Development and Validation of a Novel Real-time Assay for the Detection and Quantification of *Vibrio cholerae*

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*Vibrio cholerae* O1 and O139 has been known for its ability to cause epidemics. These strains produce cholera toxin which is the main cause of secretory diarrhea. *V. cholerae* non-O1 and non-O139 strains are also capable of causing gastroenteritis as well as septicemia and peritonitis. It has been proven that virulence factors such as T6SS, *hapA*, *rtxA*, and *hlyA* are present in almost all *V. cholerae* strains. It is imperative that viable but non-culturable cells of *V. cholerae* are also detected since they are also known to cause diarrhea. Thus, the aim of this study was to develop an assay that detects all *V. cholerae* regardless of their serotype, culturable state, and virulence genes present, by targeting the species specific conserved *ompW* sequence. The developed assay meets these goals with 100% specificity and is capable of detecting as low as 5.46 copy number of *V. cholerae*. Detection is rapid since neither lengthy incubation period nor electrophoresis is required. The assay had excellent repeatability (CV%: 0.24–1.32) and remarkable reproducibility (CV%: 1.08–3.7). Amplification efficiencies in the 89–100% range were observed. The assay is more economical than Taqman-based multiplex real-time PCR assays. Compared to other real-time assays, the *ompW* assay is specific and sensitive, has better repeatability and reproducibility, and is more economical.

**Keywords:** *Vibrio cholerae*, OmpW, Cq value, sensitivity and specificity, gene copy number, real-time PCR

**INTRODUCTION**

*Vibrio cholerae* is a Gram-negative, comma shaped facultative pathogen responsible for causing cholera. The global incidence of cholera was about 2.8 million cases per year, with 91,000 deaths (1). *V. cholerae* O1 has been the etiological agent for several cholera epidemics. The serogroup O139 was responsible for cholera outbreaks in India and other countries in Asia during 1992 (2) and was also isolated during the outbreak in November 2000 in India (3) and March–April 2002 in Bangladesh (4). *Vibrio cholerae* O1 and O139 serogroups express toxin coregulated pilus which confers the bacteria the ability to colonize the intestine while the cholera toxin is associated with secretory diarrhea (5). Depending on severity, the infectious dose for *V. cholerae* varies from 10⁶ to 10¹¹ cells (6).

Toxigenic and non-toxigenic non O1, non-O139 have been documented as incriminating in several outbreaks in developing countries (7–10). In non-CT-producing vibrios, virulence factors such as type 3 secretion systems, hemolysin (HlyA), repeat in toxin (RTX), and heat-stable enterotoxin have
major roles in causing infections (11). Hasan et al. (10) reported 98% *V. cholerae* strains carried hemagglutinin protease *hap* (98%) irrespective of their source, i.e., clinical or environmental. Other virulence factors present are T6SS (94–99%), *txsA* (96%), *toxR* (87%), and *hlyA* (83%), and all these virulence factors might be responsible for diarrhea caused by non-toxigenic non-O1/non-O139 variants.

*Vibrio cholerae* in the viable but non-culturable (VBNC) state can express virulence factors required to produce infection (12). The VBNC cells have the capacity to revert to the culturable state and colonize the intestine (13) the mechanism of which is largely unknown (14). These organisms may go undetected if conventional culture based methods are used (15). Conventional identification of *V. cholera*, which may be done by biochemical tests, is time consuming and laborious. Available commercial biochemical identification systems, such as dipstick test used for the detection of O1 and O139 strains, are not always accurate (16). *V. cholerae* has been shown to possess similar biochemical properties with other species in the Genus *Vibrio* and *Aeromonas*, hence complicating an accurate identification (17).

Compared to conventional PCR, real-time PCR is less labor intensive, more safe, and rapid due to the elimination of gel electrophoresis. It has greater sensitivity and can detect minute amounts of target amplicons that might be missed by the conventional PCR. Real-time PCR can directly target genomic DNA and thus eliminate extensive incubation periods (18). Furthermore, VBNC cells can be detected which might be missed by culture-based methods. The *ompW* sequence is highly conserved among *V. cholerae* species belonging to different biotypes and/or serogroups (17). Hence, the *ompW* gene could be used as a target for species-specific detection, identification, and quantification.

A number of assays exist for the detection of *V. cholerae* (19–24) but many of these assays lack empirical data for reproducibility and repeatability. Some of these assays have not been validated in terms of detecting non-specific products that might accompany the amplification reaction. Furthermore, a number of assays are based on virulence factors that might not be present in certain strains and might yield false negative results.

The aim of this study was to develop an assay that detects and quantifies both O1/O139 and non O1/O139 disease causing strains of *Vibrio* spp. In addition, the assay would be able to quantify VBNC cells that cannot be detected or quantified by conventional methods.

**MATERIALS AND METHODS**

**Assay Controls and Growth Conditions**

A total of 28 bacterial strains were used as assay controls. *V. cholerae* strains were grown in alkaline peptone water for enrichment, and all other strains were grown in nutrient broth for 24 h in 37°C. Genomic DNA from overnight cultures controls were extracted and purified according to the manufacturer’s instructions by QIAamp DNA mini kit (Qiagen, Hilden, Germany).

**Sample Preparation and Spiking**

Four different types of samples were taken for experiment: (i) drinking water, (iii) pond water, (ii) boiled rice, and (iii) shrimp.

Rice sample were prepared by homogenization of 25 g of boiled rice with 225 μL of phosphate-buffered saline (1 L distilled H2O, 10 g L−1 NaCl, 0.25 g L−1 KCl g L−1, 1.8 g L−1 NaHPO4, 0.3 g L−1 KH2PO4; pH 7.4) in a Stomacher Lab Blender (Seward Stomacher® 80, Lab Biomaster, UK). Shrimp sample was also prepared by following the same procedure for rice. All of the samples were spiked with different concentrations of *V. cholerae* CT+ O139, *V. cholerae* CT+ O1, and *V. cholerae* CT− non-O1/non-O139. Prior to the evaluation of this assay for these environmental samples, absence of *V. cholerae* was confirmed by qPCR. DNA extraction was conducted using QiaAmp® DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instruction.

**PCR Primer Design**

The Outer Membrane Protein W-OMPW Sequence of eight reference strains (Table 1) was downloaded from the NCBI database. The primer design was accomplished by FastPCR 6.05 (PrimerDigital, Helsinki, Finland). Primers that conformed to the default and ideal range as stated by Kalendar et al. (25).

**Table 1 | *Vibrio cholerae ompW* sequences with their GenBank accession numbers used for primer designing.**

| Strain                                                    | Accession number |
|-----------------------------------------------------------|------------------|
| *Vibrio cholerae* strain 08-5735 ompW gene, partial cds  | FJ462446         |
| *V. cholerae* strain 08-5739 ompW gene, partial cds      | FJ462447         |
| *V. cholerae* strain 08-5738 ompW gene, partial cds      | FJ462448         |
| *V. cholerae* strain 08-5737 ompW gene, partial cds      | FJ462449         |
| *V. cholerae* strain ATCC 27070 ompW gene, partial cds   | FJ462450         |
| *V. cholerae* strain ATCC 5506 ompW gene, partial cds    | FJ462451         |
| *V. cholerae* strain 08-5742 ompW gene, partial cds      | FJ462453         |
| *V. cholerae* O1 strain N16961 ompW gene, complete cds   | KJ722608         |

**Table 2 | ompW gene primers used for real-time PCR along with their properties.**

| Sequence (5′–3′) | Length (nt) | Tm (°C) | PCR Fragment Size (bp) | Tend (°C) |
|-----------------|-------------|---------|------------------------|-----------|
| Forward         | 22          | 56.8    | 191                    | 61        |
| Reverse         | 20          | 55.8    |                        |           |

**Table 3 | Primer parameters obtained for the designed primers together with the default and ideal range as stated by Kalendar et al. (25).**

| Criteria       | Default | Ideal | Obtained |
|----------------|---------|-------|----------|
| Length (nt)    | 20–24   | >21   | Forward (22 nt) Reverse (20 nt) |
| Tm range (°C)  | 52–68   | 60–68 | Forward (56.8) Reverse (55.8) |
| Tm 12 bases at 3′ end | 30–50 | 41–47 | Forward (42.9) Reverse (41.3) |
| CG (%)         | 45–65   | 50    | Forward (47.7) Reverse (50.0) |
| Linguistic complexity (LC%) | >75    | >90   | Forward (95) Reverse (89) |
| Sequence quality (PQ%) | >70    | >90   | Forward (93) Reverse (87) |
TABLE 4 | Comparison of sensitivity of detection and precision of two replicate runs.

| Copy number | SD (n = 4) | Mean (n = 4) | Coefficient of variation (CV%) |
|-------------|------------|--------------|------------------------------|
| $5.46 \times 10^5$ | 0.229951 | 18.806 | 1.185533 |
| $5.46 \times 10^4$ | 0.04455 | 18.91 | 0.235588 |
| $5.46 \times 10^3$ | 0.099654 | 22.7365 | 0.438001 |
| $5.46 \times 10^2$ | 0.347915 | 27.26575 | 1.276015 |
| $5.46 \times 10^1$ | 0.175279 | 31.034 | 0.564796 |
| $5.46 \times 10^0$ | 0.280861 | 34.67725 | 0.809206 |
| $5.46 \times 10^{-1}$ | 0.517502 | 39.26467 | 1.317984 |

| SD (n = 4) | Mean (n = 4) | Coefficient of variation (CV%) | Inter-assay CV% |
|------------|--------------|-------------------------------|----------------|
| 0.196337 | 17.90175 | 1.096748 | 2.838649 |
| 0.089388 | 18.55025 | 0.481871 | 1.084382 |
| 0.382781 | 22.2215 | 1.722573 | 1.68125 |
| 0.190516 | 26.42575 | 0.720947 | 1.932086 |
| 0.196558 | 30.3475 | 0.647623 | 1.321016 |
| 0.382505 | 33.864 | 1.129533 | 1.558894 |
| 0.332131 | 36.731 | 0.904225 | 3.792876 |

**Figure 1** | Amplification plot ($\Delta Rn$ vs Cycle) for testing the sensitivity and precision of the first replicate run.

The forward and reverse primer sequences were checked, and the pair that had the highest identity with the Query Sequences (reference sequences) was selected for further analysis (Table 2).
Calculation of the Physical Parameters of Primers
Primer quality was calculated by the consecutive summation of the points according to the parameters: total sequence and purine–pyrimidine sequence complexity, the melting temperatures of the whole primer, and of the 12 bases from each of the terminal 3’ and 5’. The melting temperature of the 12 bases at the 3’ terminus is calculated by nearest neighbor thermodynamic parameters (26). Linguistic complexity measurements (Eqs 1–3) were performed using the alphabet-capacity L-gram method (27, 28). The Tm was calculated by the nearest neighbor thermodynamic parameters (26, 29). The optimal annealing temperature (Ta) was calculated by the Eq. 4 (30).

Real-time PCR Conditions
A Mastermix consisted of 12.5 µL 2x Power SYBR green® PCR master mix containing passive reference of ROX dye (Applied Biosystems, Life Technologies, Warrington, UK), 2.5 µL of 100 nM each sense and antisense primer, 2.5 µL of DEPC treated H2O, and 5 µL of template DNA. The thermal conditions were maintained under the following conditions: polymerase activation at 95°C for 5 min, followed by 40 cycles of 30 s at 95°C for
and 1 min at 60°C. The real-time PCR was performed using the machine Applied Biosystems StepOne™ (48-well).

**Specificity of the qPCR Assay**

In order to investigate the capability of the assay to distinguish between target and non-target, DNA from 10 isolates of *E. coli*, 5 isolates of *Enterococcus* spp., 6 isolates of *Salmonella* spp., 3 isolates from *Vibrio* spp., and 7 isolates of *V. cholerae* were used as templates. The concentration of all DNA samples from the isolates was kept almost same (approximately 10 ng/µL) by diluting with DEPC-treated water or concentrating by DNA concentrator (Eppendorf Concentrator 5301).

**Melt Curve Analysis and Detection of Non-Specific Products**

Four dilutions of two *V. cholerae* strains were subjected to qPCR as stated above, and the reaction mixtures containing the SYBR Green PCR products were gradually warmed to 95°C at a ramp rate of 0.3°C/s with continuous fluorescence acquisition. The melting curves were created by plotting the derivative reporter vs the temperature. The melting curve analysis was performed with duplicates of four serial dilutions of template DNA ranging from $10^0$ to $10^2$ gene copies per reaction using the ABI software version 2.2.2. The SYBR green PCR products were also resolved for identity in 1.5% agarose gel by electrophoresis.

**Sensitivity and Limits of Detection (LOD)**

The DNA sample of *V. cholerae* was then serially diluted (10-fold) upto 7-log$_{10}$ (5.46 × 10$^5$ copy numbers down to 5.46 × 10$^{-3}$) in DEPC-treated water. Five microliters from each dilution were used as template for detection. Distilled water was used as no template control.

**Calibration Standards for Standard Curves**

To estimate the number of cells in a reaction, the mass of a single bacterial genomic DNA was calculated. The genome size of one *V. cholerae* was 4,033,460 bp (NCBI Genbank10952301). The molecular mass of the genome was found by multiplying the genome size with the mass of base pair. The molecular mass
of *V. cholerae* was found to be 4.52 fg. The starting concentration of each stock DNA was measured by ColibriMicrovolume Spectrometer (Titertek-Berthold, Berthold Detection Systems GmbH, Bleichstrasse, Pforzheim, Germany) at absorbance 260 nm. To establish the number of cells in final reaction mixture, the stock concentration was divided by the molecular mass of the specific bacteria. The 7-log serial dilution (1:10) of the stock DNA was prepared in triplicate and the corresponding cell numbers were calculated in the final PCR reaction mixture.

### Repeatability and Reproducibility

The precision of the PCR assays was evaluated for dilutions ranging from $5.46 \times 10^5$ gene copies per reaction down to $5.46 \times 10^{-1}$ copy numbers. The dilutions were tested in four replicates in two separate PCR runs. The SD of the $C_T$ values of each concentration was then calculated by using Eqs 1 and 2.

$$SD = \sqrt{\frac{\sum (C_T - \overline{C_T})^2}{n}}$$  \hspace{1cm} (1)

where $\overline{C_T}$ is the mean $C_T$ value and $n$ is the number of observations. The value obtained was used to calculate the coefficient of variation, CV, with Eq. 2.

$$CV = \frac{SD}{\overline{C_T}}$$  \hspace{1cm} (2)

The intra-assay precision (repeatability) was assessed by calculating the coefficient of variation (CV%) for individual runs. The inter-assay precision (reproducibility) was calculated...
by determining the coefficient of variation (CV%) of both runs combined.

**Ethical Clearance**
The study did not involve any human or animal related issues. Therefore, we did not seek any ethical clearance in this study. Besides, the lab is facilitated with biosafety level II functions. The test and control strains of this study fall under the BSL II category.

**RESULTS**

**Physical Parameters of Primers**
The physical parameters of the primers obtained are summarized in Table 3. Sequence quality and $T_m$, 12 bases at 3’ end of both forward and reverse primers, LC and length of forward primer, and CG% of reverse primer were all in the ideal range (see Table 3). All the others parameters were within the default range.

**Repeatability and Reproducibility**
The intra- and inter-run precision obtained has been summarized in Table 4. The coefficient of variation for the first replicate varied from 0.24 to 1.32 and for the second replicate the CV% ranged from 0.48 to 1.1. The CV% for the inter-run reproducibility varied from 1.08 to 3.79. The amplification plot and standard curve have been shown (Figures 1–4).

**Sensitivity and LOD**
The LOD or analytical sensitivity was found to be 5.46 copies since among 8 replicates. The 5.46 was the lowest gene copies that were consistently detected. For higher dilution, i.e., 0.546 copy number, the assay failed to register a $C_T$ value in 2 of the 8 replicates.

**Specificity**
The assay registered $C_T$ values which ranged from 18.778 to 19.697 for the 4 *V. cholerae* strains and was detectable in the amplification plot (Figure 5). Two *E. coli* strains, EHEC and EIEC, had $C_T$ values of 35.073 and 38.439, respectively. The $C_T$ values for all other strains were undetermined. Strains which had $C_T$ values of less than 35 were considered as *ompW* positive. Hence, the assay was able to correctly detect *V. cholerae* and gave a negative result for all other strains, thus proving the assay was *V. cholerae* specific. The results have been summarized in Table 5.
Melt Curve Analysis and Detection of Non-Specific Products

In the melt curve (Figure 6), a single distinct peak was seen, indicating that all the PCR products had similar Tm values which was approximately 78.46°C. Agarose gel electrophoresis of SYBR green PCR products gave a single distinct band of about 191 bp (Figure 7). It could be concluded that neither secondary non-specific products nor primer dimers were formed.

DISCUSSION

We have developed a real-time assay with designed primers for the detection and quantification of *V. cholerae*. The assay was based on SYBR Green PCR Mastermix and targeted the *ompW* gene, which is present in all species of *V. cholerae*. Initially, the physical properties of primers were assessed, followed by validation of sensitivity, precision, specificity, and melt curve analysis.
Table 5 | Detection of ompW gene for specificity test.

| Sr. No. | Species | Collection or isolation number | Function of the strains | Origin | Ct value | ompW presence |
|---------|---------|--------------------------------|-------------------------|--------|----------|---------------|
| 1       | *E. coli* (E. coli) | ATCC AN33859 | Test strain | Clinical | U | – |
| 2       | *E. coli* EPEC | ATCC B170 | Test strain | Clinical | U | – |
| 3       | *E. coli* EAEC | ATCC MG1214C2 | Test strain | Clinical | U | – |
| 4       | *E. coli* ETEC | ATCC MGL-IC1 | Test strain | Clinical | U | – |
| 5       | *E. coli* EHEC | NF 9422 | Test strain | Clinical | U | – |
| 6       | *E. coli* | MMLA | Test strain | Clinical | 38.439 | – |
| 7       | *E. coli* EIEC | 2 V | Test strain | Clinical | U | – |
| 8       | *E. coli* ETEC | C600 | Test strain | Clinical | U | – |
| 9       | *E. coli* EIEC | H2 | Test strain | Clinical | U | – |
| 10      | *E. coli* EHEC | BH29 | Test strain | Clinical | 35.073 | – |
| 11      | *Enterococcus faecium* | T7 | Test strain | Environmental | U | – |
| 12      | *E. faecium* | B10 | Test strain | Environmental | U | – |
| 13      | *E. faecium* | B4 | Test strain | Environmental | U | – |
| 14      | *Enterococcus faecalis* | T11 | Test strain | Environmental | U | – |
| 15      | *E. faecalis* | B4PE | Test strain | Environmental | U | – |
| 16      | *Salmonella* spp. | 29 | Test strain | Food | U | – |
| 17      | *Salmonella* spp. | 36 | Test strain | Soil | U | – |
| 18      | *Salmonella* spp. | 19 (b) | Test strain | Food | U | – |
| 19      | *Salmonella enteritidis* | A | Test strain | Environmental | U | – |
| 20      | *Salmonella typhimurium* | Ito-3313 | Test strain | Environmental | U | – |
| 21      | S. typhimurium | S1 | Test strain | Environmental | U | – |
| 22      | *Vibrio parahaemolyticus* | 1 | Test strain | Environmental | U | – |
| 23      | *V. parahaemolyticus* | 3 | Test strain | Environmental | U | – |
| 24      | *Vibrio mimicus* | 1 | Test strain | Environmental | U | – |
| 25      | *V. cholerae* serotype O1 CT* | ATCC O6706 | Control strain | Clinical | 19.624 | + |
| 26      | *V. cholerae* (VC) serotype O1 CT* | ATCC N16961 | Control strain | Clinical | 19.324 | + |
| 27      | VC serotype O1 CT | ATCC SA 317 | Control strain | Clinical | 19.697 | + |
| 28      | VC serotype CT* O139 | ATCC NHOC0270 | Control strain | Clinical | 18.778 | + |
| 29      | *V. cholerae* non-O1 CT* | Lab isolate-2P-16 | Test strain | Environmental | 22.201 | + |
| 30      | *V. cholerae* non-O1 CT* | Lab isolate-2P-203 | Test strain | Environmental | 21.329 | + |
| 31      | *V. cholerae* non-O1 CT* | Lab isolate-M-299 | Test strain | Environmental | 23.706 | + |

*Reference strains: American Type Culture Collection, ATCC were collected from Laboratory of Molecular Genetics, International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B). Other isolates were obtained from clinical laboratories of ICDDR,B and Environmental Microbiology Laboratory of University of Dhaka. U, undetermined.
The LC describes nucleotide arrangement and composition of a sequence and the likelihood of PCR success of each primer. LC values of 80 and higher serve as excellent candidate primers. The primers developed had LC values of 89 and 95 for reverse and forward primers, respectively. Low-complexity regions such as Simple Sequence Repeats, imperfect direct or inverted repeats, triple-stranded DNA structures, and G/C quadruplexes were unlikely to be formed if primers with high LC values are present in the designed primers. C/G bases, recommended for increased PCR efficiency (33) were used for reverse and forward primers, respectively. Thus, these high values suggest that self-complementarity was not apparent. Two terminal C/G bases, recommended for increased PCR efficiency (33) were present in the designed primers.

The efficiency of a PCR assay is the amount of DNA that is amplified in each cycle. An efficiency of 100% indicates the target DNA has been doubled. The efficiencies obtained for the replicates 1 and 2 were 89.16 and 97.37%, respectively. Generally, efficiencies ranging from 90 to 100% are considered to be satisfactory. Inadequate primer design, production of non-specific amplicons and primer dimers may be responsible for reduced efficiencies (34). This is, however, only an estimate of the PCR efficiency and a real test sample, such as food, may contain inhibitory substances that decrease the PCR efficiency (35).

The precision of the assay was assessed by calculating both repeatability (intra-assay precision) and reproducibility (inter-assay precision). The coefficient of variation (CV%) for the repeatability ranged from 0.24 to 1.32 for both replicates. The CV% for the reproducibility varied from 1.08 to 3.79. The reproducibility is an important parameter since changed conditions such as different equipment and operators might affect the outcome. Pipetting and other human errors might account for poor precision. The precision usually increases with decreasing gene copy concentration (34) but this pattern was not observed for the developed assay. Retesting is required if the % CV of the PCR replicates exceeded 30% (36). All the CV% values for the assay were acceptable.

Specificity is ability to detecting chosen gene in the presence of non-specific DNA (34). The specificity is an important parameter since, in clinical and food samples, DNA from a wide range of organisms might be present. The developed assay was able to correctly detect the 7 V. cholerae and gave Ct values that ranged from 18.778 to 23.706. Though the assay did not give any Ct values for the 22 non-V. cholerae strains (Table 5), two E. coli strains—E. coli EIEC 2V, E. coli EHEC BH29 showed Ct values of 38.439 and 35.073 respectively. Since the cut point Ct value for ABI StepOne real-time machine is between >8 and <35, these Ct values of E. coli strains can be considered as negative results.

The LOD is the lowest gene copy number that the assay is able to consistently detect (37). A satisfactory LOD is 10 gene copies per reaction, and the assay was able to meet this requirement by consistently detecting 5.46 copies of the gene. The LOD sheds light on how sensitive the assay is.

The assay was evaluated for its ability to detect V. cholerae O1/O139 and non-O1/non-O139 in food and environmental samples over different dilutions. It was observed that drinking water, pond water, shrimp, and boiled rice spiked with these strains registered Ct values that ranged from 16.33 to 26.78 (Table 6).

To assess if the assay is affected by interference from non-target DNA, unspiked drinking water, pond water, shrimp, and boiled rice were examined by qPCR. Before this assessment, absence of V. cholerae was confirmed. Results showed that no Ct values were obtained for these unspiked food and water samples. Thus, this assay is suitable for detecting both V. cholerae O1/O139 and non-O1/non-O139 in food and environmental samples since non-specific amplification was not seen in negative controls.

Melt curve analysis was done to assess whether secondary products such as primer dimers or non-specific products were formed. The melt curve gave a single peak with a Tm value of about 78.46°C. Agarose gel electrophoresis of SYBR Green PCR products gave a single band at 191 bp. These results suggest that

| Sl. No. | Strain | Dilution | Ct | Sample type         |
|--------|--------|----------|----|---------------------|
| 1      | V. cholerae CT* O139 | 10^5 | 16.88 | Spiked drinking water |
| 2      | V. cholerae CT* O1  | 10^6 | 16.87 | Spiked drinking water |
| 3      | V. cholerae CT* O1  | 10^5 | 20.18 | Spiked drinking water |
| 4      | V. cholerae CT* O1  | 10^5 | 24.67 | Spiked drinking water |
| 5      | V. cholerae CT* non-O1/ non-O139 | 10^6 | 20.15 | Spiked drinking water |
| 6      | V. cholerae CT* non-O1/ non-O139 | 10^6 | 23.97 | Spiked drinking water |
| 7      | –      | –        | –  | Unspiked pond water |
| 8      | –      | –        | –  | Unspiked pond water |
| 9      | V. cholerae CT* O139 | 10^6 | 16.84 | Spiked pond water |
| 10     | V. cholerae CT* O1  | 10^6 | 16.84 | Spiked pond water |
| 11     | V. cholerae CT* O1  | 10^6 | 20.85 | Spiked pond water |
| 12     | V. cholerae CT* non-O1/ non-O139 | 10^6 | 26.81 | Spiked pond water |
| 13     | V. cholerae CT* non-O1/ non-O139 | 10^6 | 20.29 | Spiked pond water |
| 14     | V. cholerae CT* non-O1/ non-O139 | 10^6 | 24.83 | Spiked pond water |
| 15     | –      | –        | –  | Unspiked pond water |
| 16     | –      | –        | –  | Unspiked pond water |
| 17     | V. cholerae CT* O139 | 10^6 | 16.75 | Spiked boiled rice |
| 18     | V. cholerae CT* O1  | 10^6 | 16.37 | Spiked boiled rice |
| 19     | V. cholerae CT* O1  | 10^6 | 20.67 | Spiked boiled rice |
| 20     | V. cholerae CT* O1  | 10^6 | 24.19 | Spiked boiled rice |
| 21     | V. cholerae CT* non-O1/ non-O139 | 10^6 | 20.34 | Spiked boiled rice |
| 22     | V. cholerae CT* non-O1/ non-O139 | 10^6 | 26.78 | Spiked boiled rice |
| 23     | –      | –        | –  | Unspiked boiled rice |
| 24     | –      | –        | –  | Unspiked boiled rice |
| 25     | V. cholerae CT* O139 | 10^6 | 16.75 | Spiked shrimp |
| 26     | V. cholerae CT* O1  | 10^6 | 16.33 | Spiked shrimp |
| 27     | V. cholerae CT* O1  | 10^6 | 21.00 | Spiked shrimp |
| 28     | V. cholerae CT* O1  | 10^6 | 23.97 | Spiked shrimp |
| 29     | V. cholerae CT* non-O1/ non-O139 | 10^6 | 20.37 | Spiked shrimp |
| 30     | V. cholerae CT* non-O1/ non-O139 | 10^6 | 25.36 | Spiked shrimp |
| 31     | –      | –        | –  | Unspiked shrimp |
| 32     | –      | –        | –  | Unspiked shrimp |
| 33     | –      | –        | –  | No template control |
| 34     | V. cholerae CT* O1  | 10^6 | 10.13 | Positive control |
the amplification was specific and only one type of amplicon was produced. Non-specific products hamper the efficiency of the assay and affect precision. Non-specific products were absent suggests that the primer design was adequate. The primers were specific and intended amplicons were produced. We can conclude the primers were not complementary to one another since primer dimers were not produced.

Many assays have been developed for detection and quantification of \textit{V. cholerae} (19–24). Though impressive none of these presented any statistical figures (such as coefficient of variation) which would inform us about the reproducibility and repeatability. Many of these assays did not undergo melt curve analysis or the PCR products were not subjected to agarose gel electrophoresis and hence we do not know their status regard the formation of non-specific products. Since they are multiplex in nature, they add to the cost and hence are not suitable for purposes. For instance, during quality control testing of seafood where only quantification is required to see if the levels in food is acceptable to the standards set by the governing bodies.

An extremely impressive multiplex real-time assay has been developed by Bliem and colleagues (38). The assay is multiplex in nature, and hence the use of multiple primers might add to the cost. The assay developed by Bliem and colleagues had inter-assay variance of 2–28% for \textit{ompW}. But our assay, which utilizes a primer for \textit{ompW} gene with different sequence, was more precise with inter-assay variance of 1.08–3.79.

Future objectives of our study might include the optimization of this assay to detect and quantify \textit{V. cholerae} in food, water, and clinical samples. Some samples might contain inhibitory substances that decrease PCR efficiency (35) and hence optimization of the methods involving sample processing, DNA extraction, and assay itself might be required.

**AUTHOR CONTRIBUTIONS**

PJ and AB are the principal investigators of the project and contributed to the manuscript revision and final version approval to be published. RR conducted the study in the laboratory, performed statistical analysis, and wrote the first draft of the manuscript. ST contributed to revising the manuscript critically for important intellectual content. JF contributed to the study designing, implementation, manuscript reviewing, and revising it critically. The authors have agreed to be accountable for answering questions related to the accuracy and integrity of the work appropriately done.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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