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Optimization and Validation of Real Time PCR Assays for Absolute Quantification of toxigenic *Vibrio cholerae* and *Escherichia coli*

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Abstract. Quantitative real-time PCR (qPCR) is a dynamic and cogent assay for the detection and quantification of specified nucleic acid sequences and is more accurate compared to both traditional culture based techniques and 'end point' conventional PCR. Serial dilution of bacterial cell culture provides information on colony forming unit (CFU) counts. This is crucial for obtaining optimal standard curves representative of DNA concentration. This approach eliminates variation in the standard curves caused by loss of DNA by serial dilution of nucleic acid elute. In this study, an assay was developed to detect and quantify DNA by real-time PCR for two pathogenic species, *Escherichia coli* (*E. coli*) and *Vibrio cholerae* (*V. cholerae*). In order to generate a standard curve, total bacterial DNA was diluted in a 10-fold series and each sample was adjusted to an estimated cell count. The starting bacterial DNA concentration was 11ng/µL. An individual *E. coli* cell has approximately 5.16 femtograms of DNA. Therefore, 11 ng/µL of DNA would indicate 2.48×10^7cells. Both SYBR Green and TaqMan assays were validated for *uidA* region in *E. coli* and *ctxA* region in *V. cholerae*, respectively and was based on previously published assays for this standard curve experiment. PCR efficiency for *uidA* gene and *ctxA* gene were obtained 103.8% and 99.21%, respectively. Analysis of Variance (ANOVA) and coefficient of variation (CV %) indicated that standard curve generated by genomic DNA dilution had higher repeatability. Although not statistically significant, low F ratios indicated that there was some variation in C_T values when genomic DNA dilution was compared to dilution of cell suspension in media. Different water samples spiked with pure cultures of *E. coli* and *V. cholerae* were used as unknown samples. The standard curve constructed by the serial dilution of genomic DNA exhibited greater efficiency when compared to that of the standard curve obtained from serial dilution of cell suspension since in the former method DNA is not lost during extraction from culture dilutions.

INTRODUCTION

Diseases caused by consumption of water contaminated with bacteria such as *Escherichia coli* (*E. coli*) and *Vibrio cholerae* (*V. cholerae*) may result in serious illnesses like diarrhea, cholera, and may even lead to death (Hunter, 1997; Momba, Malakate & Theron, 2006; Swerdlow *et al.*, 1992). Studies suggest that adverse health effects associated with contaminated drinking-water are mainly due to the presence of human and animal fecal materials. Therefore, from a public health perspective, an indicator is necessary to confirm fecal pollution in water. Since monitoring the presence of all pathogens is not feasible. *E. coli*, a common inhabitant of the gastro intestinal (GI) tract of warm blooded animals (Hartl & Dykhuizen, 1984), is used as a surrogate for fecal contamination. In addition, *Vibrio cholerae*, needs to be

monitored for their presence in the environment due to their ability to cause epidemics of cholera.

Quantitative PCR (qPCR) allows the measuring of bacterial loads efficiently and rapidly with specific and sensitive detection compared to culture-based methods. It is designed to quantify microorganisms by directly targeting genomic DNA and can yield results within a few hours (Noble & Weisberg, 2005) by eliminating steps requiring lengthy incubation. In this study, estimation of fecal indicator bacteria (*E. coli*) was obtained by targeting *uidA* gene (encoding β-glucuronidase) and the assay was developed by using SYBR green qPCR assay. Likewise, for detection of *Vibrio cholerae*, *ctxA*-gene (encoding the A subunit of cholera toxin present in Serogroup O1 andO139) was targeted with TaqMan qPCR assay. This assay was found more specific, sensitive and rapid for detection of toxigenic *Vibrio cholerae* compared to conventional-PCR and culture-dependent methods (Chapela *et al.*, 2010).

Generally, typical qPCR methods for quantification of microorganisms, are performed on the genomic DNA isolated from serial dilution of cell suspensions. A disadvantage of this method is that the standard curve attained from serial dilutions of the samples produce R^2 value which is not a 'best fit' for the quantification of unknown samples. So, in this study, we assessed the relative performance of standard curves by direct 10-fold serial dilution of genomic DNA without the dilution of cell suspension. The objectives of this study were to generate a standard curve for *E. coli* thereby enabling one to quantify indicator microorganism in water, to implement a standard curve for the quantification of toxigenic *Vibrio cholera*e, and to compare the accuracy and efficiency of quantification of DNA templates by dilution of DNA and dilution of cell suspension. This study involved simultaneous application of qPCR analysis and culture based quantification. The two methods were compared to identify the best approach for the quantification of bacteria in the original samples.

METHODS AND MATERIALS

Study Design

The qPCR method involved 21 DNA samples consisting triplicates of each of seven variable amounts of DNA samples for standard curve calibration. This method was applied for both *Escherichia coli* B170 and *Vibrio cholerae* O1 biotype el Tor N16961 which produced two different standard curves with different efficiencies and R^2 values. The quantity of bacteria in the unknown samples was estimated from the standard curve. In the culture based method, 1mL bacterial culture of target organism was serially diluted 10-fold to yield 10^7 down to 1 CFU/mL and plated on to Nutrient Agar (NA) in triplicate. After overnight incubation, colony forming units (CFU) were recorded. The starting stock bacterial culture was the same for both, qPCR and culture based method.

DNA extraction

Genomic DNA from overnight cultures of *Vibrio cholerae* and *E. coli* strains were extracted and purified according to the manufacturer's instructions by QIAamp DNA mini kit DNA mini kit (Qiagen, Hilden, Germany). The concentrations and purity of extracted DNA samples were measured at 260nm using Colibri Microvolume Spectrometer (Titertek-Berthold, Berthold Detection Systems GmbH, Bleichstrasse, Pforzheim, Germany). DNA templates were stored at -20°C until further use.

Calibration standards, controls, and standard curves

Prior to qPCR, a range of calibration standards and controls were prepared. Strains used for calibration standard were *E. coli* (ATCC B170) and *Vibrio cholerae* (N16961). Relative standards were prepared using 7-$\log_{10}$ serial dilution (1:10) of DNA isolated from these strains. The starting concentration of each stock DNA was measured by Colibri Microvolume Spectrometer. This value was then divided by the dilution factor of each consecutive DNA sample to find the concentrations of the remaining diluted DNA standards using the standard dilution formula, $C_1V_1=C_2V_2$.

To estimate the number of cells in a reaction, the mass of a single bacterial genomic DNA was calculated. Genome size of one *E. coli* and *Vibrio cholerae* were 4527247 bp (NCBI Genbank HG738867.1) and 4,033,460 bp (NCBI Genbank10952301), respectively. To calculate molecular mass, we used the following formula:

E. coli genome size = 4527247 bp

Average mass of a base pair = 675 Dalton

Mass of one *E. coli* genome
= (4527247 × 675) Dalton = 3055891725 Dalton
= $3055891725 \times 1.66 \times 10^{-24}$ gram
[*Note: 1 Dalton = 1.66×10^{-24} gram]
= $5072780263.5 \times 10^{-24}$ gram = 5.07×10^{-15} gram = 5.07 fg

Using this formula molecular mass of *Vibrio cholerae* was found 4.52 fg. Our measured concentration in the starting stock bacterial DNA was 11ng/µL which measured 0.1pg/µL in the final PCR reaction mixture. To find the number of cell number in reaction mixture, this 11ng/µL concentration was then divided by the molecular mass of the specific bacteria. After triplicate 7-log serial dilution (1:10) of the stock DNA, the equivalent cell numbers were calculated in the PCR reaction mixtures.

Reference genes, qPCR Assays and Reaction Conditions

Genome annotation report for *uidA* and *ctxA* were found only once on GenBank where *uidA* and *ctxA* genes were present as 1 copy number per genome (http://www.ncbi.nlm.nih.gov/nuccore/556503834?report=genbank). Sequences of primers and probes for *ctxA* and *uidA* genes were obtained from previous studies (Table 1). The probe for *ctxA* was validated by labelling FAM at the 52 end and a Black Hole Quencher 1 at the 32 end (Tag, Copenhagen-Oligo, Denmark).

In our experiment, we used existing TaqMan Universal Master Mix and SYBR Green Master Mix (Applied Biosystems, Life Technologies, Warrington, UK) for two of the target organisms. The qPCR was performed on Applied Biosystems StepOne™ (48-well) Real Time PCR systems.

The final *ctxA* reaction mixture (25µL) contained 12.5 µL 2XTaqMan® Universal Master Mix II (pre-mixed with passive reference dye ROX), 2.5µL of 100nM each

Table 1. List of primers, probes and their sequences

Target species and gene	qPCR Assay Mix	Sequences of Primers and Probe (52 to 32) and size	Amplicon length (bp)	References
Escherichia coli ATCCB170 (*uidA*)	*Power SYBR green® PCR master mix*	UAL1939b (terminal sense) 5'-ATGGAATTTCGCCGATTTTGC-3' (21-mer) UAL2105b (terminal antisense) 5'-ATTGTTTGCCTCCCTGCTGC-3' (20-mer)	187	(Heijnen & Medema, 2006
Vibrio cholera serotype O1 CT+ATCC N16961 (*ctxA*)	*TaqMan® Universal Master MixII with UNG*	ctxA (terminal sense) 5'-TTTGTTAGGCACGATGATGGAT-3' (22-mer) ctxA (terminal antisense) 5'-ACCAGACAATATAGTTTGACCCAC TAAG-3' (28-mer) ctxA (probe) FAM-TGT TTC CAC CTC AAT TAG TTT GAG AAG TGC CC- BHQ-1 (32-mer)	84	(Blackstone *et al.*, 2007)

sense and antisense primer, 2.5 µL 250nM probe and 5 µL of template DNA. PCR was performed under the following thermal conditions: UNG incubation at 50ºC for 2 min, polymerase activation at 95ºC for 10 min, followed by 40 cycles of 15 sec at 95ºC, and for extension, 1 min at 60ºC.

Similarly, the reaction mixture (25µL) for *uidA* contained 12.5 µL 2XPower SYBR green® PCR master mix (contains a propriety version of ROX dye), 2.5µL of 100nM each sense and antisense primer, 2.5 µL of Diethylpyrocarbonate treated H_2O and 5 µL of template DNA. The thermal conditions were maintained as following: polymerase activation at 95ºC for 5 min, followed by 40 cycles of 30 sec at 95ºC and extension for 1 min at 60ºC.

All primers and probes were purchased from Tag Copenhagen-Oligo, Denmark.

Analytical Sensitivity and Limits of Detection

The DNA sample was then serially diluted (10-fold) up to 7- $\log_{10}$ (10^7 CFU/mL down to 1 CFU/mL) in DEPC treated water. Five microliters from each dilution was used as template for detection. DNA from *E. coli* and distilled H_2O were used as negative control and no template control (NTC), respectively. For sensitivity, detection of *E. coli* possessing the *uidA* gene, the limit of detection was obtained by using the aforementioned procedure and DNA template from *Vibrio cholerae* O1 cells was used as negative control.

Analytical Specificity of the qPCR assay

In order to investigate the specificity of developed qPCR assay for detecting the chosen genes in presence of non-specific DNA, 27 DNA from isolates (as shown in Table 6) were used as templates.

Statistical analysis

In order to test the variation between the assays, Analysis of Variance (ANOVA) was performed using SPSS Version 16 (IBM, USA). The F ratio and corresponding P values were found to test the significance of variation.

RESULTS

Quantification of target organism in unknown samples

To verify the reliability of the qPCR assays used in this study, water sample spiked with 10µL of culture was used as unknown sample. Initially, copy number of each bacteria was determined by mass conversion of total DNA and the standard curve was obtained for 7-log 10-fold dilution of DNA. From this standard curve, a set of 10-fold dilution of bacterial DNA from the estimated copy number of bacteria from each dilution of pure culture of bacterial DNA was used as standard. Acquired number of copies for the unknown samples were 3.2×10^4 (*Vibrio cholerae*) and 7.6×10^3 (*E. coli*) from the developed standard curves.

Sensitivity and limit of detection

The limit of detection and sensitivity of the designed qPCR assays for both of the target organisms were determined. For both of the assays, lowest amount of DNA were restricted to approx. 0.1 pg in the diluted DNA from a starting amount of 11ng. For the successive increase of DNA concentration, C_T value increased by 3 cycles which allowed detection of 0.1pg of DNA to 11ng of DNA. The results obtained is summarized in Table 2 & Table 3.

Specificity of the two qPCR assays

Primers and probes employed in the TaqMan and SYBR green assay demonstrated PCR efficiencies of 99.21% and 103.80%, respectively (Table 2). Amplification was observed for *ctxA* harbouring *Vibrio cholerae* strains. The results have been summarized in Table 6. To observe the specificity of this assay, melt curve analysis was carried out for the 6-log 10-fold dilutions of *E. coli* DNA, which gave dissociation at the same temperature (82.80ºC). No other peaks were observed for *E. coli* which implies that neither non-specific products nor primer dimers were present. Multiple peaks would indicate that more than one product was formed.

Table 2. Variation of regression correlation, amplification efficiency, sensitivity of qPCR for two different organisms

Species	Correlation Co-efficient (R^2)	Amplification Efficiency (%)	Slope	Specificity	Limits of Detection
Vibrio cholerae	0.99	99.21	-3.34	100%	0.112 pg DNA
E. coli	0.99	103.80	-3.23	100%	0.114 pg DNA

Table 3. Comparison of sensitivity of detection from 10-fold dilution series of pure genomic DNA and 10-fold dilution of cell suspension DNA for *Vibrio cholerae* (A) and *E. coli* (B)

Vibrio cholerae

Genomic DNA (g-DNA)				Cell suspension		
Weight/25µl reaction mix	CT ± SD (n = 3)	Coefficient of variation (CV %)	Estimated copy number	CFU/ml	CT ± SD (n = 3)	Coefficient of variation (CV %)
11.2 ng	17.50±0.10	0.57	2.48×10^{7}	5.2×10^{5}	19.47±1.28	8.62
1.12 ng	20.29±0.09	0.45	2.48×10^{6}	5.2×10^{4}	20.01±0.72	3.6
112 pg	23.67±0.04	0.17	2.48×10^{5}	5.2×10^{3}	24.10±1.98	8.22
11.2 pg	27.09±0.05	0.18	2.48×10^{4}	5.2×10^{2}	30.15±1.73	5.73
1.12 pg	30.54±0.09	0.29	2.48×10^{3}	5.2×10^{1}	34.43±0.84	2.44
0.112 pg	33.82±.09	0.27	2.48×10^{2}	5.2×10^{0}	37.51±0.11	0.29

(A)

E. coli

Genomic DNA (g-DNA)				Cell suspension		
Weight/25µl reaction mix	CT ± SD (n = 3)	Coefficient of variation (CV %)	Estimated copy number	CFU/ml	CT ± SD (n = 3)	Coefficient of variation (CV %)
11.4 ng	14.71±0.08	0.54	2.21×10^{7}	2.3×10^{5}	12.11±1.32	10.9
1.14 ng	17.01±0.02	0.12	2.21×10^{6}	2.3×10^{4}	14.94±0.43	2.88
114 pg	20.17±0.05	0.24	2.21×10^{5}	2.3×10^{3}	20.77±3.32	15.98
11.4 pg	23.92±0.07	0.29	2.21×10^{4}	2.3×10^{2}	28.46±2.84	9.98
1.14 pg	26.55±0.08	0.3	2.21×10^{3}	2.3×10^{1}	29.47±1.85	6.28
0.114 pg	29.39±0.02	0.07	2.21×10^{2}	2.3×10^{0}	30.68±1.79	5.83

(B)

Table 4. Reproducibility of Real Time Assays

	Genomic DNA dilution	Cell suspension
Vibrio cholerae TaqMan Assay	F = 6.126E-4 P value = 0.999 Up to dilution 10E-7	F = 0.121 P value = 0.887 Up to dilution 10E-5
Escherichia coli SYBR Green assay	F = 1.681E-5 P value = 0.999 Up to dilution 10E-8	F = 0.026 P value = 0.974 Up to dilution 10E-8

Table 5. Comparison between Genomic DNA dilution and Cell Suspension in Real Time Assays

	Replicate 1	Replicate 2	Replicate 3
Vibrio cholerae Taqman assay	F = 0.561 P value = 0.475 Up to dilution 10E-5	F = 0.038 P value = 0.849 Up to dilution 10E-5	F = 0.107 P value = 0.752 Up to dilution 10E-5
Escherichia coli SYBR green assay	F = 0.354 P value = 0.561 Up to dilution 10E-8	F = 0.342 P value = 0.568 Up to dilution 10-8	F = 0.663 P value = 0.429 Up to dilution 10E-8

Table 6. Detection of *ctxA* gene and *uidA* gene for specificity test

Sr#.	Species	Collection or Isolation number	Origin	*ctxA* presence	*uidA* presence
1	*Escherichia coli* (*E. coli*)	[a]ATCC AN33859	Clinical	–	+
2	*E. coli* EPEC	ATCC B170	Clinical	–	+
3	*E. coli* EAEC	ATCC MG1214C2	Clinical	–	+
4	*E. coli* ETEC	ATCC MGL-IC1	Clinical	–	+
5	*E. coli* EPEC	AE3171	Clinical	–	+
6	*E. coli* EHEC	NF 9422	Clinical	–	+
7	*E. coli*	MMLA	Clinical	–	+
8	*E. coli* EIEC	2V	Clinical	–	+
9	*E. coli* EHEC	NF 9877	Clinical	–	+
10	*E. coli* ETEC	C600	Clinical	–	+
11	*Enterococcus faecium*	T7	Environmental	–	–
12	*Enterococcus faecium*	B10	Environmental	–	–
13	*Enterococcus faecium*	B4	Environmental	–	–
14	*Enterococcus faecalis*	T11	Environmental	–	–
15	*Salmonella* spp	29	Food	–	–
16	*Salmonella* spp	36	Soil	–	–
17	*Salmonella* spp	19 (b)	Food	–	–
18	*Salmonella enteritidis*	A	Environmental	–	–
19	*Salmonella typhimurium*	Ifo-3313	Environmental	–	–
20	*Vibrio parahaemolyticus*	1	Environmental	–	–
21	*Vibrio parahaemolyticus*	2	Environmental	–	–
22	*Vibrio parahaemolyticus*	3	Environmental	–	–
23	*Vibrio cholerae* serotype O1 CT^+	ATCC C6706	Clinical	+	–
24	*Vibrio cholerae* (*VC*) serotype O1 CT^+	ATCC N16961	Clinical	+	–
25	*VC* serotype nonO1 CT^-	ATCC 4460	Clinical	–	–
26	*VC* serotype O1 CT^-	ATCC SA 317	Clinical	–	–
27	*VC* serotype O1 CT^+ O139	ATCC NIHC0270	Clinical	+	–

[a]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.

DISCUSSION

To assess the microbiological quality water, real-time quantitative PCR demonstrates quantification of gene targets with higher sensitivity, specificity, and is more time efficient compared to traditional end-point PCR or conventional culture based methods. One of the disadvantages of culture-based methods is that injured cells or cells that have

evolved into viable but non-culturable state are not detectable and therefore cannot be enumerated by culture based methods (Pommepuy *et al.*, 1996). Another limitation is that a lengthy incubation period renders the protocol cumbersome. In comparison, while traditional end-point PCR improves detectability, an additional step of gel electrophoresis is required and accurate quantification cannot be achieved.

Quantification of bacterial cells by current qPCR methods primarily depends on the correlation between CFU counts obtained from culture plates with the C_T values associated with the DNA from culture suspension. Our study investigated both the correlations of C_T versus CFU counts by plate method and C_T versus serial dilutions of genomic DNA. A critical parameter to evaluate PCR efficiency is measured by a regression coefficient (R^2 value) that defines the closeness of data to the fitted regression line. R^2 value close to 1 indicates good PCR efficiency. We found higher R^2 value of C_T versus serial dilutions of genomic DNA than the R^2 value (Figure 2, 3) the former one for both of the target bacteria. Moreover, the estimated bacterial counts we found from the g-DNA dilution were higher (Table 3) in several magnitudes (i.e copy number ranged from 2.48×10^7 to 248) than the plate counts (corresponding CFU ranged from 5.2×10^5 to 52) for each bacterium since stress-induced VBNC cells are undetectable in plate counts. This result indicates that dependence on traditional plate count might result in the underestimation of potentially infectious bacterial cells in food and water (Lyon, 2001). Higher sensitivity of detection from direct g-DNA dilution was observed when compared with dilution of cell suspension DNA. The C_T value of the lowest concentration of DNA (0.1 pg) was 33.82±0.09 for genomic DNA whereas the C_T value of the lowest concentration of cell suspension exceeded the cut-off value for positive sample detection

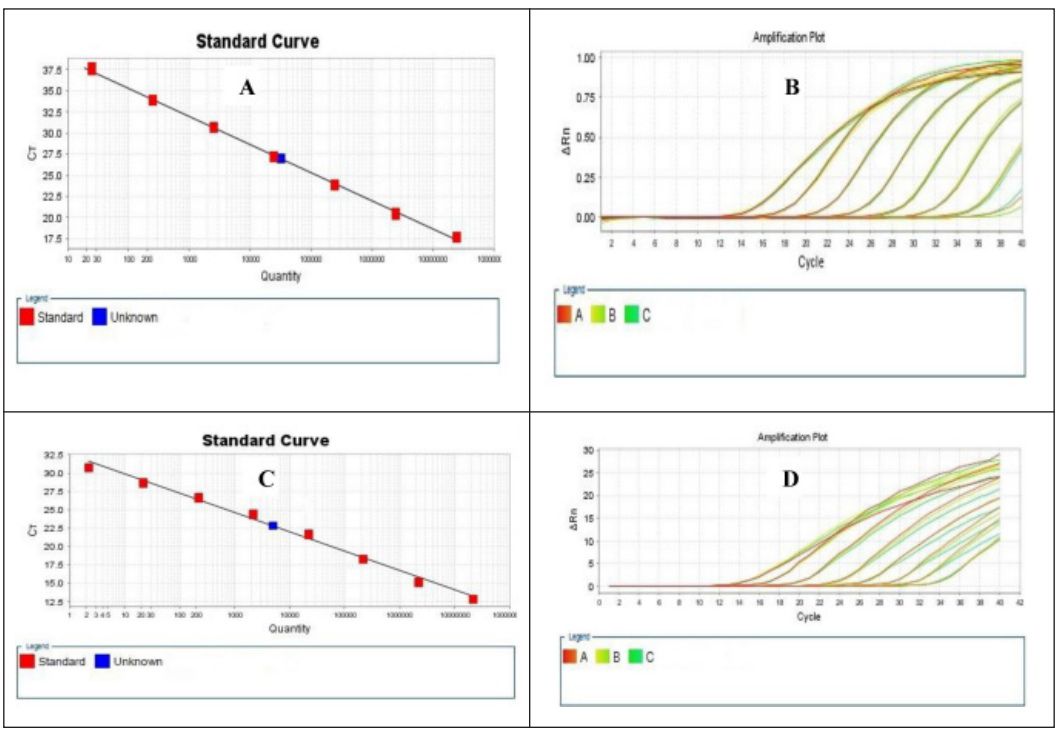

Figure 1. Standard curve and amplification plot of 10-fold series dilution for *Vibrio cholerae* and *E. coli*: Standard curves are plotted in C_T (cycle threshold) vs estimated copy number of each dilution. Amplification plots are in cycle vs "Rn. Standard curve and amplification plot of *uidA* - A, B and *ctxA*-C, D.

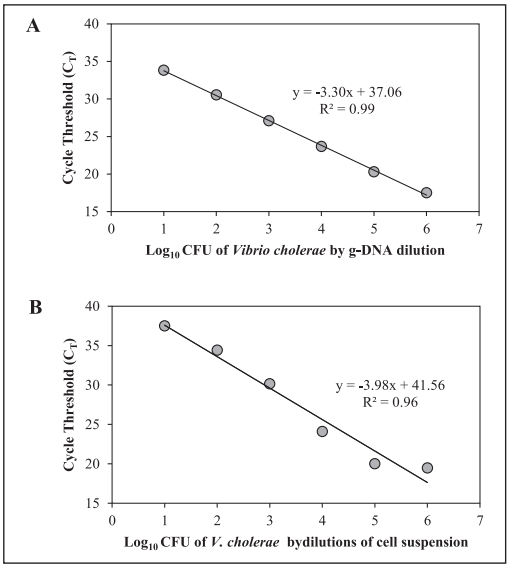

Figure 2. Analysis and comparison of two different types of dilutions for standard curve generation using target organism *Vibrio cholerae* N16961. (A) g-DNA of *Vibrio cholerae*. (B) Cell suspension of *Vibrio cholerae* Standard samples are represented by grey circles (A, B).

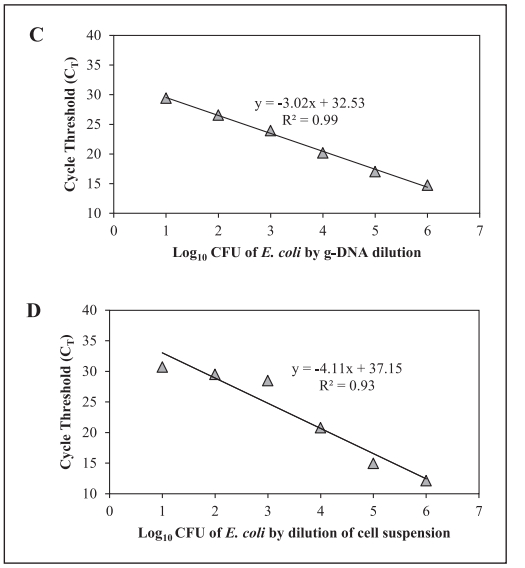

Figure 3. Regression analysis of standard curves generated by two different types of dilutions for *E. coli* B170. (C) g-DNA of *E. coli* (D) Cell suspension of *E. coli*. Standard samples are represented by grey triangles (C, D).

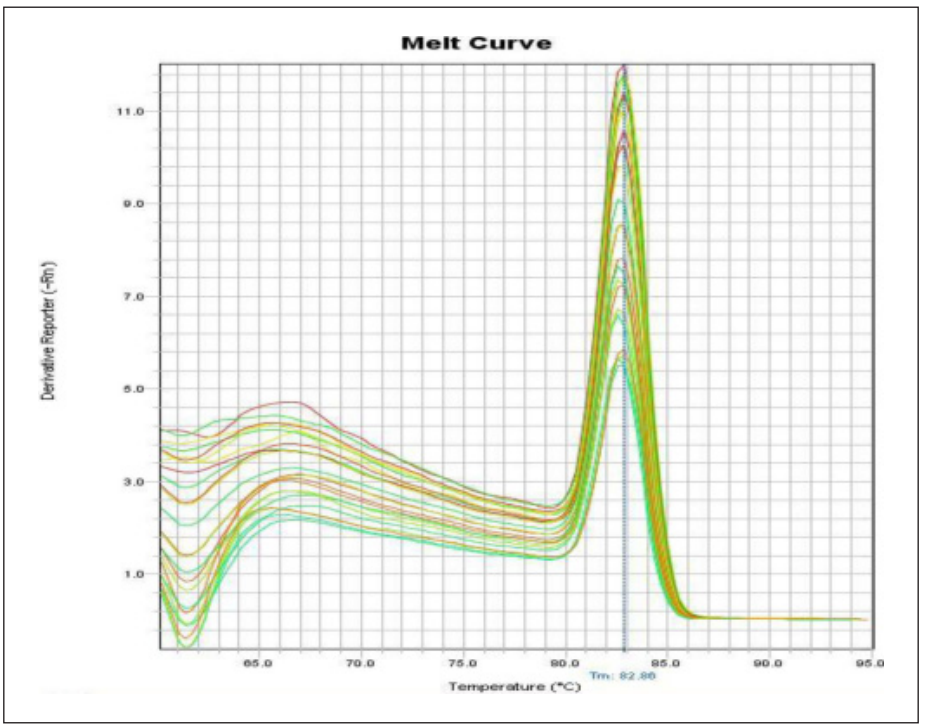

Figure 4. Melt curve analysis for *E. coli*. Dissociation was observed at 82.80°C for all dilutions of *E. coli* DNA.

(37.51±0.11>35). This difference may be due to the loss of DNA during sample processing for DNA extraction.

For analyzing the repeatability of assay, Analysis of Variance (ANOVA) was carried out to see if there were variations in the C_T values between the replicates. A lower F ratio indicates lower variation between the replicates and hence higher repeatability. In the case of the *ctxA* TaqMan assay, the F ratio observed for the genomic DNA dilution and cell suspension dilution was 6.126E-4 and 0.121, respectively. The coefficient of variation ranged from 0.17–0.57 for the genomic dilution method and for the cell suspension dilution method it ranged from 0.29–8.62. For the *uidA* SYBR Green assay the observed F ratio for the genomic DNA dilution and cell suspension dilution was 1.681E-5 and 0.026 respectively. The coefficient of variation ranged from 0.07–0.54 for the genomic dilution method and for the cell suspension dilution method it ranged from 2.88–15.98. In both cases it was seen that the F ratio and the CV% were lower for the genomic DNA dilution compared to the cell suspension dilution. The genomic DNA dilution method for the TaqMan assay exhibited higher repeatability at higher dilutions (10E-7) compared to the cell suspension dilution which was not reproducible beyond a dilution of 10E-5. This shows the former method can detect DNA at lower concentrations. However the consistency of detection for the SYBR green assay was same for *Escherichia coli* and *Vibrio cholerae*.

In case of variation between the genomic DNA dilution and cell suspension dilution, some variation was seen. Hence there is evidence to suggest that there will be no variation between replicates TaqMan and SYBR green assay. For the *ctxA* TaqMan assay, the F ratios that were obtained when comparing the genomic DNA dilution with the cell suspension dilution for replicates 1, 2, 3 were 0.561, 0.038 and 0.107 respectively. For the *uidA* SYBR green assay, the F ratios that were obtained when comparing the genomic DNA dilution with the cell suspension dilution for replicates 1, 2, and 3 were 0.354, 0.343 and 0.663 respectively.

For the health and well-being of individuals in a community, quantitative assessment of potentially hazardous pathogens is essential (Haas, Rose & Gerba, 1999). Our study sought an effective quantification technique of bacterial number which relied on only g-DNA dilution and excluded the necessity of quantitative approach by culture suspension. The calibrator control equivalents achieved by direct dilution of DNA for standard curve generation reduced the time of analysis since it only requires the preparation of stock DNA followed by serial dilution. In case of standard curve generated from cell suspension, each of the dilutions of culture suspension needs to be processed for DNA extraction. This study was also found to be suitable for analysis of wide range of samples for example rice, PBS, water and can be implemented when the number of samples is high (data not shown here).

The choice of the gene target is also an important factor for precise quantification of bacterial cells in samples. *Vibrio cholerae* is commonly present in many tropical and temperate regions of aquatic environments (R. Colwell, Kaper & Joseph, 1977; R. R. Colwell *et al.*, 1981; Islam *et al.*, 1994; Kaper, Lockman, Colwell, & Joseph, 1979) and strains harboring *ctxA* gene is a major public health concern. Some of the previous works on *Vibrio cholerae* by qPCR involved detection of multiple genes, for instance sequences encoding repeat in toxin, extracellular secretory protein, mannose-sensitive pili and the toxin coregulated pilus (Gubala, 2006) while our study targeted single gene *ctxA*, since it discriminates between toxigenic and non-toxigenic strains. Moreover, database searches demonstrated the presence of single copy per genome of *ctxA* gene and has high specificity for the toxigenic strains (Blackstone *et al.*, 2007). Similarly, for detection of *E. coli* by qPCR, many of the studies targeted 16S rDNA gene (Nadkarni, Martin, Jacques & Hunter, 2002) the internal transcribed spacer region and the 23S rRNA gene (16S-ITS-23S gene region; (Khan *et al.*, 2007). In all known human bacterial pathogens, 16S rRNA gene is present in multiple copies (Brosius, Dull,

Sleeter & Noller, 1981) and thus species-specific discrimination and quantification remains questionable in a heterogeneous DNA sample. Another study, revealed that the internal transcribed region (ITS), flanking the conserved regions of 16S rRNA gene and 23S rRNA gene having poor specificity of 85.9% (Maheux *et al.*, 2009). In the present study, our gene of interest for detection and quantification of *E. coli* was *uidA* which was found single copy per genome by database search of previously published study (Taskin, Gozen & Duran, 2011) and exhibited 100% specificity (Maheux *et al.*, 2009). In order to calculate the exact number of bacterial cell, single copy gene was chosen for each of the target organisms.

The use of TaqMan assay for detection of pathogen was more sensitive compared to SYBR Green assays. This is because additional probe is used in the reaction system. Furthermore, non-specific amplified products can increase the fluorescence due to the non-specific incorporation of the SYBR Green dye into double-stranded DNA (Bel, Ferré & Escriche, 2011). The purpose of our study was to quickly screen samples *E. coli* and *Vibrio cholerae* and estimate cell numbers, thereby allowing rapid analysis before consumption of food and water samples. Hence, optimization of protocols for the generation of the standard curve was imperative to quantify the bacterial load in samples.

Our investigation suggests the need for a standard curve generated from dilution of genomic DNA over the standard curve generated from cell suspension dilution as this method is capable of accurate and rapid quantification of bacterial pathogens in a range of environmental samples.

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