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Assay for Evaluating the Abundance of *Vibrio Cholerae* and Its O1 Serogroup Subpopulation Directly from Water Without DNA Extraction

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Abstract: Cholera is a severe diarrheal disease caused by *Vibrio cholerae*, a natural inhabitant of brackish water. Effective control of cholera outbreaks depends on prompt detection of the pathogen from clinical specimens and tracking its source in the environment. Although the epidemiology of cholera is well studied, rapid detection of *V. cholerae* remains a challenge, and data on its abundance in environmental sources are limited. Here, we describe a sensitive molecular quantification assay by qPCR, which can be used on-site in low resource settings directly on water without the need for DNA extraction. 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 genome copies per reaction. The limit of detection is as low as 5×10^3 genome copies/L of water after concentrating biomass from the sample. The ability to perform qPCR directly on water samples, portable features of the equipment, stability of the reagents at 4°C and user-friendly online software facilitate fast quantitative analysis of *V. cholerae*. These characteristics make this assay extremely useful for field research in resource-poor settings and could support continuous monitoring in cholera endemic areas.

Keywords: cholera; *Vibrio cholerae*; *Vibrio cholerae* O1; endemic; toxigenic; abundance and qPCR

1. Introduction

Cholera is a life-threatening diarrheal disease caused by pathogenic strains of *Vibrio cholerae*. Today, cholera still persevere as a global threat to public health due to its high morbidity and mortality rates [1-5]. There were around 250,000 suspected cholera cases and over 3,500 deaths reported in 2021 [6] and an estimated 2.9 million cases and 95,000 deaths occur each year around the world [7]. Lack of access to safe drinking water and inadequate management of sanitary systems in resource-poor countries are two major reasons this disease remains a significant public health problem. Current data on the global disease burden of cholera identify 47 countries around the world affected by cholera, and over half a billion people reside in areas that are labelled as cholera hotspots [8]. Cholera outbreaks in recent years affecting Haiti, Somalia, Ethiopia, Iraq, Mozambique, Zambia, Sudan, Nepal and Zimbabwe demonstrated that devastation, from an earthquake, for example, can cause an outbreak [9]. In 2018, the epidemic in Yemen was reported to be the world's fastest-growing outbreak, where 10,000 suspected cases were

reported on a weekly basis [10]. The cumulative impact of cholera in Yemen currently sits at a death toll of nearly four thousand since October 2016, with over 2.5 million people infected so far [11].

V. cholerae is naturally found worldwide, especially in brackish riverine, coastal and estuarine environments [12]. Not all *V. cholerae* present in nature are pathogenic, and only a subset of strains is known threat to humans. Amongst the 200 serogroups of this species, only O1 and O139 are associated with cholera cases (toxigenic serogroups) and are responsible for epidemic and pandemic cholera outbreaks [13-15].

Estimation of *V. cholerae* abundance, along with that of its pandemic generating serogroups [16] in aquatic ecosystems is difficult, because of the high spatio-temporal variability exhibited by its natural populations [17].   Epidemiological studies and analysis of cholera outbreaks revealed that the disease occurs in a regular seasonal pattern in cholera-endemic areas [18-20] 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 [21-24].

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 [25-28], but the disease rapidly spread after the 2010 earthquake which devastated infrastructure in the country and with the arrival of United Nations' (UN) troops carrying the bacteria from Nepal [28,29]. The country then faced a decade of the cholera epidemic before the disease faded in 2019 – 2020 [30]. Because of these epidemiological dynamics, cholera has been categorized as an emerging and re-emerging infectious disease [31,32].

In addition, surveillance of cholera outbreaks through clinical diagnosis provides an estimation of the associated disease burden but it is unable to provide quantitative information of the pathogen or its abundance in source environments. To achieve a better understanding of the cholera prevalence and to direct appropriate control measures and treatment, it is necessary to identify and promptly quantify its causative agent in its reservoir. However, one of the major obstacles 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 [33].

Conventional culture methods remains the gold standard for laboratory diagnosis of cholera [34,35] and often requires pre-enrichment of the sample [36]. Moreover, two to three days are required to conduct testing even with modern laboratory infrastructure. With these methods, isolation and identification are possible, but the total abundance quantified is often underestimated, as a considerable proportion of most populations exists as viable but non-culturable cells (VBNC), which do not revive upon culturing on microbiological media [36,37]. In areas with limited or no laboratory facilities, simple dark-field microscopy is used to detect characteristic movement of *V. cholerae* in stool samples but this method is not feasible on water sources where the bacterium is much more dilute [38]. The Crystal® VC Rapid Diagnostic Test (RDT) is also used for point-of-care detection to predict potential cholera outbreaks where this diagnostic test uses stool sample and is mainly based on detection of either cholera toxin [39,40] or a lipopolysaccharide antigen [39,41,42]. It is useful to detect serogroups O1 and O139. However, due to the poor sensitivity and specificity of this method, additional tests are required to confirm the presence of toxigenic *V. cholerae* in stool sample [39]. 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 [36,43]. Despite these methods facilitating cholera diagnosis, most of them are qualitative, only detect *V. cholerae* O1 or O139 serogroup and have been optimized for clinical but not environmental specimens.

To improve cholera surveillance, it is essential to accurately determine the abundance of the total and disease-causing serogroups of *V. cholerae* in its reservoir. To achieve this goal, we designed two species-specific primers and probes to precisely detect and quantify total *V. cholerae* and *V. cholerae* O1 serotype directly from environmental water samples

without DNA extraction. Employing the Chai Open qPCR equipment – a low cost, porta-ble qPCR machine – we demonstrate the potential for our designed set of qPCR primers to be utilized during field sampling. In-field quantification of prokaryotic and eukaryotic constituents of interest using Open qPCR have been recently performed on recreational waters in Alberta and present comparable results to conventional qPCR equipment [44]. Altogether, this method can determine the absolute abundance of this bacterium and the main serogroup associated with pathogenicity across hundreds of samples in a spatio-temporal gradient, making it possible to pinpoint the source of cholera outbreaks and warn of potential outbreaks.

2. Results

2.1. Analytical validation

The qPCR assay was validated by using a blind panel of filter-sterilized environmen-tal water samples collected from the Gabtoli area (Dhaka, Bangladesh) (FIGURE 1) and spiked with *V. cholerae* reference strains.
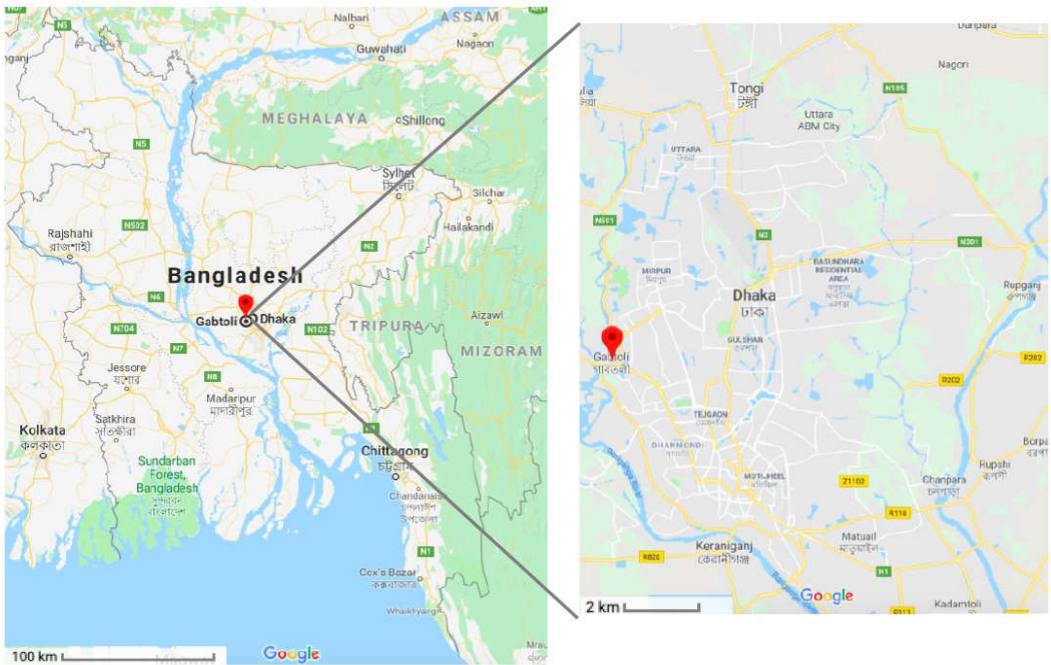

Figure 1. Environmental water sampling sites in Bangladesh. An endemic site for *V. cholerae* is shown on the map of Bangladesh. Environmental water samples were collected from the river ba-sin of Turag river in Gabtoli area in Dhaka city, an inland region of Bangladesh. Samples were col-lected in triplicate from three locations that were 5 metres apart (Image source: Google).

Forty-six bacterial isolates of known concentration of 3×10^4 CFU/mL (CFU – colony forming unit) were tested, including non-O1 *V. cholerae*, *V. cholerae* O1, other *Vibrio* species (*V. parahaemolyticus*, *V. vulnificus*, *V. metoecus*, *V. mimicus)*, as well as non-*Vibrio* species (*Escherichia coli and Pseudomonas aeruginosa*) (TABLE 1). All seventeen non-O1 *V. cholerae* and eight *V. cholerae* O1 were positive for *viuB* gene. Only samples spiked with *V. cholerae* O1 strains were positive for the *rfbO1* gene. The other *Vibrio* spp. and non-*Vibrio* species were negative for these two gene targets. Thus, the analytical specificity (i.e. the ability of an assay to detect and/or measure a specific organism in a sample) of this method was 100% (TABLE 1). Analytical sensitivity was also found to be 100% based on detection of 3 CFU/reaction (TABLE 2).

Table 1. Bacterial strains used in the analytical validation of this assay

Species	No. of strains	Strain	Target genes		Source	Reference
			viuB	*rfbO1*		
V. cholerae						
V. cholerae non O1	17	OYP1G01	+	−	Environmental	This study
		OYP2A12	+	−	Environmental	This study
		OYP2E01	+	−	Environmental	This Study
		OYP3B05	+	−	Environmental	[45]
		OYP3F10	+	−	Environmental	This study
		OYP4B01	+	−	Environmental	This study
		OYP4C07	+	−	Environmental	[45]
		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	[46]
		V52	+	+	Clinical	[47]
		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
Other *Vibrio* species						
V. parahaemolyticus	1	ATCC 17802	−	−	Clinical	[48]
V. vulnificus	3	ATCC 27562	−	−	Clinical	[49]
		MO6-24	−	−	Clinical	[50]
		CECT 5769	−	−	Environmental	[51]
V. metoecus	10	RC341	−	−	Environmental	[52]
		OP3H	−	−	Environmental	[53]
		OYP4D01	−	−	Environmental	[54]
		OYP4E03	−	−	Environmental	This study
		OYP5B04	−	−	Environmental	[55]
		OYP5B06	−	−	Environmental	[55]
		OYP5H08	−	−	Environmental	This study

		OYP8G05	–	–	Environmental	This study
		OYP8G09	–	–	Environmental	This study
		OYP8G12	–	–	Environmental	This study
V. mimicus	3	ATCC 33653	–	–	Clinical	[56]
		ATCC 33654	–	–	Environmental	[56]
		ATCC 33655	–	–	Clinical	[56]
Other bacterial species						
Escherichia coli	2	CU1	–	–	Clinical	[57]
		CU2	–	–	Clinical	[58]
Pseudomonas aeruginosa	2	PA103	–	–	Clinical	[59]
		PA14	–	–	Clinical	[60]

Table 2. Reproducibility and repeatability of qPCR assays.

No. of CFU/Reaction	Assay for Total *V. cholerae* (*viuB*)				Assay for *V. cholerae* O1 (*rfbO1*)			
	Intra-assay Mean	% CV[1]	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

[1] %CV: percent coefficient of variation

2.2. Limit of Detection (LOD)

The LOD of the assay was determined as 3 copies per reaction from the standard curve constructed using serially diluted standards of the *V. cholerae* El Tor O1 N16961 reference strain (FIGURE 2). We also determined a Sample Limit of Detection (SLOD) of 5×10^3 CFU/L for the filter-sterilized environmental water samples (5 mL) spiked with a known number of *V. cholerae* N16961 concentrated to ~10 μL with an Amicon ultra-0.5 centrifugal filter device (TABLE S1).
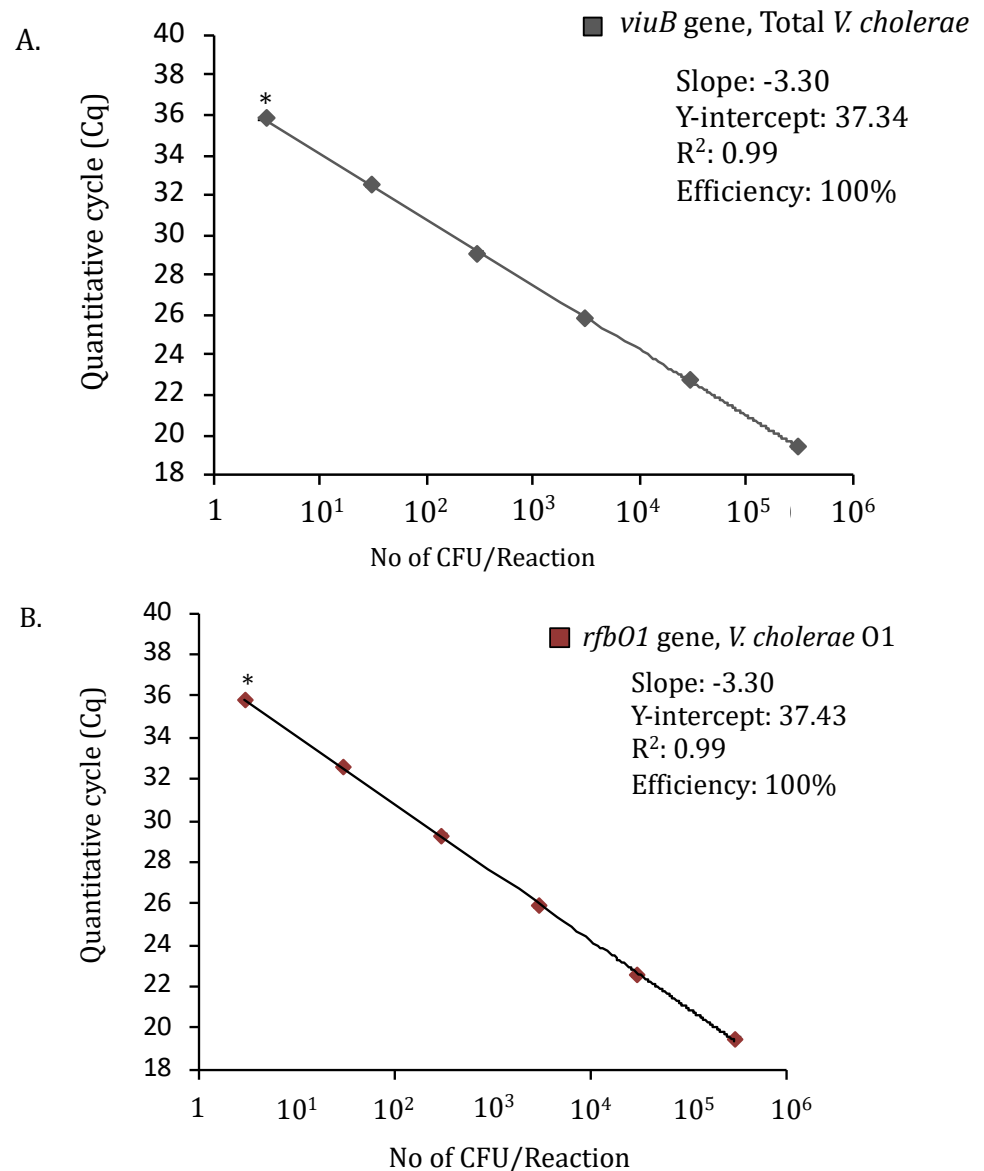
Figure 2. Standard curves for detection and quantification of total V. cholerae and V. cholerae O1 by qPCR. Two gene markers with fluorogenic probes, A) *viuB* (*V. cholerae* specific) and B) *rfbO1* (*V. cholerae* O1 specific) were used. Cells of reference culture (*V. cholerae* N16961 El Tor O1) were serially diluted 10-fold to yield concentrations ranging 3 to 3×10^6 copies per reaction (from left to right.) Fluorescence was measured in relative units.

2.3. Assay precision and efficiency

Following the Minimum Information for Publication of Quantitative qPCR Experiments (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 measurements 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 0.20% for *viuB* and 0.03 to 0.21% for *rfbO1* (TABLE 2). The efficiency of both

assays was 100% based on the standard curve generated from a serial dilution of *V. cholerae* N16961 (FIGURE 2) with $R^2 = 0.99$ and slope of -3.3.

To test for qPCR inhibition, we compared the quantitative cycle value (Cq) values for different dilutions of filter-sterilized environmental water samples spiked with the reference *V. cholerae* strains. The 10× diluted samples shifted the Cq values by 3.3 ± 0.07 cycles (TABLE S1), indicating no significant inhibition.

2.4. 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 (FIGURE 3). 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 between 3.7×10^4 to 3.9×10^4 genome copies/L (FIGURE 4). *V. cholerae* O1 was found at 4.7×10^3 to 5.4×10^3 genome copies/L, representing approximately 13% of the total *V. cholerae* population (FIGURE 4).
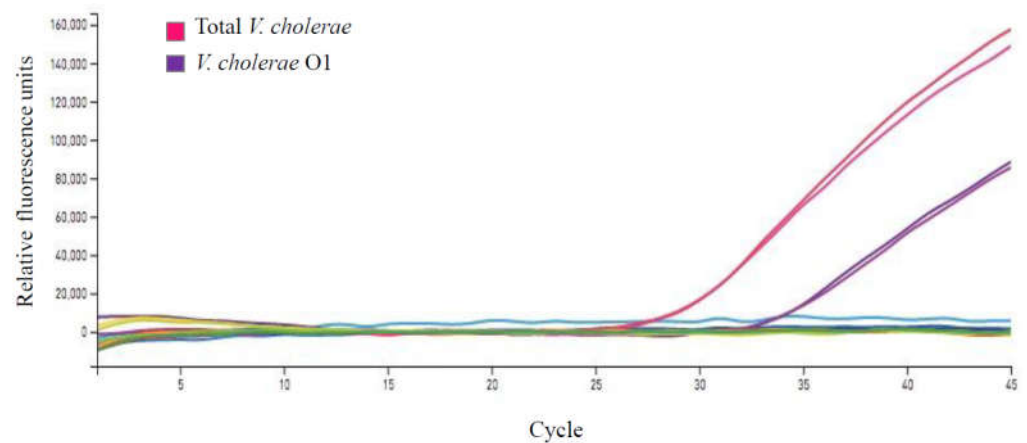

Figure 3. Detection and quantification of total *V. cholerae* and *V. cholerae* O1 in the same qPCR experiment. Filter-sterilized environmental water samples collected from the Gabtoli site were inoculated with known concentrations of cells of reference strain *V. cholerae* N16961 El Tor O1. Quantification was performed with both *viuB* and *rfbO1* primers in the same PCR run to detect total *V. cholerae* and O1 serogroup *V. cholerae*, respectively.
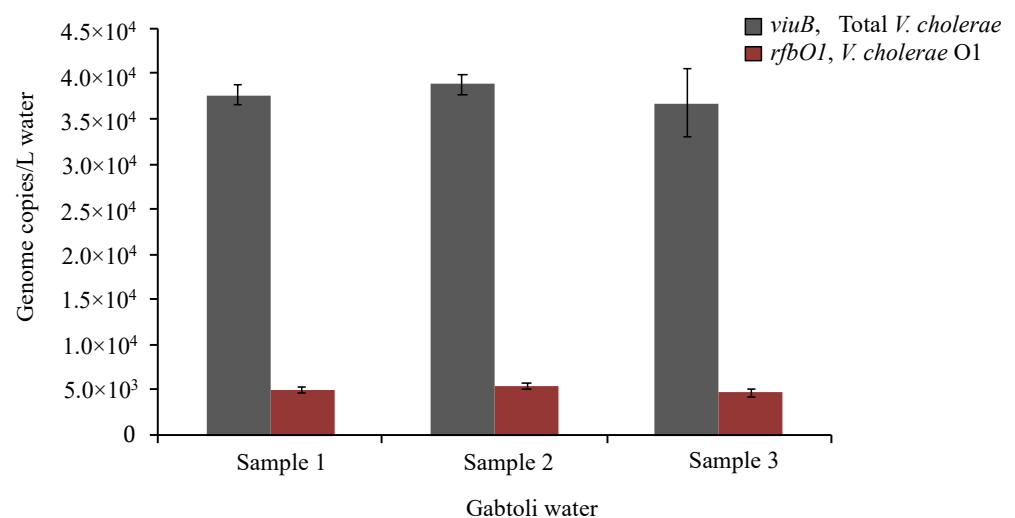

Figure 4. Abundance of *V. cholerae* along with its O1 serogroup subpopulation in water from an inland urban region (Gabtoli, Dhaka) of Bangladesh. Water samples were collected in August 2018 and analyzed using the developed qPCR assay. The *viuB* gene marker was used to quantify total *V. cholerae*, and *rfbO1* was used to quantify *V. cholerae* O1. Each qPCR reaction was run in

triplicate and evaluated with corresponding standards. Error bars represent the standard deviation of means from biological replicates.

3. 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 [61]. As a survival strategy, *V. cholerae* goes into a viable but non-culturable state (VBNC) under unfavorable environmental conditions, in which it assumes a coccoid shape and cannot be revived using traditional culturing methods [21,62]. Non-culturable *V. cholerae* in biofilm were reported in 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 [63]. Therefore, passing through the human host could be a means of revival from the VBNC state, contributing to the amplification of *V. cholerae* prior to an outbreak [64,65] and its 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 water 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. There is also recent evidence that indicates that environmental *V. cholerae* may play a role in reviving VBNCs through the over production of products associated with quorum sensing [35]. As the body of literature regarding environmental *V. cholerae* increases [12,17,24,66], we expect the interactions between pathogenic and non-pathogenic *V. cholerae* to be resolved in greater detail.

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 increased the challenge 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 [67]. However, this problem was overcame by increasing the concentration of the water sample 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. 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 expected for a well-designed and sensitive assay, as described in the MIQE guidelines [68]. The limit of detection for concentrated water samples was 5×10^3 CFU/L, which is at the lower limit of an infectious dose of *V. cholerae*, reported to be 10^3 - 10^9 cells based on a range of factors, such as the health of the exposed individual [69]. More specifically, the infectious dose for toxigenic *V. cholerae* O1 is typically around 10^4 - 10^6 cells, whereas the infective dose for non-O1 strains is around 10^6 - 10^9 [70]. Without the concentration procedure, the limit of detection of *V. cholerae* in environmental water samples would be 3×10^6 CFU/L, which is still useful to determine if a strong risk of contracting cholera exists. 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 toxigenic *V. cholerae* O1 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 [63,71].

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) facilitates the use of this method in field-level surveillance and also avoids 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 [72]. 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 [73]. The number of total *V. cholerae* detected was about 3.9×10^4 genome copies/L, 13% of which was *V. cholerae* O1 (~5×10^3 genome copies/L). This is of concern as it is within the range of the infectious dose for cholera if someone were to ingest a few hundred milliliters of water (>10^3 cells) [69]. Previous studies based on the Crystal VC® dipstick test after sample enrichment for 18 hours 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* [74,75]. From our observations during sampling, which are 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 [76]. It is therefore likely that local rivers and ponds serve as important reservoirs of the cholera pathogen in Dhaka city. The importance of local ecosystems in maintaining toxigenic cholera is known [77,78], but because of the difficulty in monitoring at sufficient frequency in a number of locations, a direct link has never been established 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 [13,79]. 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 Bangladesh water bodies during the period between outbreaks when reported cases of cholera are low [80].

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 [69]. 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 [74].

Our assay is specific, sensitive, and convenient for field studies, making 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 possible. Thus, it is possible to optimize a multiplex assay where *V. cholerae* O1 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 [81,82], further treatment of the sample to remove inhibitors may be required. A 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 [72].

In conclusion, we developed a species-specific qPCR method, which can be conveniently 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.

4. Materials and Methods

4.1. Preparation of bacterial cultures

Forty-six different strains of *Vibrio* spp. and other gammaproteobacteria were used to validate analytical sensitivity and specificity of the assay in this study (TABLE 1). Seventeen 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 1). Multiple strains from three other *Vibrio* species [*V. parahaemolyticus* (1), *V. vulnificus* (2) and *V. mimicus* (3)] were tested along with two strains of *Pseudomonas aeruginosa* and *Escherichia coli* each. The *Vibrio* strains were grown in tryptic soy broth (TSB) (Becton Dickinson) with 1.0% NaCl (BDH) at 30°C and 200 rpm overnight in a shaking incubator.

4.2. Collection and processing of environmental samples

Environmental water samples were collected from the river basin of the Turag river in the Gabtoli area (the coordinates of the latitude and longitude are 23.783726, 90.344246) [83] of Dhaka, Bangladesh (FIGURE 1) during August 2018. Three locations, approximately 5 meters 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). Five milliliters of surface water was collected directly in Thermo Scientific Nunc 50 mL conical centrifuge tubes (Thermo Fisher Scientific, Korea). When needed, collected water samples were concentrated using Amicon Ultra-0.5 centrifugal filter device (Merk KGaA, Darmstadt, Germany) (FIGURE 5) according to the user's manual. Ten Amicon tubes, each containing 500 µL of water sample, were centrifuged at 14000 × g for 20 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.
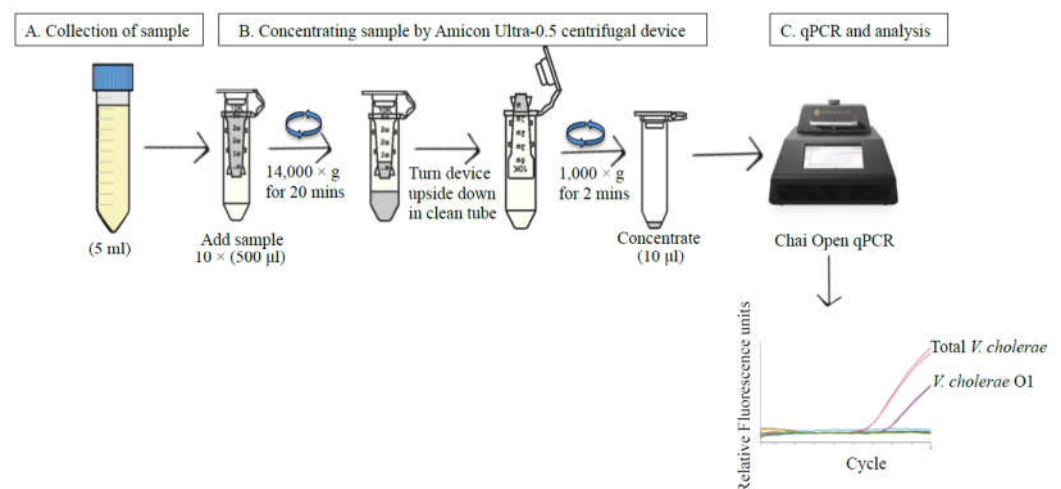

Figure 5. Method for processing of environmental samples for qPCR. (A) Sample collection. (B) Concentrating sample by Amicon Ultra-0.5 centrifugal device. (C) qPCR in the Chai Open qPCR thermocycler (*https://www.chaibio.com/openqpcr*). 5 mL of water was collected from each location. Ten Amicon tubes, each with 500 µL of water sample, were centrifuged at 14000 × g for 20 minutes in a

mini spin plus centrifuge, and all the concentrates were pooled in a total volume of concentrate of 100 μL.

4.3. Amplification using the Chai open qPCR platform

Previously developed species-specific primers [73] were used to detect and quantify *V. cholerae* in this assay. Briefly, the *viuB* gene encoding vibriobactin utilization protein B was used to quantify total *V. cholerae* (all serogroups), as it is a single copy gene present in all *V. cholerae* with only divergent 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) [73]. 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 has a single channel to detect wavelengths of 513-555 nm.

Table 3. 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	[73]
	Forward primer	TCGGTATTGTCTAACGGTAT		
	Reverse Primer	CGATTCGTGAGGGTGATA		
rfbO1	Probe	56-FAM/AGAAGTGTG/ZEN/TGGGCCAGGTAAAGT/3IABkFQ	113	[73]
	Forward primer	GTAAAGCAGGATGGAAACATATTC		
	Reverse primer	TGGGCTTACAAACTCAAGTAAG		

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, which 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 in separate tubes but in the same thermocycler run. Standards of a known number of cells and no template control were included in every assay.

4.4. 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 copies and 3 copies per 10 μL of filter sterilized water. Inoculum concentration were quantified

using a standard drop plate method [84]. 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 2). A standard curve was generated by plotting the log value of the calculated CFU per reaction against the Cq. The Cq value is the cycle at which the fluorescence from amplification exceeded the background fluorescence in the MIQE guidelines [68].

4.5. Determination of the LOD and qPCR efficiency

The LOD of the assay was determined from the standard curve constructed from serially diluted standards of *V. cholerae* N16961 as mentioned above (FIGURE 2). 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 centrifugal filter device. 1 mL of spiked environmental water sample was concentrated to 20 μL after 30 mins of spinning. The qPCR assay was carried out on these concentrated samples containing known bacterial cell numbers in the Chai Open qPCR system (TABLE S1). 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 at which all replicates were positive across ten repeated experiments.

The qPCR efficiency of the assay was calculated using the following formula: Efficiency = $10^{[-1/Slope]}$ [13,85] in Excel.

References

1. Huang, J.; Zhu, Y.; Wen, H.; Zhang, J.; Huang, S.; Niu, J.; Li, Q. Quadruplex real-time PCR assay for detection and identification of *Vibrio cholerae* O1 and O139 strains and determination of their toxigenic potential. *Appl Environ Microbiol* **2009**, *75*, 6981-6985.

2. Albert, M.J.; Siddique, A.K.; Islam, M.S.; Faruque, A.S.; Ansaruzzaman, M.; Faruque, S.M.; Sack, R.B. Large outbreak of clinical cholera due to *Vibrio cholerae* non-O1 in Bangladesh. *Lancet* **1993**, *341*, 704.

3.   Bakhshi, B.; Barzelighi, H.M.; Adabi, M.; Lari, A.R.; Pourshafie, M. A molecular survey on virulence associated genotypes of non-O1 non-O139 *Vibrio cholerae* in aquatic environment of Tehran, Iran. *Water research* **2009**, *43*, 1441-1447.

4.   Chhotray, G.; Pal, B.; Khuntia, H.; Chowdhury, N.; Chakraborty, S.; Yamasaki, S.; Ramamurthy, T.; Takeda, Y.; Bhattacharya, S.; Nair, G.B.J.E.; et al. Incidence and molecular analysis of *Vibrio cholerae* associated with cholera outbreak subsequent to the super cyclone in Orissa, India. **2002**, *128*, 131-138.

5.   Jesudason, M.; Samuel, R.; John, T.J. Reappearance of *Vibrio cholerae* 01 and concurrent prevalence of 01 and 0139 in Vellore, South India. *The Lancet* **1994**, *344*, 335-336.

6.   European Centre for Disease Prevention and Control (ECDC). Cholera worldwide overview. Available online: https://www.ecdc.europa.eu/en/all-topics-z/cholera/surveillance-and-disease-data/cholera-monthly (accessed on 13th February).

7.   Centers for Disease Control and Prevention, N.C.f.E.a.Z.I.D.N., Division of Foodborne, Waterborne, and Environmental Diseases (DFWED). Cholera - Vibrio cholerae infection. Available online: https://www.cdc.gov/cholera/general/index.html (accessed on 13th February).

8.   WHO. *Ending Cholera - A Global Roadmap to 2030*; 2019; pp. 1-32.

9.   Ratnayake, R.; Finger, F.; Edmunds, W.J.; Checchi, F. Early detection of cholera epidemics to support control in fragile states: estimation of delays and potential epidemic sizes. *BMC Med* **2020**, *18*, 397, doi:10.1186/s12916-020-01865-7.

10.   Reuters. Yemen cholera outbreak accelerates to 10,000+ cases per week: WHO. *Reuters* 2018.

11.   WHO. *Cholera situation in Yemen, December 2020*; 2021.

12.   Bliem, R.; Schauer, S.; Plicka, H.; Obwaller, A.; Sommer, R.; Steinrigl, A.; Alam, M.; Reischer, G.H.; Farnleitner, A.H.; Kirschner, A. A novel triplex quantitative PCR strategy for quantification of toxigenic and nontoxigenic *Vibrio cholerae* in aquatic environments. *Appl Environ Microbiol* **2015**, *81*, 3077-3085, doi:10.1128/AEM.03516-14.

13.   Blackstone, G.M.; Nordstrom, J.L.; Bowen, M.D.; Meyer, R.F.; Imbro, P.; DePaola, A. Use of a real time PCR assay for detection of the *ctxA* gene of *Vibrio cholerae* in an environmental survey of Mobile Bay. *J Microbiol Methods* **2007**, *68*, 254-259, doi:10.1016/j.mimet.2006.08.006.

14.   Islam, M.; Hasan, M.; Miah, M.; Yunus, M.; Zaman, K.; Albert, M. Isolation of *Vibrio cholerae* O139 synonym Bengal from the aquatic environment in Bangladesh: implications for disease transmission. *Appl Environ Microbiol* **1994**, *60*, 1684-1686.

15.   Ramamurthy, T.; Garg, S.; Sharma, R.; Bhattacharya, S.; Nair, G.B.; Shimada, T.; Takeda, T.; Karasawa, T.; Kurazano, H.; Pal, A.J.T.L. Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. **1993**, *341*, 703-704.

16.   Islam, M.T.; Alam, M.; Boucher, Y. Emergence, ecology and dispersal of the pandemic generating *Vibrio cholerae* lineage. *Int Microbiol* **2017**, *20*, 106-115.

17.   Bliem, R.; Reischer, G.; Linke, R.; Farnleitner, A.; Kirschner, A. Spatiotemporal dynamics of *Vibrio cholerae* in turbid alkaline lakes as determined by quantitative PCR. *Applied and environmental microbiology* **2018**, *84*, e00317-00318.

18.   Glass, R.I.; Becker, S.; Huq, M.I.; Stoll, B.J.; Khan, M.; Merson, M.H.; Lee, J.V.; Black, R.E. Endemic cholera in rural Bangladesh, 1966–1980. *American journal of epidemiology* **1982**, *116*, 959-970.

19.   Kaper, J.; Morris, J.L.M. Cholera. *Clin Microbiol Rev* **1995**, *8*, 48-86.

20.   Faruque, S.M.; Albert, M.J.; Mekalanos, J.J. Epidemiology, genetics, and ecology of toxigenic *vibrio cholerae*. *Microbiol Mol Biol Rev* **1998**, *62*, 1301-1314.

21.   Colwell, R.R. Viable but nonculturable bacteria: a survival strategy. *J Infect Chemother* **2000**, *6*, 121-125, doi:10.1007/s101560000026.

22.   Huq, A.; Colwell, R.R.; Chowdhury, M.A.R.; Xu, B.; Moniruzzaman, S.M.; Islam, M.S.; Yunus, M.; Albert, M.J. Coexistence of *Vibrio cholerae* 01 and 0139 Bengal in plankton in Bangladesh. *The Lancet* **1995**, *345*, 1249.

23.   Epstein, P.R. Algal blooms in the spread and persistence of cholera. *Biosystems* **1993**, *31*, 209-221.

24.   Islam, M.S.; Zaman, M.H.; Islam, M.S.; Ahmed, N.; Clemens, J.D. Environmental reservoirs of *Vibrio cholerae*. *Vaccine* **2020**, *38*, A52-A62.

25.   Lee, K. The global dimensions of cholera. *Global change and human health* **2001**, *2*, 6-17.

26.   Piarroux, R.; Barrais, R.; Faucher, B.; Haus, R.; Piarroux, M.; Gaudart, J.; Magloire, R.; Raoult, D. Understanding the cholera epidemic, Haiti. *Emerging infectious diseases* **2011**, *17*, 1161.

27.   Zuckerman, J.N.; Rombo, L.; Fisch, A. The true burden and risk of cholera: implications for prevention and control. *The Lancet infectious diseases* **2007**, *7*, 521-530.

28.   Orata, F.D.; Keim, P.S.; Boucher, Y. The 2010 cholera outbreak in Haiti: how science solved a controversy. *PLoS Pathog* **2014**, *10*, e1003967.

29.   Guillaume, Y.; Ternier, R.; Vissieres, K.; Casseus, A.; Chery, M.J.; Ivers, L.C. Responding to cholera in haiti: Implications for the national plan to eliminate cholera by 2022. *The Journal of infectious diseases* **2018**, *218*, S167-S170.

30.   Pan American Health Organization (PAHO). Haiti reaches one-year free of Cholera. Available online: https://www3.paho.org/hq/index.php?option=com_content&view=article&id=15684:haiti-reaches-one-year-free-of-cholera&Itemid=1926&lang=en (accessed on 28th Jan).

31.   Satcher, D. Emerging infections: getting ahead of the curve. *Emerg Infect Dis* **1995**, *1*, 1.

32.   Poirier, M.J.; Izurieta, R.; Malavade, S.S.; McDonald, M.D. Re-emergence of Cholera in the Americas: Risks, Susceptibility, and Ecology. *J Glob Infect Dis* **2012**, *4*, 162-171, doi:10.4103/0974-777X.100576.

33.   Almagro-Moreno, S.; Taylor, R.K. Cholera: environmental reservoirs and impact on disease transmission. *Microbiology spectrum* **2013**, *1.2*.

34.   Colwell, R.; Brayton, P.; Grimes, D.; Roszak, D.; Huq, S.; Palmer, L. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. *Nature biotechnology* **1985**, *3*, 817.

35.   Naser, I.B.; Shishir, T.A.; Faruque, S.N.; Hoque, M.M.; Hasan, A.; Faruque, S.M. Environmental prevalence of toxigenic Vibrio cholerae O1 in Bangladesh coincides with V. cholerae non-O1 non-O139 genetic variants which overproduce autoinducer-2. *PLoS One* **2021**, *16*, e0254068, doi:10.1371/journal.pone.0254068.

36.   Hasan, J.; Huq, A.; Tamplin, M.L.; Siebeling, R.J.; Colwell, R.R. A novel kit for rapid detection of *Vibrio cholerae* O1. *J Clin Microbiol* **1994**, *32*, 249-252.

37.   Colwell, R.R. Nonculturable but still viable and potentially pathogenic. *Zentbl. Bacteriol.* **1993**, *279*, 154-158.

38.   Benenson, A.S.; Islam, M.R.; Greenough Iii, W.B. Rapid identification of *Vibrio cholerae* by darkfield microscopy. *Bulletin of the World Health Organization* **1964**, *30*, 827.

39.   Alam, M.; Hasan, N.A.; Sultana, M.; Nair, G.B.; Sadique, A.; Faruque, A.; Endtz, H.P.; Sack, R.; Huq, A.; Colwell, R. Diagnostic limitations to accurate diagnosis of cholera. *Journal of clinical microbiology* **2010**, *48*, 3918-3922.

40.   Almeida, R.; Hickman-Brenner, F.; Sowers, E.; Puhr, N.; Farmer, J.; Wachsmuth, I. Comparison of a latex agglutination assay and an enzyme-linked immunosorbent assay for detecting cholera toxin. *Journal of clinical microbiology* **1990**, *28*, 128-130.

41.   Harris, J.R.; Cavallaro, E.C.; De Nóbrega, A.A.; Dos S. Barrado, J.C.; Bopp, C.; Parsons, M.B.; Djalo, D.; Fonseca, F.G.d.S.; Ba, U.; Semedo, A. Field evaluation of Crystal VC® Rapid Dipstick test for cholera during a cholera outbreak in Guinea-Bissau. *Tropical medicine international health* **2009**, *14*, 1117-1121.

42.   Moyenuddin, M.; Rahman, K.M.; Sack, D.A. The aetiology of diarrhoea in children at an urban hospital in Bangladesh. *Transactions of the Royal Society of Tropical Medicine Hygiene* **1987**, *81*, 299-302.

43.   Xu, H.-S.; Roberts, N.; Adams, L.; West, P.; Siebeling, R.; Huq, A.; Huq, M.; Rahman, R.; Colwell, R. An indirect fluoresent antibody staining procedure for detection of *Vibrio cholerae* serovar 01 cells in aquatic environmental samples. *Journal of Microbiological Methods* **1984**, *2*, 221-231.

44. Rudko, S.P.; Reimink, R.L.; Peter, B.; White, J.; Hanington, P.C. Democratizing water monitoring: Implementation of a community-based qPCR monitoring program for recreational water hazards. *PLoS One* **2020**, *15*, e0229701, doi:10.1371/journal.pone.0229701.

45. Kirchberger, P.C.; Orata, F.D.; Barlow, E.J.; Kauffman, K.M.; Case, R.J.; Polz, M.F.; Boucher, Y. A small number of phylogenetically distinct clonal complexes dominate a coastal *Vibrio cholerae* population. *Appl Environ Microbiol* **2016**, AEM. 01177-01116.

46. Heidelberg, J.F.; Eisen, J.A.; Nelson, W.C.; Clayton, R.A.; Gwinn, M.L.; Dodson, R.J.; Haft, D.H.; Hickey, E.K.; Peterson, J.D.; Umayam, L. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* **2000**, *406*, 477.

47. Chun, J.; Grim, C.J.; Hasan, N.A.; Lee, J.H.; Choi, S.Y.; Haley, B.J.; Taviani, E.; Jeon, Y.S.; Kim, D.W.; Lee, J.H.; et al. Comparative genomics reveals mechanism for short-term and long-term clonal transitions in pandemic *Vibrio cholerae*. *Proc Natl Acad Sci U S A* **2009**, *106*, 15442-15447, doi:10.1073/pnas.0907787106.

48. Yang, N.; Liu, M.; Luo, X.; Pan, J. Draft genome sequence of Strain ATCC 17802(T), the type strain of Vibrio parahaemolyticus. *Mar Genomics* **2015**, *24 Pt 3*, 203-205, doi:10.1016/j.margen.2015.05.010.

49. Li, Z.; Chen, H.; Chen, X.; Zhou, T.; Zhao, L.; Zhang, C.; Jin, W. Genome sequence of the human-pathogenic bacterium Vibrio vulnificus type strain ATCC 27562. *J Bacteriol* **2012**, *194*, 6954-6955, doi:10.1128/JB.01890-12.

50. Stine, O.C.; Sozhamannan, S.; Gou, Q.; Zheng, S.; Morris, J.G., Jr.; Johnson, J.A. Phylogeny of Vibrio cholerae based on recA sequence. *Infect Immun* **2000**, *68*, 7180-7185, doi:10.1128/IAI.68.12.7180-7185.2000.

51. Fouz, B.; Roig, F.J.; Amaro, C. Phenotypic and genotypic characterization of a new fish-virulent Vibrio vulnificus serovar that lacks potential to infect humans. *Microbiology (Reading)* **2007**, *153*, 1926-1934, doi:10.1099/mic.0.2006/005405-0.

52. Haley, B.J.; Grim, C.J.; Hasan, N.A.; Choi, S.-Y.; Chun, J.; Brettin, T.S.; Bruce, D.C.; Challacombe, J.F.; Detter, J.C.; Han, C.S. Comparative genomic analysis reveals evidence of two novel *Vibrio* species closely related to *V. cholerae*. *BMC Microbiol* **2010**, *10*, 154.

53. Kirchberger, P.C.; Turnsek, M.; Hunt, D.E.; Haley, B.J.; Colwell, R.R.; Polz, M.F.; Tarr, C.L.; Boucher, Y. *Vibrio metoecus* sp. nov., a close relative of *Vibrio cholerae* isolated from coastal brackish ponds and clinical specimens. *Int J Syst Evol Microbiol* **2014**, *64*, 3208-3214, doi:10.1099/ijs.0.060145-0.

54. Orata, F.D.; Kirchberger, P.C.; Méheust, R.; Barlow, E.J.; Tarr, C.L.; Boucher, Y. The dynamics of genetic interactions between *Vibrio metoecus* and *Vibrio cholerae*, two close relatives co-occurring in the environment. *Genome Biol Evol* **2015**, *7*, 2941-2954.

55. Orata, F.D.; Kirchberger, P.C.; Meheust, R.; Barlow, E.J.; Tarr, C.L.; Boucher, Y. The Dynamics of Genetic Interactions between Vibrio metoecus and Vibrio cholerae, Two Close Relatives Co-Occurring in the Environment. *Genome Biol Evol* **2015**, *7*, 2941-2954, doi:10.1093/gbe/evv193.

56. Davis, B.R.; Fanning, G.R.; Madden, J.M.; Steigerwalt, A.G.; Bradford, H.B.; Smith, H.L.; Brenner, D.J. Characterization of biochemically atypical *Vibrio cholerae* strains and designation of a new pathogenic species, *Vibrio mimicus*. *Journal of clinical microbiology* **1981**, *14*, 631-639.

57. Taniguchi, K.; Nakamura, A.; Tsurubuchi, K.; O'Hara, K.; Sawai, T. Identification of Escherichia coli clinical isolates producing macrolide 2'-phosphotransferase by a highly sensitive detection method. *FEMS Microbiol Lett* **1998**, *167*, 191-195, doi:10.1111/j.1574-6968.1998.tb13227.x.

58. McGilvray, D.; Umbarger, H.E. Regulation of transaminase C synthesis in Escherichia coli: conditional leucine auxotrophy. *J Bacteriol* **1974**, *120*, 715-723, doi:10.1128/jb.120.2.715-723.1974.

59. Ohman, D.E.; Cryz, S.J.; Iglewski, B.H. Isolation and characterization of Pseudomonas aeruginosa PAO mutant that produces altered elastase. *J Bacteriol* **1980**, *142*, 836-842, doi:10.1128/jb.142.3.836-842.1980.

60. Lee, D.G.; Urbach, J.M.; Wu, G.; Liberati, N.T.; Feinbaum, R.L.; Miyata, S.; Diggins, L.T.; He, J.; Saucier, M.; Deziel, E.; et al. Genomic analysis reveals that Pseudomonas aeruginosa virulence is combinatorial. *Genome Biol* **2006**, *7*, R90, doi:10.1186/gb-2006-7-10-r90.

61.   Takemura, A.F.; Chien, D.M.; Polz, M.F. Associations and dynamics of Vibrionaceae in the environment, from the genus to the population level. *Front Microbiol* **2014**, *5*, 38-38, doi:10.3389/fmicb.2014.00038.

62.   Colwell, R.R.; Huq, A. Vibrios in the Environment: Viable but Nonculturable *Vibrio cholerae*. In *Vibrio cholerae and Cholera*; American Society of Microbiology: 1994; pp. 117-133.

63.   Alam, M.; Sultana, M.; Nair, G.B.; Siddique, A.; Hasan, N.A.; Sack, R.B.; Sack, D.A.; Ahmed, K.; Sadique, A.; Watanabe, H. Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. *Proc Natl Acad Sci U S A* **2007**, *104*, 17801-17806.

64.   Colwell, R.R.; Brayton, P.; Herrington, D.; Tall, B.; Huq, A.; Levine, M.M. Viable but non-culturable *Vibrio cholerae* O1 revert to a cultivable state in the human intestine. *World J Microbiol Biotechnol* **1996**, *12*, 28-31, doi:10.1007/BF00327795.

65.   Faruque, S.M.; Islam, M.J.; Ahmad, Q.S.; Faruque, A.; Sack, D.A.; Nair, G.B.; Mekalanos, J. Self-limiting nature of seasonal cholera epidemics: role of host-mediated amplification of phage. *Proceedings of the National Academy of Sciences* **2005**, *102*, 6119-6124.

66.   Hussain, N.A.S.; Kirchberger, P.C.; Case, R.J.; Boucher, Y.F. Modular Molecular Weaponry Plays a Key Role in Competition Within an Environmental Vibrio cholerae Population. *Front Microbiol* **2021**, *12*, 671092, doi:10.3389/fmicb.2021.671092.

67.   Huq, A.; Colwell, R.R.; Rahman, R.; Ali, A.; Chowdhury, M.; Parveen, S.; Sack, D.; Russek-Cohen, E. Detection of *Vibrio cholerae* O1 in the aquatic environment by fluorescent-monoclonal antibody and culture methods. *Appl Environ Microbiol* **1990**, *56*, 2370-2373.

68.   Bustin, S.A.; Benes, V.; Garson, J.A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.L.; et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **2009**, *55*, 611-622, doi:10.1373/clinchem.2008.112797.

69.   Schmid-Hempel, P.; Frank, S.A.J.P.p. Pathogenesis, virulence, and infective dose. *J PLoS pathogens* **2007**, *3*, e147.

70.   Kothary, M.H.; Babu, U.S. Infective dose of foodborne pathogens in volunteers: a review. *Journal of food safety* **2001**, *21*, 49-68.

71.   Alam, M.; Sultana, M.; Nair, G.B.; Sack, R.B.; Sack, D.A.; Siddique, A.K.; Ali, A.; Huq, A.; Colwell, R.R. Toxigenic *Vibrio cholerae* in the aquatic environment of Mathbaria, Bangladesh. *Appl Environ Microbiol* **2006**, *72*, 2849-2855, doi:10.1128/AEM.72.4.2849-2855.2006.

72.   Ahmed, F.; Fakhruddin, A.N.M.; Imam, M.D.T.; Khan, N.; Abdullah, A.T.M.; Khan, T.A.; Rahman, M.M.; Uddin, M.N. Assessment of roadside surface water quality of Savar, Dhaka, Bangladesh using GIS and multivariate statistical techniques. *Applied Water Science* **2017**, *7*, 3511-3525, doi:10.1007/s13201-017-0619-0.

73.   Nasreen, T.H., N.A.S.; Islam, M.T.; Orata, F.D.; Kirchberger, P.C.; Case, R.J.; Alam, M.; Yanow, S.K.; Boucher, Y.F. Simultaneous Quantification of Vibrio metoecus and Vibrio cholerae with Its O1 Serogroup and Toxigenic Subpopulations in Environmental Reservoirs. *Pathogens* **2020**, *9, 1053*.

74.   Rashid, M.u.; Rahman, Z.; Burrowes, V.; Perin, J.; Mustafiz, M.; Monira, S.; Saif-Ur-Rahman, K.; Bhuyian, S.I.; Mahmud, M.T.; Sack, R.B. Rapid dipstick detection of *Vibrio cholerae* in household stored and municipal water in Dhaka, Bangladesh: CHoBI7 trial. *Tropical Medicine International Health* **2017**, *22*, 205-209.

75.   George, C.M.; Monira, S.; Sack, D.A.; Rashid, M.-u.; Saif-Ur-Rahman, K.; Mahmud, T.; Rahman, Z.; Mustafiz, M.; Bhuyian, S.I.; Winch, P. Randomized controlled trial of hospital-based hygiene and water treatment intervention (CHoBI7) to reduce cholera. *Emerging infectious diseases* **2016**, *22*, 233.

76.   Aktar, P.; Moonajilin, M.S. Assessment of Water Quality Status of Turag River Due to Industrial Effluent. *International Journal of Engineering and Information Systems (IJEAIS)* **2017**, *1*, 105-118.

77.   Sakib, S.N.; Reddi, G.; Almagro-Moreno, S. Environmental role of pathogenic traits in Vibrio cholerae. *J Bacteriol* **2018**, *200*, e00795-00717, doi:10.1128/JB.00795-17.

78.   Jutla, A.; Whitcombe, E.; Hasan, N.; Haley, B.; Akanda, A.; Huq, A.; Alam, M.; Sack, R.B.; Colwell, R. Environmental factors influencing epidemic cholera. *Am J Trop Med Hyg* **2013**, *89*, 597-607, doi:10.4269/ajtmh.12-0721.

79.   Vezzulli, L.; Stauder, M.; Grande, C.; Pezzati, E.; Verheye, H.M.; Owens, N.J.; Pruzzo, C. *gbpA* as a novel qPCR target for the species-specific detection of *Vibrio cholerae* O1, O139, non-O1/non-O139 in environmental, stool, and historical continuous plankton recorder samples. *PLoS One* **2015**, *10*, e0123983, doi:10.1371/journal.pone.0123983.

80.   Sultana, M.; Nusrin, S.; Hasan, N.A.; Sadique, A.; Ahmed, K.U.; Islam, A.; Hossain, A.; Longini, I.; Nizam, A.; Huq, A. Biofilms comprise a component of the annual cycle of *Vibrio cholerae* in the bay of bengal estuary. *mBio* **2018**, *9*, e00483-00418.

81.   Pia, H.I.; Aktar, M.; Sarkar, S.; Rahman, A.; Rayhan, A.B.M.S.; Islam, M.M.; Hassan, M. Assessment of water quality varies between pre-monsoon and post-monsoon season of the typical contaminated river of bangladesh. A case study of shitalakhya river, Dhaka. **2018**, *3.1*, 129-141.

82.   Staff Correspondent. 6 Rivers around Dhaka: Water turning untreatable? *The Daily Star* 2016.

83.   LatLong.net. Gabtoli, Dhaka, Bangladesh. Available online: https://www.latlong.net/place/gabtoli-dhaka-bangladesh-22624.html (accessed on 2019, June 1).

84.   Herigstad, B.; Hamilton, M.; Heersink, J. How to optimize the drop plate method for enumerating bacteria. *Journal of microbiological methods* **2001**, *44*, 121-129.

85.   Pfaffl, M. Quantification strategies in real-time PCR Michael W. Pfaffl. AZ Quantitative PCR **2004**, *1*, 87–112.