Comparison of droplet digital PCR and quantitative PCR for the detection of *Salmonella* and its application for river sediments

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ABSTRACT

Despite advances in microbial detection that quantitative polymerase chain reaction (qPCR) has led to, complex environmental samples, such as sediments, remain a challenge due to presence of PCR inhibitors. Aquatic sediments accumulate particle-bound microbial contaminants and thereby reflect a cumulative microbial load over time. The relatively new droplet digital PCR (ddPCR) has emerged as a direct quantitative method, highly tolerant to PCR inhibitors and relinquishing the necessity for calibration/standard curves. Information is virtually absent where ddPCR has been applied to detect pathogenic organisms in aquatic sediments. This study compared the efficacy of ddPCR with qPCR, for quantification of *Salmonella* in sediments from the Palmiet River near an informal settlement in Durban, South Africa. ddPCR significantly improved both analytical sensitivity and detection of low concentrations of *Salmonella* as compared to qPCR. The expected copy numbers measured from both qPCR and ddPCR showed good R^2 values (0.999 and 0.994, respectively). The site mostly affected by the informal settlements exhibited *Salmonella* in the range of 255 ± 37 and 818 ± 30 *Salmonella*/g ($p \leq 0.0001$) in qPCR and ddPCR, respectively. The improved detection of *Salmonella* in sediments with ddPCR makes it a promising technical method for the quantification of *Salmonella* in multifarious environmental samples.

Key words | ddPCR, qPCR, *Salmonella*, sediments

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INTRODUCTION

Pathogenic bacteria can survive longer in aquatic sediments than in the overlying water column (Luna *et al.* 2012; Vignaroli *et al.* 2013) and will represent the particle-associated fraction, accumulating over time. The occurrence and quantification of human pathogenic bacteria in environmental regimes, like surface water or bottom sediments, still to a large extent rely on quantification on selective media or enrichment. The direct quantification of specific target genes, representing the pathogen in question with quantitative polymerase chain reaction (qPCR) has, however, progressively been accepted as the gold standard and been applied for the detection and quantification of pathogens in water environmental samples (Li & Chen 2013; Singh *et al.* 2013). In general, qPCR has several advantages as compared to classical bacteriological cultivation methods and identification schemes, in terms of speed, detection limit, potential for automation, and cost.

The application of qPCR in sediment samples is a challenge mainly due to the presence of PCR inhibitory substances. Even a small quantity of PCR inhibitors can delay the C_q (threshold cycles) of complex samples in qPCR, causing erroneously low estimates of the template

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copy number (Sidstedt *et al.* 2015). The alternative, the water emulsion technology-based droplet digital PCR (ddPCR), has emerged as a direct quantitative method with the potential of overcoming the inhibitory effects affecting qPCR (Hindson *et al.* 2011). An additional advantage with ddPCR over qPCR is the ability to enable the absolute quantification of DNA concentrations without external calibrators (Pinheiro *et al.* 2012). In digital PCR, the sample is subjected to partitioning into hundreds to millions of individual reaction chambers (depending upon the digital PCR platform) prior to the PCR cycles, so that each contains one or no copy of the sequence of interest (Baker 2012; McDermott *et al.* 2013). The partitioning of the sample in ddPCR into multiple droplets substantially reduces the susceptibility to inhibitors (Morisset *et al.* 2013). Recent studies have demonstrated the accuracy and precision of ddPCR in the quantitative detection of bacteria and viruses in clinical samples (Lui & Tan 2014; Rački *et al.* 2014; Devonshire *et al.* 2015; Zhao *et al.* 2015) but its environmental application for the detection of, for example, *Salmonella* has so far not directly been done, except for one study in commercial poultry processing water samples (Rothrock *et al.* 2013). This study was, therefore, undertaken with the objective of comparing the enumeration with ddPCR and qPCR for the detection of *Salmonella* targeting the *ttr* gene in river sediment samples collected from sites of the Palmiet River, Durban, South Africa.

MATERIAL AND METHODS

Salmonella enterica serovar *enteritidis* ATCC 13076 was procured from Microbiologics Inc, USA. The primers specific for the *ttr* gene targeting *Salmonella* were adopted from Malorny *et al.* (2004). The qPCR standard curve for the *ttr* gene was generated from the purified DNA extracted from the reference strain, *S. enteritidis* ATCC 13076 (2 to 2×10^6 gene copies (GC/PCR)) according to Jyoti *et al.* (2011). The qPCR was performed using CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA) using qPCR protocol of initial denaturation for 5 min at 95°C, followed by 45 cycles of three steps consisting of 10 sec at 95°C, 20 sec at 54°C and 20 sec at 72°C. The standard curve was automatically generated by the CFX Manager™ Software v3.1. The sample concentrations were calculated from the generated standard curve.

For ddPCR, *Salmonella enterica* serovar *enteritidis* ATCC 13076 exhibiting the *ttr* gene was grown in LB broth for 16 h at 37 ± 1°C (optical density 0.8 at 600 nm). A serial 10-fold diluted culture (20 to 2×10^4 CFU mL^{-1} to get 2 to 2×10^3 GC/PCR) was spiked, in triplicate, to 10 mL sterile Milli-Q® (Millipore, Billerica, MA, USA) water. DNA template was prepared from 1 mL spiked samples by extracting genomic DNA using the QIAamp DNA Mini Kit (Qiagen, Germany) as per the manufacturer's instructions. The 5 μL of extracted DNA (range of 2 to 2×10^3 GC/PCR) was used as template in ddPCR for the detection and quantitative enumeration of *Salmonella*.

ddPCR was performed on the BioRad QX200 ddPCR system. Briefly, *ttr* gene copies (2 to 2×10^3 copies) were added with primers and Qx200™ ddPCR™ Evagreen Supermix to the reaction mixture in a final volume of 20 μL in accordance with the manufacturer's instructions (Bio-Rad Laboratories, CA, USA). The reaction mixture was then processed with 70 μL of droplet generation oil using the droplet generator (Bio-Rad Laboratories). The droplets generated were then transferred into 96-well plates and PCR amplification was performed with a thermal profile of denaturation at 95°C for 5 min, followed by 40 cycles at 94°C for 30 s, 54°C for 1 min, 4°C for 5 min, 90°C for 5 min on a T100 thermal cycler (Bio-Rad Laboratories). Finally, the plate was loaded onto the droplet reader and the data were generated and analysed using the Quanta Soft analysis software (Bio-Rad Laboratories).

For culture-free quantitative enumeration of *Salmonella* spp., in a riverine environment, sediment samples were collected in triplicate in sterile bags from four sites of the Palmiet River and transported on ice to the laboratory.

DNA was extracted from sediments by PowerSoil® DNA Isolation Kit (Mo Bio, Laboratories) according to the manufacturer's protocol. The extracted DNA (5 μL) was used as template in qPCR assays and ddPCR as described above. Quantitative enumeration of *Salmonella* in sediment samples by qPCR was carried out using standard curve prepared by 10-fold diluted genomic DNA of *S. enteritidis* ATCC 13076 (from 2 to 2×10^6 GC/PCR), while in ddPCR DNA extracted was directly subjected to

droplet generation followed by PCR amplification to detect copies/μL of reaction mixture.

RESULTS AND DISCUSSION

The performance of both qPCR and ddPCR targeting the *ttr* gene to detect *Salmonella* was analysed in water samples spiked with a 10-fold serially diluted culture of *S. enteritidis* ATCC 13076. The sensitivity of qPCR assay targeting the *ttr* gene for detecting *Salmonella* was assessed and found to be capable of rapidly detecting 2×10^7 down to 20 CFU/PCR in water samples spiked with the *S. enteritidis* ATCC 13076. In comparison, ddPCR was able to detect as low as 2 GC/PCR *Salmonella* with DNA extracted from water samples spiked with the reference strain. Overall, the expected copy numbers measured from both the methods (qPCR and ddPCR) showed good linear regression correlation coefficients (R^2) values of 0.999 and 0.994, respectively, in spiked water samples.

The performance of ddPCR was further analysed to detect targeted bacteria in sediment samples from Palmiet River sites around an informal settlement in Durban and subsequently compared with the qPCR. The sensitivity and the linear range of ddPCR were comparable to those of qPCR in the case of spiked water samples but were significantly more sensitive in the case of sediment samples. This is most probably due to the droplet partitioning in ddPCR, in which, the whole PCR reaction is split into 20,000 droplets where ideally each droplet contains one or less copies of targeted DNA, effectively reducing the effect of PCR inhibitors (Baker 2012).

The *Salmonella* load was found to be significantly different in both qPCR and ddPCR for sites around the informal settlements of Quarry road (p value = 0.0025, unpaired 't' test). The site upstream of the informal settlement had the lowest load, while for both sites, at the start and further down within the settlement, the values were in the same range, but varied significantly between the two methods: 852 ± 35 *Salmonella* GC/g of sediment with ddPCR and the corresponding qPCR result of 355 ± 29.6 GC/g ($p \leq 0.0001$) for site #2 and for site #3 in the same range, 818 ± 29.6 and 255 ± 36.6 GC/g in ddPCR and qPCR, respectively (site differences were however statistically significant, $p \leq 0.0001$) (Table 1). The numbers were lower again in the downstream site: 341 ± 30.9 and 75 ± 4.7 GC/g of sediment in ddPCR and qPCR, respectively ($p \leq 0.0001$) (Table 1). The higher values at the sites within the informal settlements is believed to reflect direct discharge of wastes to the river streams from these communities. The presence of a significantly higher amount of *Salmonella* in the sediments of the Palmiet River also reflects a higher likelihood of the presence of other pathogens and will pose a health risk to both inhabitants and downstream localities.

One limitation of ddPCR in comparison to qPCR is the need to perform dilution of the samples, as concentrations above 75,000 copies of the target molecules lead to a significant loss of linearity at high concentrations (Hayden *et al.* 2013). This was also evident in our results, where ddPCR showed higher variability and less precision at the higher concentrations (2×10^5 or 2×10^4) while qPCR performed well at this range. In order to overcome this problem, the ddPCR was performed on DNA standards ranging from 2.0×10^0 *ttr* gene copies to 2.0×10^3 *ttr* gene copies.

ddPCR may provide an opportunity to reduce the inhibitory effects of PCR inhibitors experienced with qPCR, but the methodology needs to be further tested and applied for complex environmental samples. In conclusion, for this first comparison related to *Salmonella* and sediment samples, ddPCR is fully amenable for the quantification of *Salmonella* and offers a robust, accurate, high-throughput, affordable and more sensitive quantitation than qPCR of pathogens related to this type of environmental sample.

Table 1 | Comparison of qPCR and ddPCR performances in sediment samples collected from upstream and downstream of Quarry road informal settlements in Palmiet River

Sampling sites	*Salmonella*[a] *ttr* GC/g in qPCR	*Salmonella*[a] *ttr* GC/g in ddPCR
Site#1 (upstream of informal settlements)	59 ± 1.4	65 ± 0.77
Site#2 (start of informal settlements)	355 ± 30	852 ± 35
Site#3 (downstream of river, within informal settlements)	255 ± 37	818 ± 30
Site#4 (end of informal settlements)	75 ± 5.0	341 ± 31

[a]Values represented ($n = 3$) ± SD.

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