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The Development and Application of DDPCR Technology on Quantification of Total Coliforms in Water

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Abstract: In this research, the detection method for absolute quantification of total coliforms was established based on Droplet Digital Polymerase Chain Reaction (DDPCR) technology using *lacZ* as the target gene for coliform group detection. The experimental conditions (e.g. primer and probe concentrations, annealing temperatures, *etc*) were well optimized. Besides, the linear range, precision and limit of quantification (LOQ) of this method were investigated and evaluated. The results illustrated that the optimal primer concentration was 0.2 μmol/L, whereas the optimal probe concentration was 0.5 μmol/L. The optimal annealing temperature was 56°C. The linear relationship between the total coliform genome DNA concentrations derived from DDPCR and DNA fluorometer was quite good (R^2 = 0.999). The linear range was 3.95 ~ 7.80 × 10^4 copies/20 μL DDPCR reaction system. The LOQ for total coliforms was single copy per reaction system. Practical applications using real water samples collected from water supply system in Macao illustrated that this innovative method possessed high efficiencies and capabilities. This is probably the first research using DDPCR technology to absolutely qualify and quantify total coliforms and successfully applied it in Macao water supply system. The achievements from this research could provide with significant values for setting-up the emergency mechanism of water pollution in early stage.

Keywords: Total Coliforms, DDPCR, Absolute Quantification, Water Supply, Water-borne Pathogen

1. Introduction

Rapid economic development and its corresponding population explosion are putting incredible strains on our environment. Once water-borne pathogens enter water supply system, it may cause threats to public health and even lives [1]. Therefore, water quality and consequent safety problems have always been our top concerns. Total coliforms, as microbial indicators, have been selected and widely applied to suggest the presence of pathogens and thus to assess the microbiological quality of water [2-6].

Internationally recognized total coliforms detection methods are mainly membrane filtration [7, 8], multiple-tube fermentation [9, 10], immunomagnetic separation [11, 12], *etc*. Even though these traditional methods have been regarded as the "Golden Standards" worldwide, they pose disadvantages such as longer detection period (several days or a dozen of days), tedious operation procedures (requirement for verification experiments), failure to detect damaged microbes, lower sensitivity and potential biohazards, *etc* [13-16]. Therefore, these methods cannot meet the requirement for rapid diagnosis on water quality in water supply system, especially when the water quality suddenly changes, or customer complains. The selection and setup of a safer, faster and simpler detection method with higher specificity and sensitivity is of most significance and urgency. It has been the innovation "hotspot" among water industries [17-20].

Molecular methods, especially polymerase chain reaction (PCR) technologies are assuming a dominant place in modern diagnostic microbiology because of its rapid detection and higher specificity and sensitivity. Thus, PCR-based technologies have been applied in a wide range

of areas, such as clinics, pharmacy, environment, food, agriculture, *etc* [21-24]. So far, water quality monitoring and particularly microbial source identification rely heavily on real-time PCR (qPCR) method since the detection targets could be quantified [25-27]. In qPCR, quantification is accomplished through comparing the quantification cycle (Ct) results from a sample with unknown detection targets. Despite its wide acceptance, the accuracy and precision of this technology are highly affected due to its intrinsic constraints, such as requirement for standards of known concentration, inhibition of PCR amplification due to substances within water samples, *etc* [28, 29].

Droplet digital PCR (DDPCR) is the most advanced generation PCR without the need for calibration curves. Specifically, due to "water-in-oil" technology, each assay is partitioned into approximately 20,000 highly uniform nanoliter droplets, such that each droplet in the emulsion is an independent reaction and all the droplets are assorted in random fashion. After PCR amplification, the fluorescence of each droplet is individually measured and defined as either positive (presence of detecting target) or negative (absence of detecting target) events. The quantification results can then be obtained directly from the ratio of positive events to total partitions, based on binominal Poisson statistics. This technology can not only shorten the detection period (several hours), but also enhance the sensitivity and absolutely quantify the pathogens. Thus, DDPCR could introduce new level of advanced water-borne pathogen detections in both qualification and quantification [30-36]. However, seldom researches apply the DDPCR technology in pathogen detections in water environment.

2. Objectives

In this research, the DDPCR detection method for absolute quantification of total coliforms in Macao water supply system was established by selection of *lac*Z as the target gene for coliform group detection. By rapid diagnosis of water-borne pathogens (e.g. total coliforms), water treatment plant could take measures to improve the water quality at priority, thus avoiding unnecessary and inestimable lost. The main objectives are:

(1) to setup the DDPCR detection method by optimizing the experimental parameters, such as reagent concentrations, annealing temperatures, *etc*.,

(2) to verify the capabilities and efficiencies of optimized DDPCR detection method using practical water samples from local water supply system,

(3) to work as a demonstration role in setting up the emergency mechanism on microbiological water pollution in Macao.

3. Materials and Methods

3.1. Materials and Reagents

Primers and Taqman-MGB probes were synthesized by Integrated DNA Technologies (Coralville, IA, USA) unless otherwise mentioned. PowerSoil® DNA Isolation Kit (MOBIO, USA) was used for coliforms DNA extraction. The cellulose esters membrane which had a pore size of 0.45 μm (Merck Millipore, Germany) was used for sample filtration. All the reagents and consumables related to DDPCR were purchased from Bio-Rad (Pleasanton, CA, USA).

3.2. Instruments

The Qubit® 3.0 fluorometer (Life Technologies, USA) was used to measure the DNA concentrations after extraction. QX200^{TM} DDPCR system which includes manual droplet generator, C1000 touch thermal cycler, PX1 PCR plate sealer, droplet reader and QuantaSoft v1.7.4 software was purchased from Bio-Rad (Pleasanton, CA, USA). Rainin® E4-200XLS single-channel electronic pipettes were purchased and applied through the research (Rainin, USA).

3.3. Methods

3.3.1. Selection of Primer Set and Probe

Total coliforms contain a group of coliform species which includes the genera *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Serratia* and *Yersinia* [36]. Therefore, the design of primer set becomes the main difficulties when nucleic acid targets quantification technology is applied for multiple coliforms detections. The characteristics of primer set should not only have the capabilities to detect all the required coliform species among different genus, but also exclude those similar species of unrequired ones. In our research, the designed primer set and probe [37] was applied according to the gene sequence of *lac*Z in GenBank. The sequences of primer set and probe were listed in Table 1.

Table 1. *The primer set and probe sequences for total coliforms detection.*

Primer/Probe	Sequence (5'-3')	*Lac*Z Position	Production Size (bp)
Forward primer	GCTGATGAAGCAGAACA	1133~1250 bp	
Reverse primer	CATGCCGTGGGTTTC	1243~1257 bp	125 bp
Probe	FAM - TTTAACGCCGTGCGCT - MGB	1153~1168 bp	

3.3.2. Setup of DDPCR Detection Procedure

An aliquot of 100 mL water samples was filtered by membrane filtration method and total coliforms were concentrated on a cellulose esters membrane (0.45 μm pore size). Then total coliforms genome DNA was extracted by PowerSoil® DNA Isolation Kit according to manufacturer's instruction and used as the DNA template in DDPCR reaction system. The concentration of the DNA template was then measured by fluorometer immediately.

In DDPCR reaction system, each reaction system contained 10 μL of DDPCR^{TM} Supermix, 1 μL of total

coliform *lacZ* genome DNA template, primer set and probe (concentration to be optimized), the final volume was reached to 20 μL by sterilized distilled water.

The aliquot of 20 μL reaction system were placed into QX200™ droplet generator, which utilized droplet generation oil and microfluidics to partition the samples into 20,000 nanoliter-sized uniform droplets. Almost 40 μL of emulsified samples were pipetted to 96-well PCR plate and then the plate was sealed with the PX1™ PCR plate sealer.

The template DNA was amplified using C1000 touch thermal cycler with the following cycling conditions: 10 minutes at 95°C initial denaturation, 40 cycles each consisting of a 30 seconds denaturation at 94°C, followed by 60 seconds under the annealing temperature (to be optimized), and 10 minutes at 98°C for a final extension.

After amplification, the 96-well PCR plate was loaded into the QX200™ droplet reader, then raw fluorescence data from each well were exported from the software (QuantaSoft v1.7.4). Droplets were automatically classified as positive and negative based on Poisson theory. Then, ambiguous results or potentially spurious events were filtered out followed a custom algorithm.

3.3.3. Optimization of Experimental Parameters

i. Optimization of Probe Concentrations

In DDPCR reaction system, 0.2 μmol/L of the forward and reverse primer concentrations were applied, respectively. Different probe concentrations at 0.25 μmol/L, 0.5 μmol/L, 0.75 μmol/L, 1 μmol/L, 1.5 μmol/L, and 2 μmol/L were tested for optimization, respectively.

The template DNA with the following cycling conditions: 10 minutes at 95°C initial denaturation, 40 cycles each consisting of a 30 seconds denaturation at 94°C followed by 60 seconds at 56°C annealing temperature, and a final extension at 98°C for 10 minutes. After cycling, droplets were analyzed immediately. All the other conditions were same as in section 3.3.2.

ii. Optimization of Primer Concentrations

In DDPCR reaction system, 0.5 μmol/L of the probe concentration was applied, whereas different primer set concentrations at 0.2 μmol/L, 0.25 μmol/L, 0.35 μmol/L, 0.4 μmol/L, 0.5 μmol/L, 0.65 μmol/L, 1.0 μmol/L and 1.8 μmol/L were tested for optimization, respectively. All the other conditions were same as in section 3.3.2.

iii. Optimization of Annealing temperatures

In DDPCR reaction system, 0.5 μmol/L of the probe concentration and 0.2 μmol/L of respective forward and reverse primer concentrations were applied. Annealing temperatures gradient at 45°C, 46°C, 47.8°C, 50.5°C, 53.6°C, 56.3°C, 58.1°C and 59°C were tested for optimization, respectively. All the other conditions were same as in section 3.3.2.

3.3.4. Investigation of Linear Range

The optimized DDPCR assay was applied to investigate the method linear range. Ten-fold serial dilutions were applied to obtain total coliforms *lacZ* genome concentrations at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} ng/mL and marked as S1 ~ S7, respectively.

In DDPCR, each reaction system contained 10 μL of DDPCR™ Supermix, 1 μL of total coliform *lacZ* genome DNA template, 0.2 μmol/L of primer set and 0.5 μmol/L of probe, the final volume was reached to 20 μL by sterilized distilled water.

The template DNA with the following cycling conditions: 10 minutes at 95°C initial denaturation, 40 cycles each consisting of a 30 seconds denaturation at 94°C followed by 56°C annealing temperature for 60 seconds, and a final extension at 98°C for 10 minutes. After cycling, droplets were analyzed immediately.

Four replicates were applied in each concentration for linear range confirmation.

3.3.5. Evaluation of Precision

The method precision was evaluated based on the relative standard deviation (RSD) derived from DDPCR results. The relationship between the total coliforms *lacZ* genome concentrations derived from Qubit® 3.0 fluorometer and DDPCR was investigated. Four replicates were applied for method precision investigation.

3.3.6. Validation Study

A total of 50 water samples collected from Macao water supply system were used to verify the capability and efficiency of this innovative DDPCR detection method in the research. The water samples included raw water, treated water and water from networks (Figure 1). The quantification detection of total coliforms in subsequent samples were processed by DDPCR as described above.

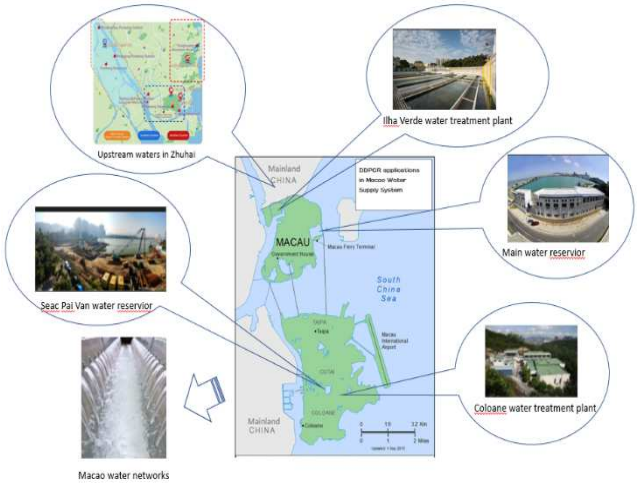


Figure 1. *Water samples collected from Macao water supply system.*

3.3.7. Data Analysis

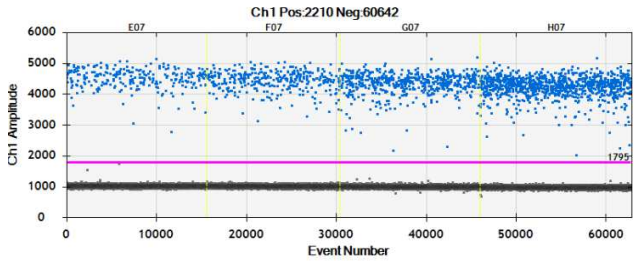
DDPCR data was initially analyzed in QuantaSoft v1.7.4 software following the manufacturer's recommendation. For the wells which had the accepted droplet less than 10,000 were excluded from analysis. Wells with less than 3 positive droplets were regarded as negative. One standard deviation above negative droplets was set as the fluorescence threshold.

Dose responses and regressions were plotted in Excel 2016. Goodness of fit was confirmed by R^2 values. The limit of quantification (LOQ) which used to compare total coliforms DNA genome concentrations were determined by a t-test. All the significant confidence levels were quoted at 95% ($p \leq 0.05$).

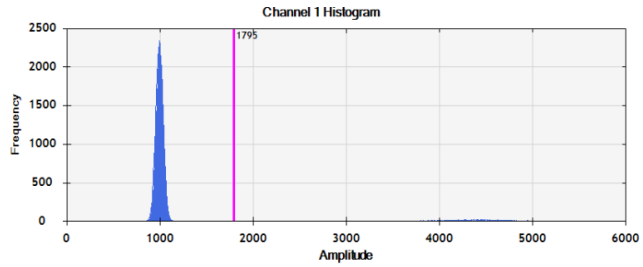
4. Results and Discussion

4.1. Amplification Performance

The interpretation of DDPCR results is based on the accumulated fluorescence of all droplets by the end of measurement. Thus, the accuracy of this detection method is dependent on the intensity of accumulated fluorescence after amplification. In this research, the DDPCR amplification performance on total coliforms genome DNA detection were shown in Figure 2.



(a) Scatter plot



(b) Histogram

Figure 2. *DDPCR amplification performance on total coliforms genome DNA detection. Where represents positive droplets and represents negative droplets.*

One-dimensional scatter plot and histogram figures showed an optimized DDPCR assay with a clear distinction between positive and negative partitions. This good DDPCR amplification performance provided with capabilities for target DNA quantification after optimization.

4.2. Confirmation of Optimized Experimental Parameters

4.2.1. Confirmation of Optimized Probe Concentration

The accumulated fluorescence (shown as fluorescent amplitudes) of total coliforms after DDPCR amplification at different probe concentrations (0.25 μmol/L, 0.5 μmol/L, 0.75 μmol/L, 1 μmol/L, 1.5 μmol/L, and 2 μmol/L) were shown in Figure 3.

Regardless of probe concentrations, fluorescent amplitudes of each droplet generated both a positive and negative cluster. The positive fluorescent amplitude increased as the probe concentration increased, thus more *lacZ* genome DNA was amplified by DDPCR method. The fluorescent amplitude was the highest when the probe concentration was 2 μmol/L. When the probe concentration was 0.5 μmol/L, it provided with the clearest separation of the negative and positive droplets. Therefore, considering the intensity of positive fluorescent amplitude, stability and the corresponding clearness of droplets separation, the optimized probe concentration was confirmed at 0.5 μmol/L.

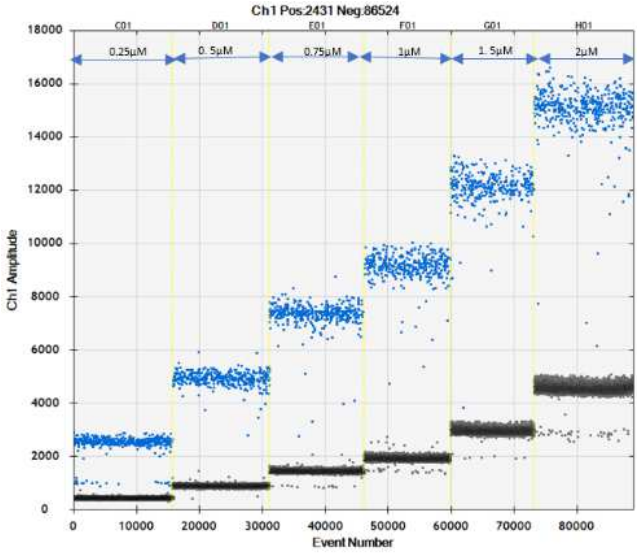


Figure 3. *The DDPCR amplification performance at different probe concentrations.*

4.2.2. Confirmation of Optimized Primer Set Concentration

The primer set concentration was optimized after the probe concentration confirmed at 0.5 μmol/L. The DDPCR amplification performance which was in the form of fluorescent amplitude at different primer concentrations was shown in Figure 4.

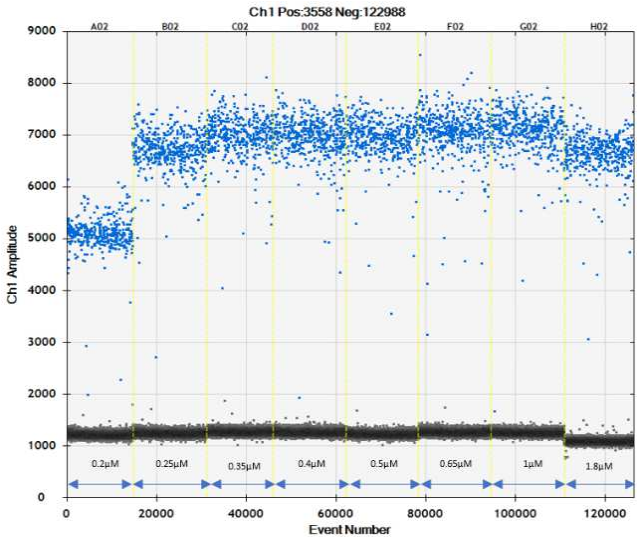


Figure 4. *The DDPCR amplification performance at different primer set concentrations.*

When the primer concentration was 0.2 μmol/L, it provided with the clearest separation of the negative and positive droplets and the positive cluster was more convergently distributed. As the primer concentration further increased from 0.25 μmol/L to 1 μmol/L, there was no significant difference between positive fluorescent amplitudes (p=0.68). The positive fluorescent amplitude was inhibited when the primer concentration further increased to 1.8 μmol/L. Considering the stability, the corresponding clearness of droplets separation and reagent cost, the optimized primer concentration was confirmed at 0.2 μmol/L.

4.2.3. Confirmation of Optimized Annealing Temperature

Annealing temperatures of DDPCR reaction system were optimized followed by the confirmation of operational primer set and probe concentrations. The DDPCR amplification performance which was in the form of fluorescent amplitude at a serial of annealing gradients was shown in Figure 5.

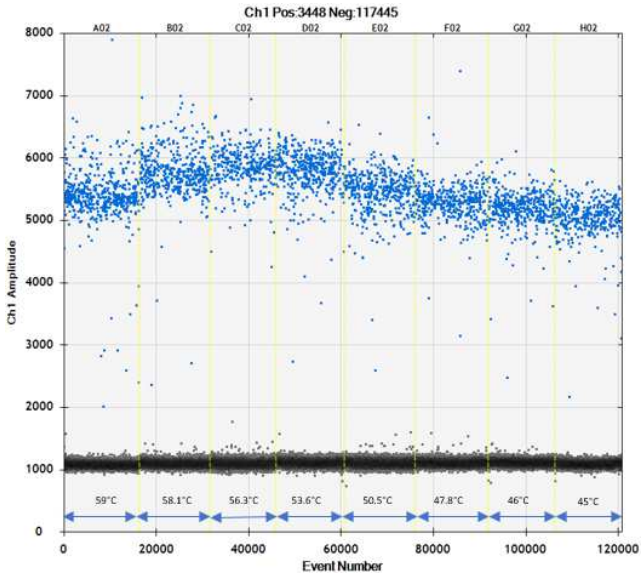


Figure 5. *The DDPCR amplification performance at different annealing temperatures.*

As annealing temperature increased, the positive fluorescent amplitude increased. When the annealing temperature increased to 56.3°C, it showed the highest fluorescent amplitude and the positive cluster was more convergent. As the annealing temperature further increased, the fluorescent amplitude decreased which indicated that higher temperature inhibited amplification. Considering the stability and corresponding clearness of droplets separation, the optimized annealing temperature was 56.3°C.

4.3. Confirmation of Method Linear Range

4.3.1. Prerequisites for Quantification

The average event (number of droplets) generated from DDPCR reactions (n=32) was 15,307±1,528. The average event of accepted droplets at each diluted concentration was above 14,000 which provided the one of the prerequisites for accuracy DDPCR quantification (Figure 6).

As the concentration of *lacZ* genome DNA increased (from S7 to S1), the event of positive droplets increased whereas the event of negative droplets decreased. In sample S1, there was no significant difference in events between accepted droplets and positive ones (p=0.52) which illustrated that most of the droplets in sample S1 (the concentration of 10^{-1} dilution) were positive droplets.

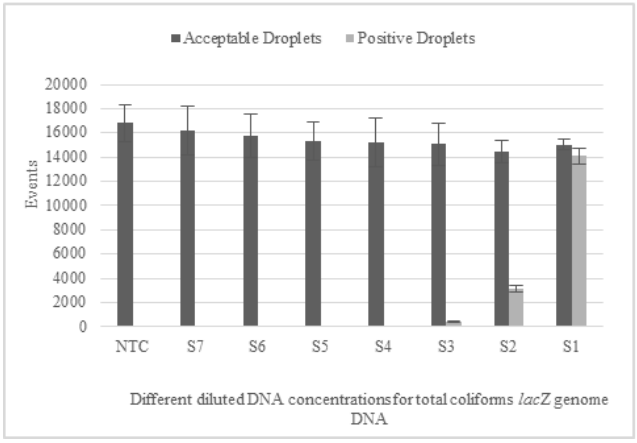


Figure 6. *The events of droplets at different lacZ genome DNA concentrations.*

All the No Template Control (NTC) samples without any *lacZ* genome DNA (worked as negative control) were tested by optimized DDPCR method. There was no single positive droplet generated in all NTC samples (Figure 7). This fluorescent amplitude results demonstrated that all the amplification systems were not contaminated and/or there was no non-specific amplification generated. Thus, this innovative DDPCR method was regarded as high specificity.

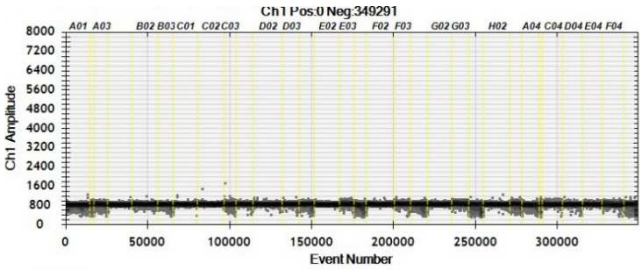


Figure 7. *The DDPCR amplification performance for NTC samples.*

4.3.2. Linear Range for Quantification

The dose response relationship between *lacZ* genome DNA concentrations derived by both fluorometer and optimized DDPCR assay was linear (Figure 8). In this research, the linear range of innovative DDPCR method was 3.95 ~ 7.80 × 10^4 copies/20 μL DDPCR reaction system (R^2=0.999).

The LOQ of the method refers to the lowest *lacZ* genome DNA concentration corresponded to a significant increase in the fluorescent amplitude ($p \leq 0.05$) relative to NTC. The *lacZ* genome DNA concentration (0.2 copies/μL DDPCR reaction system) in sample S5 was the lowest concentration

for accurate quantification. Thus, the LOQ of DDPCR method was 4 copies/20μL DDPCR reaction system.

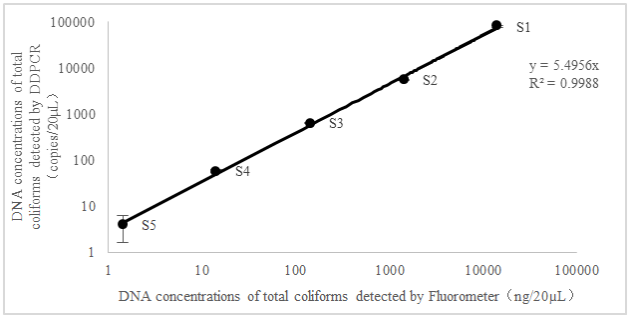


Figure 8. *The linear range of DDPCR detection method.*

4.4. Confirmation of Method Precision

Dose response relationship between four replicates of *lacZ* genome DNA concentrations derived by optimized DDPCR method and its corresponding RSD was fitted well with the resultant sigmoidal models (Sigmoidal, 4 parameters) (R^2=0.89).

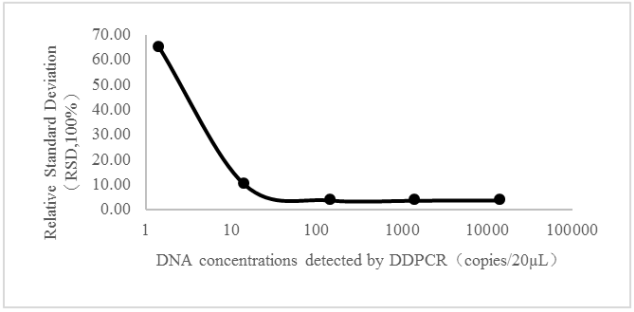


Figure 9. *The relationship between lacZ genome DNA concentration and its relative standard deviation.*

As *lacZ* genome DNA concentration increased, its corresponding RSD decreased, thus the detection precision increased (Figure 9). When *lacZ* genome DNA concentration was 4 copies/20μL DDPCR reaction system (LOQ), the RSD of was 65%. When *lacZ* genome DNA concentration within the range of 57.5 ~ 77,950 copies/20μL DDPCR reaction system, the RSD was less than 5%. Therefore, better precision and accuracy of the method would be obtained if the operational *lacZ* genome DNA concentration maintained within this range.

4.5. Validation from Practical Samples

A total of 50 water samples were collected from both up-streams (3 water reservoirs in Zhuhai, China: marked A, B and C) and Macao local water supply systems (Macao SAR, China). The Macao local water samples included 5 samples from Macao water reservoirs (marked D ~ H), 24 raw water samples from treatment plants, 4 samples from treated water, and 14 samples from networks.

The results derived from DDPCR method after *lacZ* genome DNA extraction for raw water were shown below (Table 2 and Table 3).

Table 2. *The concentration of lacZ genome DNA in reservoir water samples (copies/20μL DDPCR reaction system).*

Sample	Replicate 1	Replicate 2	Mean	Standard deviation
Reservoir A	316.0	286.0	301.0	21.2
Reservoir B	1.4	4.0	2.7	1.8
Reservoir C	22.0	16.0	19.0	4.2
Reservoir D	1.4	1.6	1.5	0.1
Reservoir E	28.0	14.0	21.0	9.9
Reservoir F	1.2	3.8	2.5	1.8
Reservoir G	1.4	1.8	1.6	0.3
Reservoir H	12.0	10.0	11.0	1.4

Table 3. *The concentration of lacZ genome DNA in raw water samples in Macao water treatment plants (copies/20μL DDPCR reaction system).*

Sample	DNA Conc.	Sample	DNA Conc.	Sample	DNA Conc.
1	1.1 ± 0.1	9	7.5 ± 0.2	17	2.8 ± 0.4
2	1.4 ± 0.0	10	6.4 ± 0.0	18	1.0 ± 0.4
3	3.1 ± 0.4	11	2.4 ± 0.6	19	1.0 ± 0.1
4	2.1 ± 1.0	12	2.8 ± 1.1	20	0.6 ± 0.1
5	1.2 ± 0.0	13	5.6 ± 0.8	21	3.6 ± 1.0
6	1.2 ± 0.0	14	1.3 ± 0.1	22	3.5 ± 0.4
7	2.5 ± 1.8	15	2.0 ± 0.8	23	1.1 ± 0.1
8	1.5 ± 0.1	16	3.2 ± 0.0	24	2.4 ± 0.1

The results showed that *lacZ* genome DNA was detected by innovative DDPCR method which indicated that total coliforms existed in all raw water samples.

In Table 2, for reservoir water samples, the *lacZ* genome DNA concentration of "sample A" from up-streams in Zhuhai was the highest (301.0 ± 21.2 copies/20μL DDPCR reaction system), whilst the *lacZ* genome DNA concentration of "sample D" from Macao was the lowest (1.5 ± 0.1 copies/20μL DDPCR reaction system).

In Table 3, for raw water samples from water treatment plants, there was no significant difference between *lacZ* genome DNA concentration derived from DDPCR method (p = 0.65).

DDPCR method was also applied to treated water samples and samples from networks after DNA extraction. The concentration of *lacZ* genome DNA concentration was below LOQ which proved that no coliforms were detected from those samples.

5. Conclusions

Even though water-borne pathogen detections using conventional culture-based methods has become the "Golden Standards", it normally takes at least 4~6 days for total coliforms detections from sample-to-result time including verification experiment. The LOQ of this method is 1 Colony Forming Unit (CFU)/100mL.

Compared to culture-based methods, qPCR provides faster sample-to-result time, higher specificity and sensitivity, thus it is a widely accepted and applied method for water-borne pathogen detections. Especially, qPCR for Legionella detection has become an internationally recognized method

[37]. However, it is still a relative quantification method based on Ct and the absolute concentration of target samples remains unknown until calibrated with standard samples. Therefore, the reliability and consistency of required standards have tremendous impact on the accuracy of quantification. Besides, qPCR is susceptible to inhibitors which might naturally exist in environmental samples and these inhibitors are normally complex and often contain substances that interfere with qPCR amplification [38].

Digital PCR, especially DDPCR, has the potential to overcome the limitations of qPCR based on its partitioning process and Poisson statistics. Thus, the target DNA copies can be estimated by counting the frequency of positive partitions and no external standards are needed for quantification of unknown samples. Most of studies regarding DDPCR have focused on food, clinical and pharmaceutical areas, little is known for DDPCR applications in environmental samples, especially in water supply system.

The interpretation of DDPCR result is based on the accumulated fluorescence of all droplets by the end of measurement. Thus, the accuracy of this detection method is dependent on the intensity of accumulated fluorescence after amplification. Very few DDPCR publications detail any information regarding PCR assay optimization. Here the optimal experimental conditions, such as reagent concentrations and annealing temperatures, were well confirmed to further increase the accuracy and consistency of the assay. In addition, DDPCR partitions are made up of water-in-oil emulsion droplets which are quite fragile. Therefore, single-channel electronic pipettes were used through all the experiments to avoid the loss of droplet events. Furthermore, the sample-to-result time was only 5 hours using the innovative DDPCR method in this research and the LOQ of this method was only single copy/20μL DDPCR reaction system which was in accordance with other studies in pathogen quantifications [39, 40]. Practical applications using real water samples collected from water supply system in Macao illustrated that this innovative method possessed high efficiencies and capabilities.

In this research, a safer, faster and simpler detection method with higher specificity and sensitivity for absolute total coliforms qualification and quantification was setup based on DDPCR technology. This is probably the first research successfully applied DDPCR technology in Macao water supply system. The achievements from this research could provide with significant values and work as a demonstration role for setting-up the emergency mechanism of water pollution in early stage. Future field case studies are needed to further evaluate its full spectrum of other water-borne pathogens and potential limitations to be concerned.

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References

[1] Ramírez-Castillo, F. Y., Loera-Muro, A., Jacques, M., Garneau, P., Avelar-González, F. J., Harel, J., and Guerrero-Barrera, A. L. (2015). Waterborne pathogens: detection methods and challenges. Pathogens, 4 (2): 307-334.

[2] McGinnis, S., Spencer, S., Firnstahl, A., Stokdyk, J., Borchardt, M., McCarthy, D. T., and Murphy, H. M. (2018). Human bacteroides and total coliforms as indicators of recent combined sewer overflows and rain events in urban creeks. Science of Total Environment, 630: 967-976.

[3] Mohammed, H., Hameed, I. A., and Seidu, R. (2018). Comparative predictive modelling of the occurrence of fecal indicator bacteria in a drinking water source in Norway. Science of Total Environment, 628-629: 1178-1190.

[4] Messner, M. J., Berger, P., and Javier, J. (2017). Total coliform and E. coli in public water systems using undisinfected ground water in the United Sates. International Journal of Hygiene and Environmental Health, 220 (4): 736-743.

[5] Wang, J., and Deng, Z. Q. (2019). Modeling and predicting fecal coliform bacteria levels in oyster harvest waters along Louisiana Gulf coast. Ecological Indicators, 101: 212-220.

[6] Noble, R. T., Moore, D. F., Leecaster, M. K., McGee, C. D., and Weisberg, S. B. (2003). Comparison of total coliform, fecal coliform and enterococcus bacterial indicator response for ocean recreational water quality testing. Water Research, 37 (7): 1637-1643.

[7] Maheux, A. F., Dion-Dupont, V., Bisson, M. A., Bouchard, S., and Rodriguez, M. J. (2014). Detection of Escherichia Coli colonies on confluent plates of chromogenic media used in membrane filtration. Journal of Microbiological Methods, 97: 51-55.

[8] Wang, D. L., and Fiessel, W. (2008). Evaluation of media for simultaneous enumeration of total coliform and Escherichia Coli in drinking water supplies by membrane filtration techniques. Journal of Environmental Sciences, 20 (3): 273-277.

[9] Eckner, K. F. (1998). Comparison of membrane filtration and multiple-tube fermentation by the Colilert and Enterolert methods for detection of waterborne coliform bacteria, Escherichia coli, and Enterococci used in drinking and bathing water quality monitoring in southern Sweden. Applied and Environmental Microbiology, 64 (8): 3079-3083.

[10] Wang, Z. D., Xiao, G. S., Zhou, N., Qi, W. H., Han, L., Ruan, Y., Guo, D. Q., and Zhou, H. (2015). Comparison of two methods for detection of fecal indicator bacteria used in water quality monitoring of the Three Gorges Reservoir. Journal of Environmental Sciences, 38: 42-51.

[11] Shelton, D. R., Higgins, J. A., Van Kessel, J. A. S., Pachepsky, Y. A., Belt, K., and Karns, J. S. (2004). Estimation of viable Escherichia Coli O157 in surface waters using enrichment in conjunction with immunological detection. Journal of Microbiological Methods, 58 (2): 223-231.

[12] Exum, N. G., Pisanic, N., Granger, D. A., Schwab, K. J., Detrick, B., Kosek, M., Egorov, A. I., Griffin, S. M., and Heaney, C. D. (2016). Use of pathogen-specific antibody biomarkers to estimate waterborne infections in population-based settings. Current Environmental Health Reports, 3: 322-334.

[13] Rompré, A., Servais. P., Baudart, J., de-Roubin, M. R., and Laurent, P. (2002). Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. Journal of Microbiological Methods, 49 (1): 31-54.

[14] Van Poucke, S. O., and Nelis, H. J. (2000). Rapid detection of fluorescent and chemiluminescent total coliforms and Escherichia coli on membrane filters. Journal of Microbiological Methods, 42 (3): 233-244.

[15] Collins, S., Stevenson, D., Walker, J., and Bennett, A. (2017). Evaluation of Legionella real-time PCR against traditional culture for routine and public health testing of water samples. Journal of Applied Microbiology, 122: 1692-1703.

[16] Maheux, A. F., Huppé, V., Boissinot, M., Picard, F. J., Bissonnette, L., Bernier, J. L. T., and Bergeron, M. G. (2008). Analytical limits of four β-glucuronidase and β-galactosidase-based commercial culture methods used to detect Escherichia Coli and total coliforms. Journal of Microbiological Methods, 75 (3): 506-514.

[17] Liu, Q., Zhang, X. L., Yao, Y. H., Jing, W. W., Liu, S. X., and Sui, G. D. (2018). A Novel microfluidic module for rapid detection of airborne and waterborne pathogens. Sensors and Actuators B: Chemical, 258: 1138-1145.

[18] Dao, T. N. T., Lee, E. Y., Koo, B., Jin, C. E., Lee, T. Y., and Shin, Y. (2018). A microfluidic enrichment platform with a recombinase polymerase amplification sensor for pathogen diagnosis. Analytical Biochemistry, 544: 87-92.

[19] Elsäßer, D., HO, J., Niessner, R., Tiehm, A., and Seidel, M. (2018). Heterogeneous asymmetric recombinase polymerase amplification (haRPA) for rapid hygiene control of large-volume water samples. Analytical Biochemistry, 546: 58-64.

[20] Martzy, R., Kolm, C., Brunner, K., Mach, R. L., Krska, R. Šinkovec, H., Sommer, R., Farnleitner, A. H., and Reischer, G. H. (2017). A loop-mediated isothermal amplification (LAMP) assay for the rapid detection of Enterococcus spp. in water. Water Research, 122: 62-69.

[21] Gunawardana, M., Chang, S., Jimenez, A., Holland-Moritz, D., Holland-Moritz, H., La VaL, T. P., Lund, C., Mullen, M., Olsen, J., Sztain, T. A., Yoo, J., Moss, J. A., and Baum, M. M. (2014). Isolation of PCR quality microbial community DNA from heavily contaminated environments. Journal of Microbiological Methods, 102: 1-7.

[22] Garofalo, C., Bancalari, E., Milanović, V., Cardinali, F., Osimani, A., Sardaro, M. L. S., Bottari, B., Bernini, V., Aquilanti, L., Clementi, F., Neviani, E., Gatti, M. (2017). Study of the bacterial diversity of foods: PCR-DGGE versus LH-PCR. International Journal of Food Microbiology, 242: 24-36.

[23] Lacout, A., Mone, Y., Franck, M., Marcy, P. Y., Mas, M., Veas, F., and Perronne, C. (2018). Blood cell disruption to significantly improve the borrelia PCR detection sensitivity in borreliosis in humans. Medical Hypothesis, 116: 1-3.

[24] Wang, Y. J., Zhu, S. Y., Hong, W. M., Wang, A. P., and Zuo, W. Y. (2017). A multiplex PCR for detection of six viruses in ducks. Journal of Virological Methods, 248: 172-176.

[25] Boehm, A. B., Van De Werfhorst, L. C., Griffith, J. F., Holden, P. A., Jay, J. A., Shanks, O. C., Wang, D., and Weisberg, S. B. (2013). Performance of forty-one microbial source tracking methods: a twenty-seven lab evaluation study. Water Research, 47 (18): 6812-6828.

[26] Griffith, J. F., and Weisberg, S. B. (2011). Challenges in implementing new technology for beach water quality monitoring: lessons from a California demonstration project. Marine Technology Society Journal, 45 (2): 65-73.

[27] Harwood, V. J., Staley, C., Badgley, B. D., Borges, K. and Korajkic, A. (2014). Microbial source tracking markers for detection of fecal contamination in environmental waters: relationships between pathogens and human health outcomes. FEMS Microbiological Reviews, 38 (1): 1-40.

[28] Cao, Y., Sivaganesan, M., Kinzelman, J., Blackwood, A. D., Noble, R. T., Haugland, R. A., Griffith, J. F. and Weisberg, S. B. (2013). Effect of platform, reference material, and quantification model on enumeration of Enterococcus by quantitative PCR methods. Water Research, 47 (1): 233-241.

[29] Sivaganesan, M., Siefring, S., Varma, M., and Haugland, R. A. (2011). MPN estimation of qPCR target sequence recoveries from whole cell calibrator samples. Journal of Microbiological Methods, 87 (3): 343-349.

[30] Joensson, H. N., and Adersson, S. H. (2012). Droplet microfluidics – A tool for single-cell analysis. Angewandte Chemie International Edition, 51 (3): 12176-12192.

[31] Floren, C., Wiedemann, I., Brenig, B., Schűtz, E., and Beck, J. (2015). Species identification and quantification in meat and meat products using droplet digital PCR (DDPCR). Food Chemistry, 173: 1054-1058.

[32] Eastburn, D. J., Huang, Y., Pellegrino, M., Sciambi, A., Ptáček, L. J., and Abate, A. R. (2015). Microfluidic droplet enrichment for targeted sequencing. Nucleic Acids Research, 43 (13): e86. http://dx.doi.org/10.1093/nar/gkv297.

[33] Scollo, F., Egea, L. A., Gentile, A., La Malfa, S., Dorado, G., and Hernandez, P. (2016). Absolute quantification of olive oil DNA by droplet digital-PCR (DDPCR): comparison of isolation and amplification methodologies. Food Chemistry, 213: 388-394.

[34] Memon, A. A., Zőller, B., Hedelius, A., Wang, X., Stenman, E., Sundquist, J., and Sundquist, K. (2017). Quantification of mitochondrial DNA copy number in suspected cancer patients by a well optimized DDPCR method. Biomolecular Detection and Quantification, 13: 32-39.

[35] Periyannan Rajeswari, P. K., Soderberg, L. M., Yacoub, A., Leijon, M., Andersson Svahn, H., and Joensson, H. N. (2017). Multiple pathogen biomarker detection using an encoded bead array in droplet PCR. Journal of Microbiological Methods, 129: 22-28.

[36] Horan, N. J. (2003). Handbook of water and wastewater microbiology: Chapter 7 – Fecal indicator organisms. Academic press, 105-112.

[37] International Organization for Standardization. ISO/TS 12869: 2012 [S/OL]. [2012-10-12]. https://www.iso.org/standard/52079.html.

[38] Cao, Y., Griffith, J. F., Dorevitch, S., and Weisberg, S. B. (2012). Effectiveness of a qPCR permutations, internal controls and dilution as means for minimizing the impact of inhibition while measuring Enterococcus in environmental waters. Journal of Applied Microbiology, 113 (1): 66-75.

[39] Dong, L. H., Meng, Y., Wang, J. and Liu, Y. Y. (2014). Evaluation of droplet digital PCR for characterizing plasmid reference material used for quantifying ammonia oxidizers and denitrifiers. Analytical and Bioanalytical Chemistry, 406 (6): 1701-1712.

[40] Cao, Y. P., Raith, M. R., and Griffith, J. F. (2015). Droplet digital PCR for simultaneous quantification of general and human-associated fecal indicators for water quality assessment. Water Research, 70: 337-349.