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Comparison of next-generation droplet digital PCR (ddPCR) with quantitative PCR (qPCR) for enumeration of *Cryptosporidium* oocysts in faecal samples

Rongchang Yang [a], Andrea Paparini [a], Paul Monis [b], Una Ryan [a,*]

[a] School of Veterinary and Life Sciences, Vector- and Water-Borne Pathogen Research Group, Murdoch University, Murdoch, Western Australia 6150, Australia
[b] Australian Water Quality Centre, South Australian Water Corporation, 250 Victoria Square, Adelaide, SA 5000, Australia

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ABSTRACT

Clinical microbiology laboratories rely on quantitative PCR for its speed, sensitivity, specificity and ease-of-use. However, quantitative PCR quantitation requires the use of a standard curve or normalisation to reference genes. Droplet digital PCR provides absolute quantitation without the need for calibration curves. A comparison between droplet digital PCR and quantitative PCR-based analyses was conducted for the enteric parasite *Cryptosporidium*, which is an important cause of gastritis in both humans and animals. Two loci were analysed (18S rRNA and actin) using a range of *Cryptosporidium* DNA templates, including recombinant plasmids, purified haemocytometer-counted oocysts, commercial flow cytometry-counted oocysts and faecal DNA samples from sheep, cattle and humans. Each method was evaluated for linearity, precision, limit of detection and cost. Across the same range of detection, both methods showed a high degree of linearity and positive correlation for standards ($R^2 \geqslant 0.999$) and faecal samples ($R^2 \geqslant 0.9750$). The precision of droplet digital PCR, as measured by mean Relative Standard Deviation (RSD;%), was consistently better compared with quantitative PCR, particularly for the 18S rRNA locus, but was poorer as DNA concentration decreased. The quantitative detection of quantitative PCR was unaffected by DNA concentration, but droplet digital PCR quantitative PCR was less affected by the presence of inhibitors, compared with quantitative PCR. For most templates analysed including *Cryptosporidium*-positive faecal DNA, the template copy numbers, as determined by droplet digital PCR, were consistently lower than by quantitative PCR. However, the quantitations obtained by quantitative PCR are dependent on the accuracy of the standard curve and when the quantitative PCR data were corrected for pipetting and DNA losses (as determined by droplet digital PCR), then the sensitivity of both methods was comparable. A cost analysis based on 96 samples revealed that the overall cost (consumables and labour) of droplet digital PCR was two times higher than quantitative PCR. Using droplet digital PCR to precisely quantify standard dilutions used for high-throughput and cost-effective amplifications by quantitative PCR would be one way to combine the advantages of the two technologies.

1. Introduction

The protozoan parasite *Cryptosporidium* is an important cause of enteric disease worldwide (Xiao, 2010) and is increasingly recognised as one of the major causes of moderate to severe diarrhoea in developing countries (Kotloff et al., 2013). After rotavirus, cryptosporidiosis is the second greatest cause of diarrhoea and death in children (Striepen, 2013). It is transmitted via the faecal oral route, with large amounts of *Cryptosporidium* oocysts excreted by infected individuals (10^5 to 10^9 oocysts/gram of stool) (Chappell et al., 2006). The parasite is a significant threat to water utilities as it has a low infectious dose (10–100 oocysts), is able to survive for long periods in the environment and is resistant to drinking water disinfectants (Fayer, 2004). Of the waterborne protozoan parasitic outbreaks that have been reported worldwide between 2004 and 2010, *Cryptosporidium* was the etiological agent in 60.3% ($n = 120$) (Baldursson and Karanis, 2011).

Enumeration of *Cryptosporidium* oocysts in samples such as stool or water is particularly important for diagnostic purposes, catchment management and water quality assessment. To this end, the advent of quantitative PCR (qPCR) (Leutenegger et al., 2001) represented a significant advance with respect to conventional PCR which is based on endpoint analyses. qPCR allows

* Corresponding author. Tel.: +61 8 93602482.
E-mail address: Una.Ryan@murdoch.edu.au (U. Ryan).

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closed-tube quantitation of template DNA by monitoring, in real-time, the progression of the reaction after each amplification cycle, using a variety of fluorescent reporter chemistries (e.g., probes or dyes) (Rahman et al., 2013). Quantitative information is obtained from the cycle threshold (Ct), a point on the fluorescence curve where the signal increases above background (Hindson et al., 2011; Baker, 2012). qPCR enables detection and quantitation of the target nucleotide sequences, initially present in the reaction mixture, down to one or a few copies (Rački et al., 2014).

A variety of qPCR-based assays have been developed for enumeration of *Cryptosporidium* oocysts in faecal, sewage and water samples (e.g. Masago et al., 2006; Alonso et al., 2011; Hadfield et al., 2011; Loganthan et al., 2012; Rolando et al., 2012; Mary et al., 2013; Yang et al., 2013, 2014). However, due to the intrinsic constraints of qPCR, standards of known concentration are required to generate calibration curves used to estimate the concentration of pathogens in a sample (Hindson et al., 2011). In addition, a variety of factors including inhibitory substances found in faecal and water samples, competing DNA and non-exponential amplification during early PCR cycles affect the Ct values limiting, in-turn, the accuracy and precision of this technique (Skotarczak, 2009; Hindson et al., 2011; Roberts et al., 2013).

Droplet digital PCR (ddPCR) (Hindson et al., 2011; Pinheiro et al., 2012) is the third-generation implementation of conventional PCR that facilitates the quantitation of nucleic acid targets without the need for calibration curves (Vogelstein and Kinzler, 1999). In ddPCR, a fluorescent probe-based PCR assay is partitioned into highly uniform one-nanolitre reverse-micelles (water-in-oil), such that each droplet in the emulsion is an independent nano-PCR, containing zero, one or more copies of the target nucleic acid, assorted in a random fashion. After PCR amplification, the fluorescence of each droplet is individually measured and defined as positive (presence of PCR product) or negative (absence of PCR product). The absolute number of target nucleic acid molecules, contained in the original sample before partitioning, can be calculated directly from the ratio of positive events to total partitions, using binomial Poisson statistics (Pinheiro et al., 2012).

In ddPCR, the ratio between target DNA molecules to PCR reagents is substantially higher, in the nano-litre volume, than in conventional microlitre-scale PCR. This entails that the likelihood of favourable primer-template interactions and, thus, the efficiency, specificity and sensitivity of ddPCR, is potentially higher in comparison with conventional PCR (Vincent et al., 2010). Similarly, the fluorescent product is confined to the droplet volume and, since each single droplet is analysed individually, small changes in fluorescence intensity are more readily detected by the instrument than a similar absolute amount of fluorescence would be by conventional qPCR platforms (Vincent et al., 2010). In addition, preliminary studies seem to suggest that ddPCR is robust against many of the factors that can negatively influence conventional PCR (Dingle et al., 2013), because the DNA template, when confined, is sequestered from cross-reacting DNA templates and inhibitory moieties (Nakano et al., 2003).

In light of these potential advantages, ddPCR is attracting considerable attention and the technique has already been used for a variety of clinical and environmental applications, including the quantitation of *Chlamydia trachomatis* infections (Roberts et al., 2013), waterborne RNA viruses (Rački et al., 2014), and human epidermal growth factor receptor 2 (HER2) expression in formalin fixed paraffin embedded (FFPE) breast cancer samples (Heredia et al., 2013). ddPCR has also shown its potential utility in the characterisation of the temporal dynamics of microbial populations in complex soil environments (Kim et al., 2014) and in the accurate quantification of DNA (Dong et al., 2014). Accurate quantitation of *Cryptosporidium* oocysts in animal faecal deposits on land is an essential starting point for estimating *Cryptosporidium* loads for a particular catchment (Davies et al., 2003). Therefore, in the present study, we compared the quantitation of *Cryptosporidium* DNA by ddPCR and qPCR, to assess the utility of ddPCR for enumerating *Cryptosporidium* oocysts in clinical or environmental samples.

2. Materials and methods

2.1. Sources of DNA

For the present study, DNA was extracted from four sources: (i) recombinant plasmids containing partial fragments of the *Cryptosporidium* 18S rRNA and actin genes, (ii) haemocytometer-counted purified *Cryptosporidium parvum* oocysts, (iii) commercial *C. parvum* oocyst standards (Easyseed™, Biotechnology Frontiers, Australia) and (iv) various animal and human faecal samples ($n = 18$) (Tables 1–4). No-template controls (NTCs) were used in all PCR assays and 1 μL of template DNA was used in all reactions.

2.1.1. Cloned plasmids

Segments of the 18S rRNA and actin genes (283 and 161 bp, respectively) were amplified separately using the primers described in Section 2.2, with *C. parvum* genomic DNA as the template. Amplicons were then cloned in the pGEM-T Easy Vector System II (Promega, NSW, Australia). After transformation of the ligation products into *Escherichia coli* JM109 competent cells, plasmid DNA from the positive colonies were extracted using a QIAprep Spin Miniprep Kit (Qiagen, Victoria, Australia) from cultured single colonies grown overnight. The DNA concentrations of the pGEMT-18S rRNA and pGEMT-actin plasmids were then measured using a BioSpectrometer (Eppendorf, NSW, Australia) and recalculated to plasmid copies/μl as previously described (Sambrook and Russel, 2001). Plasmid DNA preparations (pGEMT-18S rRNA or pGEMT-actin), quantitated using the BioSpectrometer, were normalised to 10,000 copies per μl and were used to generate two standard curves by carrying out three independent serial dilutions (i.e., $n = 3$/plasmid), so that each of the dilution steps was represented in triplicate. Each serial dilution, consisting of five 10-fold dilution steps (1:1 to 1:10,000), was then used in both the ddPCR and qPCR assays.

2.1.2. Haemocytometer-counted purified oocysts

Oocyst DNA was extracted from a *C. parvum* isolate (SC26) (Tables 1 and 2), originally obtained from an infected calf, from the Institute of Parasitology, University of Zurich, Switzerland. The oocysts were purified using a Ficoll density gradient extraction as previously described (Meloni and Thompson, 1996). Purified oocysts were enumerated with a haemocytometer and stored until required at 4 °C in 1 × PBS supplemented with antibiotics (100 IU/ml of penicillin G, 0.1 μg/ml of streptomycin and 2.5 μg/ml of amphotericin B) at a concentration of 10^7 oocysts/ml.

For DNA extraction, 100 μl of oocyst solution, at a concentration of 5,000 oocysts/μl, were centrifuged for 10 min at full speed (10,000*g*) in a bench-top microcentrifuge (500,000 oocysts total). The supernatant was carefully removed via aspiration. Thereafter, the pellet was resuspended in 100 μl of lysis mix, consisting of 4 μl of 10 × PCR buffer, 54 μl of pure sterile water and 40 μl of a 50% Chelex beads solution (Bio-Rad, NSW, Australia). The tubes were subjected to four cycles of freezing (liquid nitrogen) and thawing (~95 °C) (1 min each), followed by a 10 min boiling step (~95 °C). Proteinase K (2 μl of 600 mAU/ml) was then added and samples were incubated at 56 °C overnight. The samples were then centrifuged at 10,000 *g* for 5 min to pellet the Chelex and the supernatant was transferred to fresh tubes for storage at −20 °C. The DNA preparation was serially diluted to obtain oocyst-DNA