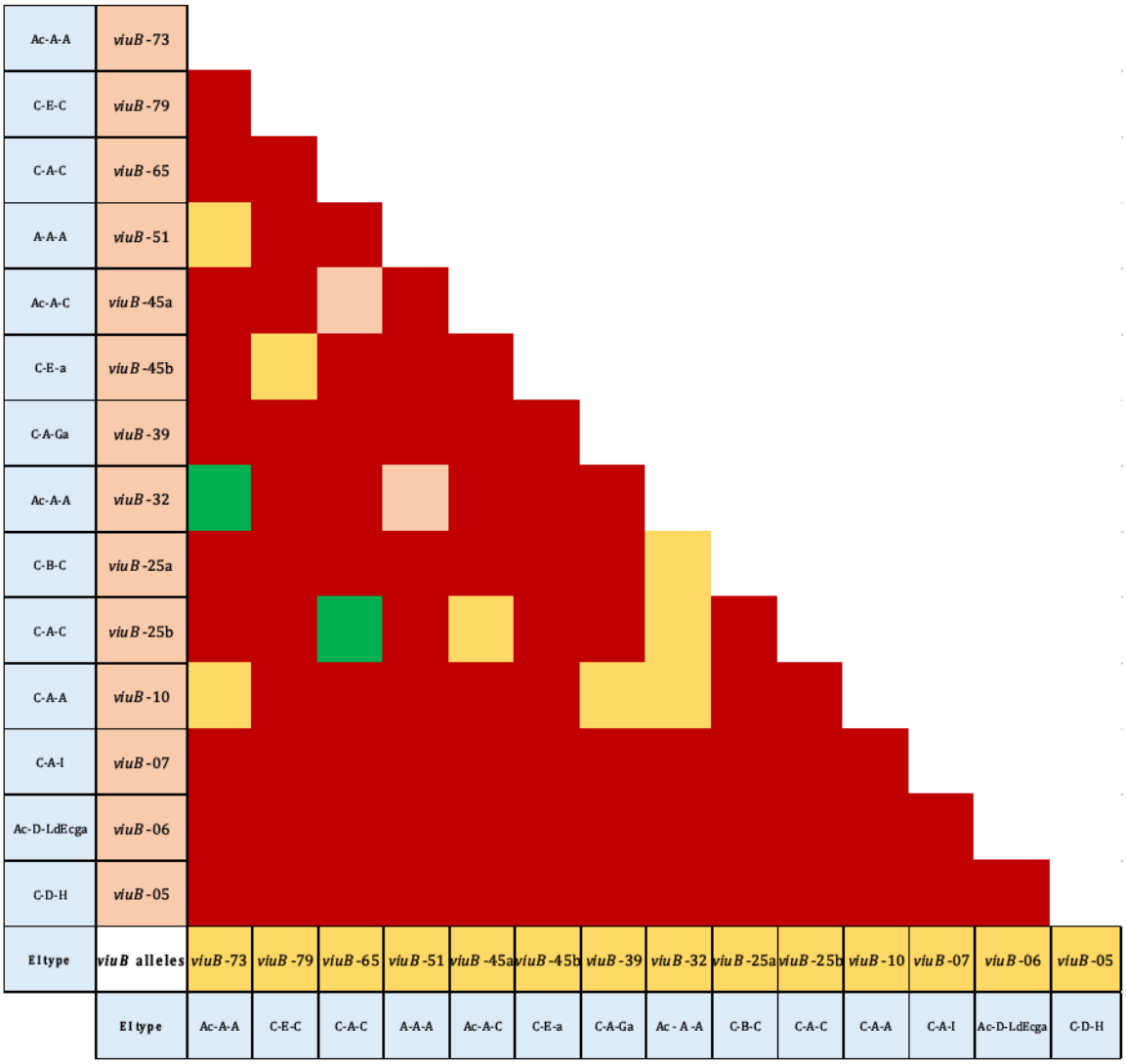

FIG D.2. Incompatible existence of *V. cholerae* corresponds to *viuB* alleles identified from environmental biomass in Dhaka. Killing matrix generated from hypothetical interaction between effector-immunity protein composition in T6SS identified in *V. cholerae* corresponding to *viuB* alleles. Self-comparison was ignored.

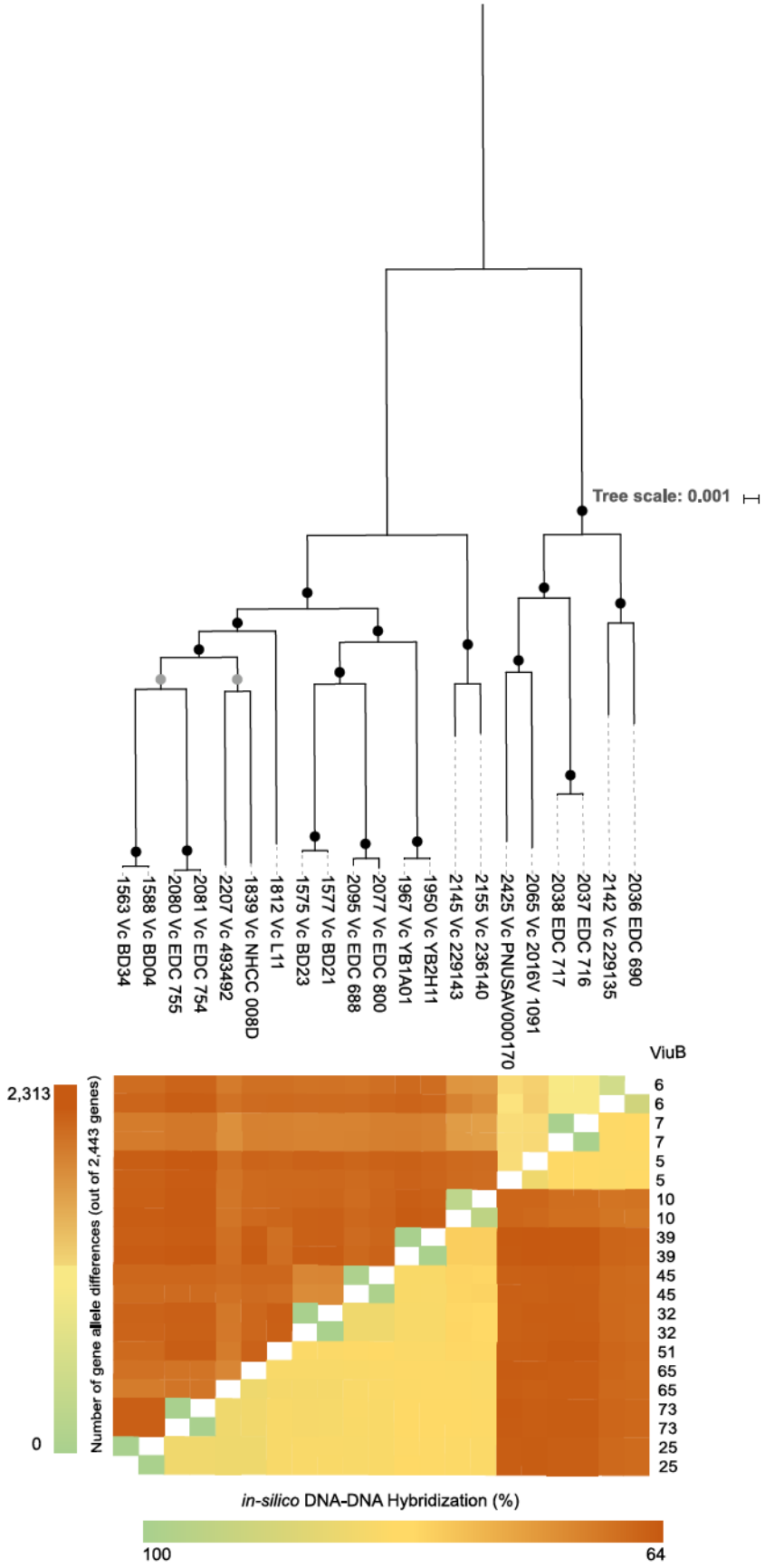
Tree scale: 0.001
1563 Vc BD34
1588 Vc BD04
2080 Vc EDC 755
2081 Vc EDC 754
2207 Vc 493492
1839 Vc NHCC 008D
1812 Vc L11
1575 Vc BD23
1577 Vc BD21
2095 Vc EDC 688
2077 Vc EDC 800
1967 Vc YB1A01
1950 Vc YB2H11
2145 Vc 229143
2155 Vc 236140
2425 Vc PNUSAV000170
2065 Vc 2016V 1091
2038 EDC 717
2037 EDC 716
2142 Vc 229135
2036 EDC 690
ViuB
6
6
7
7
5
5
10
10
39
39
45
45
32
32
51
65
65
73
73
25
25
2,313
0
Number of gene allele differences (out of 2,443 genes)
in-silico DNA-DNA Hybridization (%)
100
64

FIG D.3. Clear genomic and phylogenetic distinction between long branch *viuB* and other *viuB* alleles. RaxML was used to reconstruct the phylogenetic tree of at most two representative strains for each major *viuB* allele found in Dhaka under the GTRGAMMA model with 100 bootstrap replicates. Allelic differences were shown on the top of the matrix and *in-silico* DDH values on the bottom. Self-comparisons are ignored. *viuB*-73 (on the lower half of the phylogenetic tree) was commonly associated with the pandemic El Tor lineage. *viuB*-05, *viuB*-06 and *viuB*-07 were clearly distinct from the rest of the *viuB* alleles as they form a clear monophyletic clade by themselves. Allelic difference and *in-silico* DDH data also showed a clear boundary between isolates from these *viuB* alleles and isolates from other *viuB* alleles. *viuB*-79 is not included in this analysis as no genomes were availability.