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

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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.

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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

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 Bio-Spectrometer, 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 (10,000g) 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

Table 1
Comparison of droplet digital PCR (ddPCR) with quantitative PCR (qPCR) for the quantification of recombinant plasmids, carrying *Cryptosporidium parvum* 18S rRNA and actin gene fragments, and DNA extracted from haemocytometer-counted oocysts and flow cytometry-counted oocysts. All DNA preparations were quantified spectrophotometrically, prior to amplification. Nominal input is the amount of template DNA used in each individual PCR, expressed as the gene copy number/reaction or oocyst-equivalents/reaction, for the recombinant plasmids and oocysts, respectively. Measured concentration is also expressed as the gene copy number/reaction or oocyst-equivalents/reaction, for the recombinant plasmids and oocysts, respectively.

Source of template DNA	ddPCR		Nominal input	qPCR			
	18S rRNA	Actin		18S rRNA		Actin	
	Measured concentration	Measured concentration		Measured concentration	Corrected concentration [a]	Measured concentration	Corrected concentration[a]
Recombinant plasmid	10,625.00	15,000.00	20,000.00	23,101.00	12,012.50	19,432.30	14,515.90
	1,008.30	1,431.70	2,000.00	1,719.30	8,94.04	1,836.30	1,371.70
	99.2	162.3	200	218	1,13.36	188.3	140.7
	13.3	15.2	20	21.4	11.1	18.8	14
	4	5	10	8.3	4.3	7.6	5.7
	0	0	5	3.8	2	3.2	2.4
	0	0	2.5	1.3	0.7	0.7	0.5
Haemocytometer-counted oocysts	198.8	195.6	500	531.3	212.5	516.7	201.5
	54.1	45.8	125	107.6	43	121.7	47.5
	13.1	12	31	33.4	13.4	29.6	11.5
	3	3.4	8	7.8	3.1	8.1	3.2
	0.7	0	2	2	0.8	2.1	0.8
Flow cytometry-counted oocysts	14.9	13.6	20	19.9	14.9	20.3	14.6
	7.9	7.6	10	10.3	7.7	9.6	6.9
	3.6	3.8	5	4.5	3.4	5.5	4
	1.9	1.7	2.5	2.4	1.8	2.3	1.7

[a] Actual measured concentrations as determined by cycle threshold (Ct) values were adjusted for inaccuracies of spectrophotometer readings, pipetting errors and DNA extraction efficiency as determined by ddPCR (i.e. for recombinant plasmids the difference between the nominal input and the ddPCR readings were ~48% and 29% for the 18S rRNA and actin loci, respectively. For haemocytometer-counted oocysts, the difference was ~60% and 61% for the 18S rRNA and actin loci, respectively, and for flow-cytometry counted oocysts, the difference was 25% and 28% for the 18S and actin loci, respectively). Therefore the qPCR data was revised down by these percentages.

Table 2
Pairwise ratios between droplet digital PCR (ddPCR) and quantitative PCR (qPCR) and between the two loci (actin and 18S rRNA) for corrected qPCR data when applied to recombinant plasmids carrying *Cryptosporidium parvum* 18S rRNA and actin gene fragments, and DNA extracted from *C. parvum* haemocytometer-counted oocysts and flow cytometry-counted oocysts. Numbers in bold represent highest and lowest values within their respective column.

Source of template DNA	Ratio ddPCR vs qPCR		Ratio actin vs 18S rRNA	
	18S rRNA	Actin	ddPCR	qPCR
Recombinant plasmid	0.88	1.03	1.41	1.21
	1.13	1.04	1.42	1.53
	0.53	1.15	**1.64**	1.24
	1.19	1.08	1.14	1.26
	0.93	0.88	1.25	1.31
	–	–	–	1.21
	–	–	–	**0.76**
Average	0.93	1.04	1.22	1.37
Haemocytometer-counted oocysts	0.94	0.97	0.95	0.98
	1.26	0.96	1.10	0.85
	0.98	1.04	0.86	0.92
	0.96	1.08	1.01	1.13
	0.88	0.00	1.02	0.00
Average	1.00	1.01	0.99	0.97
Flow cytometry-counted oocysts	1.00	0.93	0.98	0.91
	1.02	1.10	0.89	0.96
	1.07	0.96	1.17	1.06
	1.06	1.03	0.92	0.89
Average	1.04	1.00	0.99	0.96

concentrations equivalent to 500, 125, 31, 8 and 2 oocysts/µl, prior to amplification by ddPCR and qPCR.

2.1.3. Commercial flow cytometry-counted oocysts (EasySeed™)

For further validation of the ddPCR assay, five vials of EasySeed™ (100 flow cytometry-counted oocysts of *C. parvum* in each vial) were purchased from Biotechnology Frontiers and DNA was extracted using the same protocol described in Section 2.1.2. Serial dilutions of the Easyseed™ DNA, from the 500 oocysts, were made to obtain the equivalent of 20, 10, 5 and 2.5 oocysts/µl (Tables 1 and 2).

2.1.4. Faecal samples

Genomic DNA was extracted from 18 faecal samples positive for *Cryptosporidium* by microscopy (six each from cattle, sheep and humans) (Tables 3 and 4). A total of 200 mg for each faecal sample were extracted using the PowerSoil DNA purification Kit (Geneworks, Adelaide, Australia), according to the manufacturer's instructions with a slight modification (prior to the DNA extraction, the samples were subjected to three cycles of freezing-thawing to ensure complete breakup of oocysts). Mock extractions (no faecal sample) were performed for each extraction group, to exclude the occurrence of contaminating

Table 3
Summary of the *Cryptosporidium*-positive faecal DNA preparations (*n* = 18) used in this study and the numbers of oocysts detected per gram of faeces, using droplet digital PCR (ddPCR) and quantitative PCR (qPCR).

Sample source	Sample code	*Cryptosporidium* Spp.	No oocysts/gram of faeces, as determined by ddPCR		No. oocysts/gram of faeces, as determined by qPCR			
			18S rRNA	Actin	Actual 18S rRNA	Corrected 18S rRNA[a]	Actin	Corrected actin[a]
Sheep	PL30	*C. ubiquitium*	15,708.00	16,250.00	17,167.00	8,926.80	20,750.00	14,732.50
(*n* = 6)	PL38	*C. ubiquitium*	86,354.00	80,990.00	94,508.00	49,144.20	86,375.00	61,326.30
	PL73	*C. ubiquitium*	16,458.00	20,052.00	24,138.00	12,551.80	24,500.00	17,395.00
	PL93	*C. ubiquitium*	8,646.00	9,323.00	9,283.00	4,827.20	11,567.00	8,212.60
	PL94	*C. xiaoi*	15,177.00	14,583.00	19,354.00	10,064.10	19,250.00	13,667.50
	Vic2C65	*C. xiaoi*	392	396	413	214.8	542	384.8
Average			23,789.20	23,599.00	27,477.20	14,288.10	27,164.00	19,286.40
Cattle	McDav4	*C. bovis*	1,835.00	1,755.00	1,783.00	927.2	2,167.00	1,538.60
(*n* = 6)	McDav6	*C. bovis*	24,115.00	22,969.00	24,583.00	12,783.20	27,167.00	19,288.60
	Latmore 1	*C. parvum*	36,771.00	25,050.00	44,676.00	23,231.50	27,435.00	19,478.90
	Latmore 4	*C. parvum*	21,942.00	13,547.00	29,166.00	15,166.30	19,333.00	13,726.40
	Ernest 1	*C. bovis*	983	1,141.00	1,033.00	537.2	1,563.00	1,109.70
	Ernest 2	*C. bovis*	2,007.00	2,224.00	2,192.00	1,139.80	2,875.00	2,041.30
Average			14,608.80	11,114.30	17,238.80	8,964.20	13,423.30	9,530.60
Human	HC03	*C. parvum*	22,813.00	22,188.00	27,942.00	14,529.80	24,250.00	17,217.50
(*n* = 6)	HC07	*C. hominis*	28,333.00	33,333.00	31,488.00	16,373.80	29,267.00	20,779.60
	HC08	*C. hominis*	29,483.00	19,757.00	39,343.00	20,458.40	33,678.00	23,911.40
	HC11	*C. parvum*	57,188.00	58,490.00	66,117.00	34,380.80	65,250.00	46,327.50
	HC13	*C. parvum*	67,708.00	61,615.00	90,167.00	46,886.80	71,417.00	50,706.10
	HC05	*C. hominis*	32,813.00	25,365.00	31,104.00	16,174.10	17,833.00	12,661.40
Average			39,723.00	36,791.30	47,693.50	24,800.60	40,282.50	28,600.60

[a] qPCR data was revised down by 48% and 29% for the *Cryptosporidium* 18S rRNA and actin loci, respectively, based on previous corrections for the recombinant plasmid standards which were used to calibrate the qPCR (Table 1). Correlation analyses were then conducted on the DNA from three sample sources (hosts), to estimate the relationships between the two loci and PCR techniques.

Table 4
Pairwise ratios between the techniques (droplet digital PCR (ddPCR) and quantitative PCR (qPCR)) and between the two loci (actin and 18S rRNA) for corrected qPCR data when applied to the *Cryptosporidium*-positive faecal DNA preparations (*n* = 18) used in this study. Numbers in bold represent highest and lowest values within their respective column.

Sample source	Sample code	*Cryptosporidium* spp.	Ratio ddPCR vs. qPCR		Ratio actin vs. 18S rRNA	
			18S rRNA	Actin	ddPCR	qPCR
Sheep (*n* = 6)	PL30	*C. ubiquitium*	1.76	1.10	1.03	1.65
	PL38	*C. ubiquitium*	1.76	1.32	0.94	1.25
	PL73	*C. ubiquitium*	1.31	1.15	1.22	1.39
	PL93	*C. ubiquitium*	1.79	1.14	1.08	1.70
	PL94	*C. xiaoi*	1.51	1.07	0.96	1.36
	Vic2C65	*C. xiaoi*	1.83	1.03	1.01	1.79
Average			1.66	1.13	1.04	1.52
Cattle (*n* = 6)	McDav4	*C. bovis*	1.98	1.14	0.96	1.66
	McDav6	*C. bovis*	1.89	1.19	0.95	1.51
	Latmore 1	*C. parvum*	1.58	1.29	0.68	0.84
	Latmore 4	*C. parvum*	1.45	0.99	**0.62**	0.91
	Ernest 1	*C. bovis*	1.83	1.03	1.16	**2.07**
	Ernest 2	*C. bovis*	1.76	1.09	1.11	1.79
Average			1.73	1.12	0.93	1.47
Human (*n* = 6)	HC03	*C. parvum*	1.57	1.29	0.97	1.18
	HC07	*C. hominis*	1.73	1.60	1.18	1.27
	HC08	*C. hominis*	1.44	**0.83**	0.67	1.17
	HC11	*C. parvum*	1.66	1.26	1.02	1.35
	HC13	*C. parvum*	1.44	1.22	0.91	1.08
	HC05	*C. hominis*	**2.03**	2.00	0.77	0.78
Average			1.65	1.37	0.92	1.14

Cryptosporidium-specific DNA from reagents, consumables and operators. The concentrations of all DNA preparations were measured spectrophotometrically using an Eppendorf BioSpectrometer. All faecal DNA samples were normalised to a total concentration of 50 ng/μl to minimise potential differences in amplification efficiency across samples due to template concentration.

2.2. Probes and primers

Primers targeting the 18S rRNA gene (5′ AGTGACAAGAAATA-ACAATACAGG 3′ and 5′ CCTGCTTTAAGCACTCTAATTTTC 3′) were described by Morgan et al. (1997), and the 6-carboxyfluorescein (FAM)-labelled TaqMan probe (5′ FAM- AAGTCTGGTGCCAG-CAGCCGC-black hole quencher 1 (BHQ1) 3′) was described by King et al. (2005). Primers (Allactin F1 5′ ATCGTGAAAGAATGACWC AAATTATGTT 3′ and Allactin R1 5′ ACCTTCATAAATTGGAACGGTG TG 3′) and probes (5′ FAM-CCAGCAATGTATGTTAATA BHQ1 3′) for the actin locus were used as previously described (Yang et al., 2014). All of the primers and probes were synthesised by Biosearch Technologies (Petaluma, CA, USA).

Each 25 μl of qPCR mixture contained 12.5 μl of 2 x qPCR master mix (Roche, NSW, Australia), 0.5 μM of each primer and the targeted probe. The qPCR was *carried out* on a *Rotor Gene Q* (Qiagen). The PCR cycling conditions consisted of 95 °C for 10 min and then 45 cycles of 95 °C for 30 s and 60 °C for 1 min.

A comparison of the specificity of ddPCR and qPCR was conducted on a range of *Cryptosporidium* and non-*Cryptosporidium*

DNA samples including *Cryptosporidium muris*, *C. parvum*, *Cryptosporidium hominis*, *Cryptosporidium meleagridis*, *Cryptosporidium felis*, *Cryptosporidium andersoni*, *Cryptosporidium serpentis*, *Cryptosporidium canis*, *Cryptosporidium suis*, *Cryptosporidium bovis*, *Cryptosporidium fayeri*, *Cryptosporidium macropodum*, *Cryptosporidium ryanae*, *Cryptosporidium xiaoi*, *Cryptosporidium ubiquitum*, *Cryptosporidium tyzzeri*, *Cryptosporidium* mouse genotype II and *Cryptosporidium scrofarum* and non-*Cryptosporidium* spp.: *Isospora* sp., *Giardia duodenalis*, *Caryospora*, *Campylobacter* spp., *Chlamydia pecorum*, *Salmonella* spp., *Toxoplasma gondii*, *Trichostrongylus* spp., *Teladorsagia circumcincta*, *Haemonchus contortus*, *Streptococcus bovis* (ATCC 33317), *Enterococcus durans* (ATCC 11576), *E. coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6633) and *Eimeria* sp., as well as human, sheep and cattle DNA (38 DNA samples in total).

2.3. Droplet digital PCR (ddPCR)

The numbers of *Cryptosporidium* oocysts in faecal samples were quantified with the primers and probes described in Section 2.2. for the 18S rRNA and actin loci, using a QX100TM droplet digital PCR system (Bio-Rad) according to the manufacturer's instructions. Briefly, the ddPCR reaction mixture (25 µl) contained 12.5 µl of a $2 \times$ ddPCR master mix (Bio-Rad) and 2 µl of primer/probe mix (12.5 µM of each primer and probe). Droplets were generated using the Droplet Generator (DG) with 70 µl of DG oil/well, with a DG8 cartridge and cartridge holder, 25 µl of PCR mix and a DG8 gasket. Droplets were dispensed into a 96 well PCR plate by aspirating 40 µl from the DG8 cartridge into each well. The PCR plate was then heat-sealed with a foil seal and placed in the thermocycler (C1000, Bio-Rad). Cycling consisted of 95 °C for 10 min, followed by 45 cycles of 94 °C for 30 s and 58 °C for 1 min, one cycle of 98 °C for 10 min with a 12 °C hold. After the reaction, the droplets were read using the Droplet Reader and QuantaSoft software was used to convert the data into the number of 18S rRNA and actin template copies/µl of PCR mixture, and then oocyst equivalents. This was done on the basis that there are five copies of the 18S rRNA gene per haploid sporozoite (Le Blancq et al., 1997), one copy of the actin gene per haploid sporozoite (Kim et al., 1992), and four sporozoites per oocyst.

2.4. Statistical analysis

Statistical analyses were performed using SPSS 21.0 for Windows (SPSS Inc. Chicago, USA). Linear coefficients of determination (R^2), percentage of Relative Standard Deviation (% RSD) and Cohen's Kappa (κ) statistics were calculated. DNA extraction efficiency was estimated for each extraction, based on the number of the gene copies/oocysts equivalents measured by ddPCR. Chi-Squared analysis was used to analyse the ratio between the measured and expected gene copies of the 18S rRNA or actin genes in the *Cryptosporidium* genome. $P < 0.05$ was considered significant.

3. Results

3.1. Specificity analysis

Specificity analysis revealed excellent agreement between ddPCR and qPCR ($\kappa = 1.0$). All of the non-*Cryptosporidium* samples tested were negative by both ddPCR or qPCR and all of the positive samples were correctly identified (data not shown).

3.2. ddPCR versus qPCR on recombinant plasmids

Regression analyses, conducted to compare nominal plasmid concentrations (i.e., assumed template copy number based on spectrophotometric measurements) and calculated concentrations measured by ddPCR and qPCR, showed highly significant agreement ($P < 0.010$) and linearly positive correlations with excellent coefficients of determination ($R^2 \geqslant 0.999$) (Tables 1 and 2). Similarly, pairwise correlations conducted between all loci and methods ($n = 4$; i.e. 18S rRNA ddPCR versus 18S rRNA qPCR, actin ddPCR versus actin qPCR, 18S rRNA ddPCR versus actin ddPCR, and 18S rRNA qPCR versus actin qPCR) showed a highly significant agreement ($P < 0.01$) and clear linearly positive correlations ($R^2 \geqslant 0.999$) (data not shown).

The precision of the assays was evaluated by analysing the mean% RSD, calculated using triplicate measurements, over three separate runs. Overall, the ddPCR precision was higher for both loci compared with qPCR (as indicated by the lower% RSD values, Fig. 1), although the% RSDs for ddPCR and qPCR at the actin locus were similar (Fig. 1B). In the case of ddPCR, the precision decreased as DNA concentration decreased (Fig. 1). For example, the% RSD values ranged from 1.4% to 5.3% for 18S rRNA ddPCR and from 6.2% to 8.6% for qPCR at the same locus (Fig. 1A). However, these differences were not significant. The precision of qPCR, although lower, appeared unaffected by the DNA concentration.

However, in the case of qPCR, the concentration of the standards (nominal input) is defined by an estimated plasmid concentration based on spectrophotometry, which is known to overestimate DNA concentrations (Anon, 2010) and does not take into account pipetting errors. For example, for a nominal input of 20,000 recombinant plasmid copies of the 18S rRNA gene, ddPCR reported a concentration of only 10,625 copies whereas qPCR reported 23,101 copies. On average, the differences between the nominal inputs and the ddPCR readings were ~52% and 71% for the 18S rRNA and actin loci, respectively. Therefore the qPCR data was revised down by 48% and 29%, respectively, for the 18S rRNA and actin loci to account for this (Tables 1 and 2).
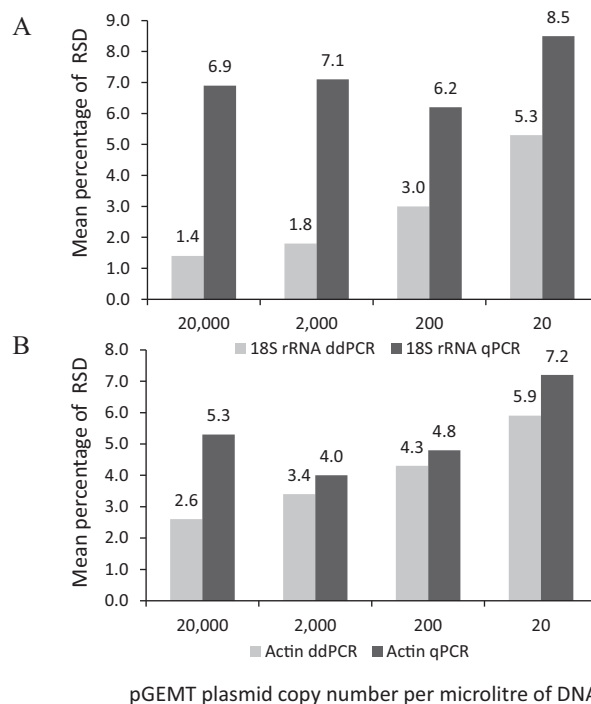
Fig. 1. Comparison of the precision of droplet digital PCR (ddPCR) and quantitative PCR (qPCR) on cloned *Cryptosporidium* targets (gene copy numbers ranged from 20 to 20,000) for (A) 18S rRNA gene plasmid (pGEMT) construct and (B) actin gene plasmid construct as measured by the percentage of relative standard deviation (RSD). The data show results from three separate runs.

3.3. ddPCR versus qPCR on haemocytometer-counted purified oocysts

For serially diluted haemocytometer-counted purified oocysts, there was significant agreement ($P < 0.001$) between all pairwise comparisons of methods (18S rRNA ddPCR nominal versus 18S rRNA ddPCR measured number; actin qPCR nominal versus actin qPCR measured number and actin ddPCR nominal versus actin ddPCR measured number), with R^2 values $\geqslant 0.995$ (data not shown). As with the recombinant plasmid standards, there were differences between the number of oocyst equivalents (calculated from known gene copy numbers) detected by ddPCR and qPCR (Table 1). For example, for a nominal oocyst concentration of eight oocysts/reaction, ddPCR detected 3.0 and 3.4 oocysts for the 18S rRNA and actin loci, respectively, whereas, by qPCR, 7.8 and 8.1 oocysts were detected for the 18S rRNA and actin loci, respectively. However, the DNA extraction efficiency (based on the ddPCR readings) on average was ~40% for the 18S rRNA and 39% for the actin locus. Therefore, when qPCR data were corrected for DNA extraction efficiency (Table 3), the sensitivity of both methods was comparable. For example, for a nominal oocyst concentration of eight oocysts/reaction, the adjusted qPCR reading was 3.1 and 3.2 oocysts for the 18S rRNA and actin loci, respectively.

3.4. ddPCR versus qPCR on flow cytometry-counted oocysts (EasySeed^{TM})

For flow cytometry-counted oocysts, all linear regressions performed ($n = 4$) showed that there was significant agreement ($P < 0.001$) between nominal and measured oocysts for both loci and both techniques ($R^2 \geqslant 0.998$). ddPCR detected fewer oocysts/μl than qPCR, but detected genomic DNA from 2.5 oocysts equivalents (Table 1). For example, at a nominal oocyst count of five oocysts/reaction, ddPCR detected 3.6 and 3.8 oocysts for the 18S rRNA and actin loci, respectively, whereas qPCR detected 4.5 and 5.5 oocysts, respectively. As flow cytometry is considered a more accurate method of counting oocysts than by haemocytometer (Reynolds et al., 1999), the DNA extraction efficiency (based on 18S rRNA ddPCR) was higher and was on average 75% and 72% at the 18S and actin loci, respectively (Table 2). Therefore at a nominal oocyst count of five oocysts/reaction, the adjusted qPCR readings were 3.5 and 4.0 oocysts, respectively (Table 1).

A pairwise analysis of the ratios between the techniques (ddPCR and qPCR) and between the two loci (18S rRNA and actin) revealed that overall the techniques compared well but there were some discrepancies (Table 2). For the techniques, the range was 0.53–1.26, demonstrating that for most samples, qPCR provided higher estimates. For the two loci, the range was 0.76–1.64, but the average ratios were close to one for both loci.

3.5. ddPCR versus qPCR on Cryptosporidium-positive faecal samples

A total of 18 Cryptosporidium-positive faecal samples were analysed, using both 18S rRNA and actin loci, by ddPCR and qPCR, and the number of oocysts per gram of faeces was calculated for each method. Samples that had oocysts numbers outside the standard curve used for qPCR were diluted 1:10 and 1:100 and re-amplified (Table 3). There was a positive linear agreement between the quantitation based on 18S rRNA ddPCR and 18S rRNA qPCR ($P = 0.130$) and between actin ddPCR and actin qPCR ($P = 0.180$), for all 18 faecal samples tested ($R^2 = 0.965$ and 0.977 for actin and 18S rRNA loci, respectively).

As the faecal samples were quantitated by qPCR, using the recombinant plasmids as standards, oocysts numbers determined by qPCR were revised down by 48% and 29% for the 18S rRNA and actin loci, respectively. When this correction was applied, the numbers of oocysts detected per gram of faeces were
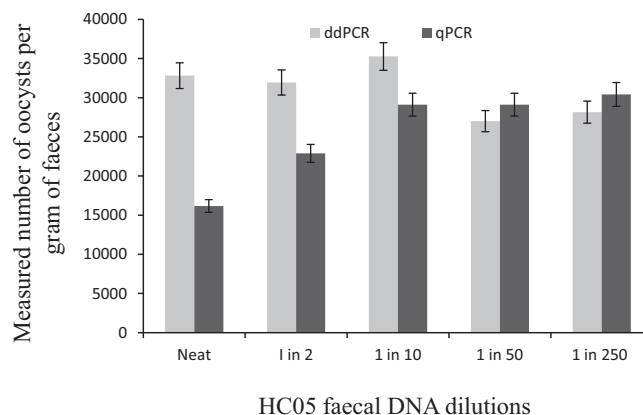

Fig. 2. Comparison of the susceptibility of droplet digital PCR (ddPCR) and quantitative PCR (qPCR) to PCR inhibitors at the Cryptosporidium 18S rRNA locus based on serial dilutions of DNA (50 ng/μl) extracted from a Cryptosporidium hominis-positive human faecal sample (HC05).

consistently lower for qPCR than ddPCR, with the exception of one sample (HC08) at the actin locus (Table 3), indicating that inhibition may have been an issue for qPCR. To test for the presence of possible PCR inhibitors, the DNA of one sample (HC05) was serially diluted and re-analysed using ddPCR and qPCR at both loci. This revealed that the number of oocysts calculated by qPCR was higher for the diluted DNA than for the neat (undiluted) preparation, indicating that some PCR inhibitors were indeed present in the HC05 DNA sample. These inhibitors negatively affected the qPCR efficiency but not that of ddPCR (Fig. 2).

A pairwise analysis of the ratios between the techniques (ddPCR and qPCR) and between the two loci (18S rRNA and actin) revealed that overall the techniques compared well but there were some discrepancies (Table 4). For the techniques, the range was 0.83 to 2.03, demonstrating that for most samples, ddPCR provided higher estimates with the exception of one sample at the actin locus (i.e. 0.83 for HC08). For the two loci, the range was 0.62–2.07 but the average ratios were closer to one for actin, indicating that actin may provide more consistent quantitation. However, further analysis is needed to confirm this.

3.6. Comparison of the costs of ddPCR and qPCR

A comparison of the direct and indirect costs of ddPCR and qPCR is summarised in Table 5. Testing one unknown sample (in duplicate) by qPCR required five DNA standards for generating a standard curve (in duplicate), plus two NTCs. For one sample, the total cost was AUD 61.00 for ddPCR and AUD 50.00 for qPCR (Table 5). On a 96 well plate, a total of 42 samples in duplicate can be run (excluding standards and controls) for qPCR. For ddPCR, 46 samples can be run on a 96 well plate (excluding controls). On a 96 well plate, the total cost of ddPCR (including labour) was two times higher than qPCR (~AUD 566 versus ~AUD 190) (Table 5), and the actual cost per sample was ~AUD 12 for ddPCR compared with ~AUD 4 for qPCR. The higher costs for ddPCR are due to both reagents and labour (calculated based on hands-on time only). ddPCR turnaround time was also longer than for qPCR. For example, approximately 6.5 h were required to prepare and run a 96 well plate for ddPCR, whereas qPCR could be completed in 2.5 h (Table 5).

4. Discussion

We believe that the present study is the first published assessment of ddPCR compared with qPCR for the quantitative

Table 5
Cost analysis (in Australian dollars) and turnaround time (h) for droplet digital PCR (ddPCR) and quantitative PCR (qPCR) assays, based on one unknown sample and a 96 well plate.

Number of samples[a]	Specifications	Item	ddPCR	qPCR
1	1 unknown sample (in duplicate), 1 non-template control (NTC), 1 ddPCR positive control or qPCR standard curve (5 dilutions)	Consumables	$18.00	$22.00
		Labour Fee	$44.00	$28.00
		Total cost	$61.00	$50.00
		Turnaround time (h)	2.7	2.2
96	46 unknown samples (in duplicate), NTC and 1 positive control (both in duplicate) for ddPCR or 42 unknown samples for qPCR including a standard curve (5 dilutions) and a NTC	Consumables	$486.00	$170.00
		Labour Fee	$ 80.00	$ 20.00
		Total cost	$566.00	$190.00
		Turnaround time (h)	6.5	2.5

Note costs for qPCR include costs for standard curve samples. Labour fee (AUD 40.00/h) has been calculated based on actual hands-on time (excluding incubations and run times). For both methods, calculations were based on positive controls, NTCs and samples run in duplicate. Positive controls for ddPCR were in also duplicate but for generating the qPCR standard curves five dilutions, each in duplicate, were considered. Thus, for one sample only, the minimum number of reactions were $n = 6$ for ddPCR and $n = 14$ for qPCR.

[a] For ddPCR, 46 samples (in duplicate) can be run on a 96 well plate excluding controls. For qPCR, 42 samples (in duplicate) can be run on a 96 well plate excluding standards and controls.

detection of *Cryptosporidium* DNA in a range of samples including animal and human faecal samples. Quantitative linearity, precision, quantitative detection and cost were assessed. The utility of a direct rather than a relative DNA-based measurement, to quantify *Cryptosporidium* oocysts or other microorganisms, is very important, particularly if proven precise and reliable.

A major limitation of qPCR is that the quantitative data generated are only as accurate as the standards used. For *Cryptosporidium*, as for several other analogous assays, DNA standards are usually obtained using dilutions of cloned *Cryptosporidium* DNA in recombinant plasmids, or dilutions of genomic DNA extracted from known numbers of oocysts.

Quantitation of nucleic acids (NAs) is usually achieved using either of two methods: by spectrophotometry to measure UV absorbance, which can be directly related to the amount of DNA or RNA present, or by measuring the fluorescence intensity of NAs in the presence of NA-binding dyes such as Picogreen. Spectrophotometric quantitations are simple, rapid and inexpensive, but recommended for relatively highly purified and concentrated DNA preparations (5–90 ng/μL), because several (contaminating) compounds show specific absorption coefficients overlapping with that of pure double-stranded DNA (dsDNA; Sambrook and Russel, 2001). This means that the low selectivity of the method can potentially lead to overestimation of template DNA in a sample or standard (Anon, 2010). Fluorescent-based methods, on the other hand, potentially allow estimation of single stranded DNA (ssDNA), dsDNA or RNA and allow higher sensitivity. However, this approach requires the use of standards and the generation of standard curves to convert the fluorescent measurement into a DNA concentration. Thus, quantitation of DNA in reference standards for qPCR could be improved by using dyes such as SYTO9, Picogreen or EvaGreen, which excite preferentially when bound to dsDNA. For example, the Qubit1.0 fluorometer (Life Technologies, Mulgrave Victoria, Australia) is highly selective for dsDNA through the use of a DNA-specific dye and has been shown to be more accurate than other systems at lower DNA concentrations (Hoffmann and Griebeler, 2013).

Similarly, manual enumeration of oocysts using a haemocytometer is relatively inaccurate (due to manual counting, pipetting and dilution errors), whereas counting by a flow-cytometer has been shown to be more reliable (Reynolds et al., 1999). This is another potential source of inaccuracy for methods, such as qPCR, which deliver relative quantitations based on standards.

In the present study, both ddPCR and qPCR showed a high degree of linearity and positive correlation for standards ($R^2 \geqslant 0.999$) and faecal samples ($R^2 \geqslant 0.975$) across their detectable ranges. However, qPCR quantifications are indirect, because

they are based on the concentrations of the standards, which are defined by the operator (after spectrophotometric quantification). On the other hand, ddPCR quantifications are direct estimations of the template concentration within the reaction mixture. As such, the differences observed between the plasmid standard spectrophotometry estimates and the ddPCR data are most likely due to an overestimate of copy number from the DNA quantitation by the spectrophotometer. Similarly, differences between the oocyst counts by haemocytometer and flow cytometry and the ddPCR result are most likely due to pipetting errors and DNA losses during extraction. Therefore the DNA/oocyst estimates determined by qPCR were revised down based on the ddPCR data to provide a more accurate reflection of the true qPCR values.

When these corrections were applied, the sensitivity of ddPCR on diluted plasmid DNA was 10 copies/reaction for ddPCR, and 2.5 copies/reaction for qPCR, at both 18S rRNA and actin loci (Table 1). On haemocytometer-counted purified oocysts, the limit was two oocyst equivalents for both ddPCR and qPCR at the 18S rRNA locus and eight and two oocyst equivalents for ddPCR and qPCR, respectively, at the actin locus (Table 1). However, when serial dilutions of flow cytometry-counted purified oocysts (a potentially more accurate method of counting oocysts) were used as templates, then the detection limits of ddPCR and qPCR were very similar (2.5 oocysts equivalents, corresponding to 50 copies of 18S rRNA and 10 copies of actin).

Although extrapolations outside the tested range of DNA concentrations to infer theoretical limits of detection (LODs) would not be appropriate, all coefficients of determination obtained during the study clearly demonstrate local positive and linear relationships across the tested range of DNA concentrations. This evidence could be used as a benchmark for selecting the technique of choice in future screenings of *Cryptosporidium*, particularly if using the same chemistries and primers used in the present study.

For faecal samples using uncorrected qPCR data, ddPCR consistently underestimated the DNA concentration compared to qPCR for both loci with the exception of human isolate HC05 (Table 3). This discrepancy has previously been reported for Cytomegalovirus in samples of human plasma (Hayden et al., 2013). However, when the corrections to the qPCR data were applied, qPCR significantly ($P < 0.05$) underestimated the DNA concentration compared with ddPCR for both loci. This suggested that ddPCR appeared to be less sensitive to inhibitors than qPCR, as one of the *Cryptosporidium*-positive human samples (HC05) was shown to contain PCR inhibitors, which affected the qPCR quantitation but not that of ddPCR, due to partitioning of DNA, inhibitors and reagents in ddPCR. Recent studies have also shown that inhibitory substances had little effect on DNA quantification using ddPCR (Hoshino and Inagaki,

2012). Further studies are required to confirm that ddPCR is less sensitive to inhibition across a range of faecal and water samples as this would be a distinct advantage when analysing these types of samples for *Cryptosporidium* as they are known to contain various PCR inhibitors (Skotarczak, 2009). Another advantage of ddPCR over qPCR is that the precision of ddPCR was higher compared with qPCR as measured by mean RSD (%). This has previously been reported in other studies (Hindson et al., 2013; Strain et al., 2013).

In our hands, the direct costs (including labour) of the current QX100 ddPCR system was ~AUD 12/sample for ddPCR compared with ~AUD 4/sample for qPCR when running high throughput (96 well) assays. For the same assay, ddPCR also took longer to perform (6.5 h versus 2.5 h). This is clearly an issue when large numbers of samples need to be analysed, particularly in the context of a commercial laboratory.

In conclusion, both ddPCR and qPCR showed a high degree of linearity across the samples tested; ddPCR was less sensitive to inhibitors, had higher reproducibility and offered highly precise, absolute quantitative detection, without the need of standard curves.

The cost benefit analysis presented in this paper seems to suggest that qPCR is cheaper and provides better throughput. However, results from the present study show that ddPCR is a useful technique for calibrating qPCR standards to produce much more accurate standard curves, while qPCR can be used for screening of samples. Individual samples showing high inhibition can be successfully assayed by ddPCR. This combination is expected to offer a more robust, accurate, high-throughput, affordable and sensitive quantitation. Moreover, as the newer ddPCR technology matures, its costs are also expected to reduce. For instance, while performing the experiments presented in the present study a new ddPCR platform was released by BioRad (QX200) which, unlike the previous model, is compatible not only with fluorescent hydrolysis probes but also with dsDNA-binding dyes (e.g., EvaGreen). While the cost of the second chemistry is potentially lower, its specificity and applicability to future *Cryptosporidium* screenings remains to be tested.

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