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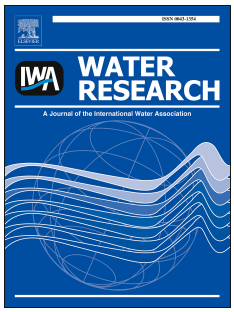

Variably improved microbial source tracking with digital droplet PCR

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Variably Improved Microbial Source Tracking with Digital Droplet PCR

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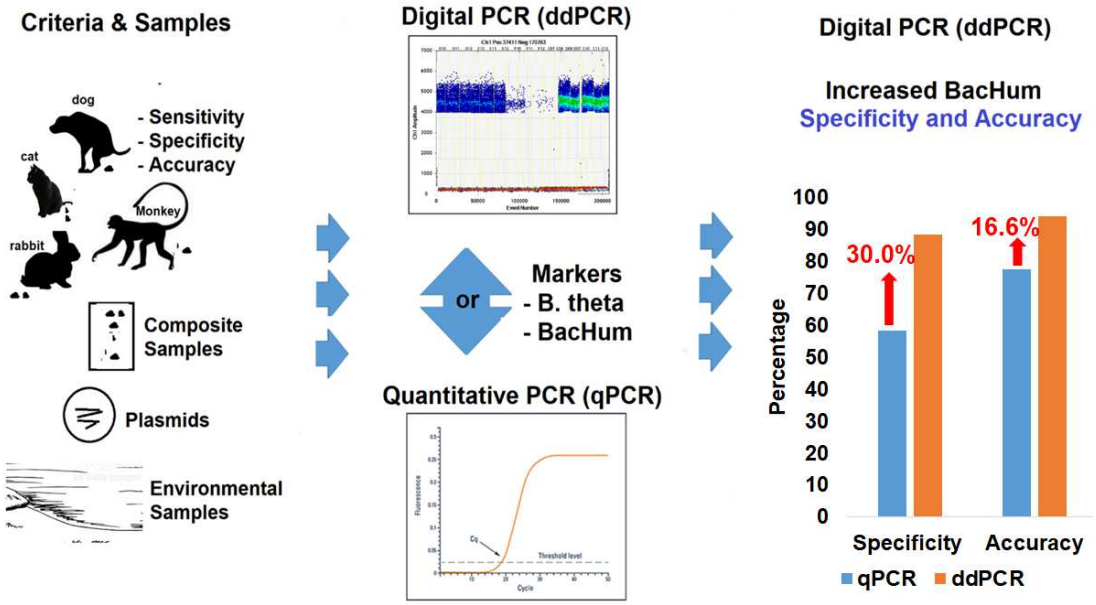
Highlights:

1. Digital droplet (dd) PCR was validated for *Bacteroidales*-based microbial source tracking
2. Sensitivity of quantitative (q) PCR for *Bacteroidales* human markers in feces was superior to ddPCR
3. Assay specificity and reproducibility in feces by ddPCR were greater than or nearly equal to those by qPCR
4. qPCR and ddPCR platform performance may vary with assay

Graphical Abstract



Abstract

This study addressed whether digital droplet PCR (ddPCR) could improve sensitivity and specificity of human-associated *Bacteroidales* genetic markers, BacHum and B.theta, and their quantification in environmental and fecal composite samples. Human markers were quantified by qPCR and ddPCR platforms obtained from the same manufacturer. A total of 180 samples were evaluated by each platform including human and animal feces, sewage, and environmental water. The sensitivity of ddPCR and qPCR marker assays in sewage and human stool was 0.85 to 1.00 with marginal reduction in human stool by ddPCR relative to qPCR (<10%). The prevalence and distribution of markers across complex sample types was similar (74-100% agreement) by both platforms with qPCR showing higher sensitivity for markers in environmental and composite samples and ddPCR showing greater reproducibility for marker detection in fecal composites. Determination of BacHum prevalence in fecal samples by ddPCR increased specificity relative to qPCR (from 0.58 to 0.88) and accuracy (from 0.77 to 0.94), while the B.theta assay performed similarly on both platforms (specificity = 0.98). In silico analysis indicated higher specificity of ddPCR for BacHum was not solely attributed to reduced sensitivity relative to qPCR. Marker concentrations measured by ddPCR for all sample types were consistently lower than those measured by qPCR, by a factor of 2.6 ± 2.8 for B.theta and 18.7 ±10.0 for BacHum. We suggest that differences in assay performance on ddPCR and qPCR platforms may be linked to the characteristics of the assay targets (that is, genes with multiple versus single copies and encoding proteins versus ribosomal RNA) however further work is needed to validate these ideas. We conclude that ddPCR is a suitable tool for microbial source tracking, however, other factors such as cost-effectiveness and assay-specific performance should be considered.

Keywords: Microbial source tracking, digital droplet PCR, quantitative PCR, genetic markers

1. Introduction

Several significant technological advances have improved the application of microbial source tracking (MST) to identify origins of fecal contamination in waterways and water bodies. Use of the polymerase chain reaction (PCR) to identify genetic markers in environmental samples accelerated the development of cultivation-independent methods for MST. PCR-based MST was improved by adoption of real-time quantitative PCR (qPCR) that enabled quantification of targeted fecal sources (Dick and Field, 2004, McQuaig et al., 2009, Seurinck et al., 2005, Bernhard and Field, 2000). This improvement led to the development of multiple qPCR assays to determine the relative contributions from humans and various animal sources of fecal contamination (Shanks et al., 2008, Shanks et al., 2009, Kildare et al., 2007). However, uncertainty in qPCR has been linked to poor MST performance. For example, recent MST studies using qPCR have reported challenges with quantification errors due to PCR inhibition (Noble et al., 2010, Cao et al., 2012), variability in standard curves and low reproducibility over time (Shanks et al., 2012), performance variability associated with different sources of cellular reference materials and reagents (Cao et al., 2013), or different batches of reagents from the same vendor (Sivaganesan et al., 2011), and lack of consistent standard material (Cao et al., 2013). Recently, the United States Environmental Protection Agency (USEPA) approved two qPCR methods as standard methods for water quality assessment (USEPA, 2013, USEPA, 2012a, USEPA, 2012b) highlighting the utility of the technology despite these concerns.

Introduction of a next-generation quantitative tool, the digital droplet PCR (ddPCR) (Pinheiro et al., 2012, McDermott et al., 2013, Hindson et al., 2013) promises to address some limitations of qPCR and further improve the implementation of MST. The workflow for ddPCR includes partitioning the reagent mix containing a DNA binding fluorescent dye into thousands

of individual reaction droplets in an oil emulsion followed by amplification (McDermott et al., 2013). The target concentration is determined by counting the droplets that score a positive or a negative for target amplification based on fluorescence of the DNA-binding dye. The distribution of positive droplets is processed using Poisson statistics to generate concentrations of the target in each sample without the use of a standard curve (Hindson et al., 2011).

Several reports indicate ddPCR is superior to qPCR in terms of sensitivity, specificity, accuracy, reproducibility near the limit of detection, and an overall reduction of inhibitor effects (Pinheiro et al., 2012, McDermott et al., 2013, Hindson et al., 2013, Whale et al., 2013). These characteristics have led to the application of ddPCR for monitoring bacterial and viral targets in multiple fields including medicine (Hayden et al., 2013), food safety (Floren et al., 2015, Morisset et al., 2013) and water quality (Racki et al., 2014, Cao et al., 2015, Te et al., 2015). Given its increasing application to water quality assessment (Cao et al., 2015, Te et al., 2015, Nshimyimana et al. 2018), we sought to evaluate whether ddPCR delivered significant improvements relative to qPCR for performance of two *Bacteroidales* assays recently validated for MST in tropical environments.

Human-associated *Bacteroidales* genetic marker assays targeting 16S ribosomal RNA or functional genes have been developed for MST to identify, quantify and monitor levels of human fecal pollution by qPCR (Yampara-Iquise et al., 2008, Shanks et al., 2009, Shanks et al., 2010, Sauer et al., 2011, Green et al., 2014, Molina et al., 2014, Kildare et al., 2007). *Bacteroidales* were preferred due to their ubiquity in human and animal guts, and because their obligate anaerobic metabolism limits survival in surface water environments (Fogarty and Voytek, 2005). Validation of human-associated *Bacteroidales* 16S rRNA genetic markers requires testing markers against fecal samples from animals and humans to determine the sensitivity and cross-reactivity of the assays. Fecal samples, and environmental water samples,

110 are often associated with high levels of inhibitors and low levels of targets (near the limit of
111 detection) leading to quantification errors by qPCR (Grgicak et al., 2010, Cao et al., 2012). In
112 this study, we compared ddPCR and qPCR-based detection and quantification of the human-
113 associated *Bacteroidales* markers B.theta and BacHum using assay conditions established by
114 previous multi-laboratory validation for qPCR (Kildare et al., 2007, Yampara-Iquise et al.,
115 2008, Odagiri et al., 2014, Nshimyimana et al., 2017a) and conditions recommended by the
116 manufacturer for compatibility with emulsion chemistry for ddPCR. Environmental and fecal
117 composite samples were characterized to compare assay performance in complex samples and
118 we determined how the sensitivity, specificity, and accuracy of ddPCR compared to qPCR
119 using fecal samples collected from human volunteers, wild and domestic animals common in
120 Southeast Asia (Nshimyimana et al., 2017a), and sewage of mixed origin.

2. Methods

2.1 Sample selection and preparation

A total of 180 genomic DNA (gDNA) samples were extracted from human and animal fecal samples (n=105) and sewage samples (n=20) previously used in a validation study of *Bacteroidales* markers for microbial source tracking in Southeast Asia (Nshimyimana et al., 2017a), environmental water samples collected in tropical urban residential areas (n=20), and composite samples containing both human and animal feces (n=35). Animal fecal samples were obtained from domesticated animals (cats n=10, rabbits n=10, dogs n=10 and chickens n=10); wild animals (wild boars n=10, monkeys n=10, and birds n=10); while 35 human stool samples were obtained from volunteers as previously described (Nshimyimana et al., 2017a). Environmental water samples were filtered onto a 0.22-µm membrane Sterivex brand cartridge filter (MilliporeSigma, Burlington, MA, USA) followed by DNA extraction, using the PowerFecal® kit (Mo Bio, Carlsbad, CA, USA) and OneStep™ PCR inhibitor removal kit (Zymo Research Cops, Irvine, CA, USA) as described in (Nshimyimana et al., 2017b). Procedural control blanks consisted of Sterivex filters that were subjected to DNA extraction and were identified as NTD (no-target detected) in all downstream analyses. The concentration of each DNA sample was measured by NanoDrop nd-1000 (NanoDrop Technologies, Wilmington, DE, USA) and was diluted to a concentration of 2 ng/µL using real-time PCR grade water (Qiagen, Hilden, Germany) for use throughout this study. Composite fecal samples (n=35) were prepared under a blind mixing protocol where aliquots from human and animal fecal DNAs were combined by one member of the study team at a volumetric ratio of approximately 1:1, while the identities and compositions of the mixtures remained unknown to the rest of the team until after qPCR- and ddPCR-based quantification was complete. The

145 expected detection of markers in composite samples was determined based on marker levels
146 measured in the individual samples and taking into account dilution due to mixing.
147
148 **2.2 Construction of *B. thetaiotaomicron*-specific alpha-1-6, mannanase (B.theta) plasmid**
149 **and preparation of plasmid stock solutions**
150 A B.theta plasmid was constructed for this study as a qPCR standard. The TaqMan
151 qPCR assay for *Bacteroides thetaiotaomicron*-specific alpha-1-6, mannanase (average
152 concentration of B. *thetaiotaomicron* is 1.39 x 10^8 cells/g of human feces (Yampara-Iquise et
153 al., 2008)) was performed using the StepOnePlus Real-Time PCR System (Applied
154 Biosystems®, Foster City, CA, USA). Each 25-µl qPCR reaction mixture contained 100 pg of
155 *B. thetaiotaomicron* (VPI 5482) nucleic acid extract, 1x TaqMan® Environmental Master Mix
156 2.0 (Applied Biosystems®, Foster City, CA, USA), 900 nM (each) of B.theta-F and B.theta-R
157 primers, and 250 nM B.theta-P probe #62 (Roche, Mannheim, Germany). The thermal cycling
158 conditions applied were 2 min at 50°C and 10 min at 95°C, followed by 45 cycles at 94°C for
159 15 s and 60°C for 1 min. The amplicons were run on 1.5% agarose gel and the 63-bp target
160 bands were extracted using a QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA, USA).
161 Purified amplicons were inserted into the PCR™4-TOPO vector by use of the TA
162 Cloning® Kit for sequencing (Invitrogen, Carlsbad, CA, USA). Plasmid DNA containing the
163 target *B. thetaiotaomicron* sequences was extracted using a QIAprep Spin MiniPrep Kit
164 (Qiagen, Valencia, CA, USA). Sequences of inserted target product were analyzed by ABI
165 Prism® 3730 Genetic Analyzer in the DNA Sequencing Facility at the University of California,
166 Davis.
167 Construction of plasmid DNA containing the BacHum assay standard was previously
168 described by our laboratory (Kildare et al., 2007) and fresh plasmid DNA containing the target

BacHum sequences was extracted using a QIAprep Spin MiniPrep Kit for this study (Qiagen, Valencia, CA, USA). DNA concentrations of plasmid standards for B.theta and BacHum were measured by Qubit 2.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA) and converted to plasmid copies based on the molecular weight of plasmid and insert. Plasmid DNA stock solutions were diluted to 10^8 copies/ µl in deionized water and stored as single-use aliquots at -80°C.

2.3 qPCR and ddPCR assays

2.3.1 qPCR assays

A Bio-Rad CFX96 (Bio-Rad, Hercules, CA, USA) (qPCR) machine was used to quantify concentrations of human-associated *Bacteroidales* gene markers (BacHum and B.theta). All assays were conducted in 20-µL reactions containing 10 µL of SsoAdanced™ Universal Probes Supermix 2X (Bio-Rad, Hercules, CA, USA). Primer and probe concentrations were respectively 400nM and 80nM for BacHum, and 900 nM and 250 nM for B.theta based on published assay conditions (Yampara-Iquise et al., 2008, Kildare et al., 2007, Odagiri et al., 2014) (Table S1). Sample DNAs were added in 2 µl volumes containing either 4 ng sample DNA, or water for no template controls (NTC), diluted plasmid standards were added in 1 µL volumes, molecular quality deionized water was added to reach the final reaction volume. qPCR reactions were subjected to hot start enzyme activation at 95°C for 3 min, followed by 40 cycles of 95°C for 15s, and annealing at 60°C for 1 min. After each QPCR run, data was examined using Bio-Rad CFX96 detection system (BioRad, Hercules, CA) with fluorescence threshold set at 200 RFU and auto-determination of baseline cycle (Van De Werfhorst et al., 2011). Standards and samples were analyzed in triplicate and duplicate, respectively, and the coefficient of variability (CV) for crossing point (C_p) values were ≤5.4% (average CV was 1.5% ± SD 1.9%) where replicate C_p values differed by ≤1.8 C_p (average ΔCp

193 was 0.56 ± SD 1.16) and were thus considered to be of high quality consistent with previously
194 established criteria for qPCR assay precision (Ebentier et al. 2013 and Shanks et al. 2016).

195 All qPCR and ddPCR reactions were prepared in a bench top hood fitted with a UV-
196 light for sterilization before and after use (Airclean® Systems, Creedmoor, NC, USA) using
197 electronic pipettes that were calibrated at the beginning of the study (Gilson Inc., Middleton,
198 WI, USA). At least two negative controls (no DNA template) were analyzed for each 96-well
199 plate. Reproducibility of qPCR conditions and absence of significant batch effects across
200 multiple 96-well plates was confirmed by close agreement of measured concentrations from
201 plasmid standards diluted to 10^3 or 10^5 copies/µL and included on each plate (Figure S3).

202 Potential inhibition of PCR was previously assayed for undiluted samples by spiking a
203 target marker at 10^3 copies/reaction into duplicate qPCRs for each sample, and comparing the
204 measured marker concentration to the standard quantified in the absence of sample background
205 (Nshimyimana et al., 2017a). As none of the undiluted samples were observed to cause
206 significant qPCR inhibition using either the B.theta or BacHum assay, we assumed identical
207 behavior for the diluted samples considered in this study.

208 Standard curves for qPCR were established using ten-fold serial dilutions of plasmid
209 stock solution (10^8 to 10^0 copies/µL) for either the B.theta or BacHum target sequence.
210 Standard curves of C_p values versus target DNA concentrations for each assay were generated
211 and fit by least-squares regression. Data from the lowest concentration standard (10^0
212 copy/reaction) were excluded to improve overall model fit, and the next lowest concentration
213 standard (10^1 copy/reaction) exhibiting linearity with more concentrated standards, was
214 operationally defined as the limit of quantification (LOQ). qPCR standard curves were defined
215 for BacHum by a slope of -3.37, intercept of 37.09 and for B.theta by a slope of -3.49, intercept

of 35.0. High linearity ($R^2 \geq 0.99$) of the qPCR standard dilutions was consistent with best practices (Shanks et al., 2016).

Confidence intervals of predicted target concentrations on measured C_P values were calculated based on propagation of error in the standard curve (Harris, 1995). The limit of detection (LOD) for BacHum and B.theta qPCR assays was determined as the highest C_p value significantly different from the lowest C_p value observed in no template control (NTC) wells at a 95% confidential interval as previously described (Nshimyimana et al., 2014). At least 2 NTC wells were considered per 96 well plate (a total of 22 NTC wells per assay; Table S3). NTC from qPCR runs that included samples (i.e. Plates #1 - #5 for each marker) did not show any amplification confirming absence of contamination during sample processing, consistent with NTC recommendations for *Bacteroidales* assays (Shanks et al., 2016). NTC wells on the plate used for amplification of the standard curve indicated trace levels of background contamination and the lowest NTC Cp values (Cp = 35.2 and 39.4 for B. theta and BacHum, respectively) were used for the LOD calculation. LODs at the 95% confidence level were thus determined to be 1.0 copies/reaction for BacHum and 3.0 copies/reaction for B.theta.

2.3.2 ddPCR assays

Samples were quantified using QX200™ Droplet Digital™ PCR (ddPCR) (Bio-Rad, Hercules, CA, USA) following the manufacturer's recommendations to ensure appropriate chemistry for emulsion PCR. B.theta and BacHum assay reactions were prepared by mixing 10 µL of ddPCR™ Supermix for probes (NodUTP), primers at 900 nM, probes at 250 nM and 2 µl template containing either 4 ng DNA, or sterilized water for no template controls. Diluted plasmid standards were added in 1 µL volumes as positive controls. Molecular grade deionized water was added to bring reaction volumes to 20 µL. Each sample was loaded onto a D8™ cartridge (Bio-Rad, Hercules, CA, USA) with 70 µL of ddPCR™ droplet generator oil (Bio-

Rad, Hercules, CA, USA) and subjected to droplet generation on a QX200™ Droplet generator machine (Bio-Rad, Hercules, CA, USA). Forty microliters of each resulting emulsion was loaded into a 96-well plate and sealed using a PX1™ Plate Sealer (Bio-Rad, Hercules, CA, USA). ddPCR assays were subjected to a thermocycle with enzyme activation at 95°C for 10 min; denaturation at 94°C for 30 s; annealing and extension at 60°C for 1 min; for 40 cycles, followed by enzyme deactivation at 98°C for 10 min, and a continuous hold at 4°C. Quantification results were read using ddPCR™ Droplet Reader Oil (Bio-Rad, Hercules, CA, USA); QX200™ Droplet Reader (Bio-Rad, Hercules, CA, USA) and QuantaSoft™ software (Bio-Rad, Inc., Hercules, CA, USA).

Quality control for ddPCR was implemented as previously described (Cao et al., 2015, Huggett et al., 2013, Pinheiro et al., 2012, Nshimyimana et al. 2018). Briefly, the number of accepted droplets generated for each sample run by ddPCR in this study was ≥10,000. The threshold for distinguishing positive from negative droplets was determined manually as the intensity in relative fluorescence units (RFU) above which no droplet signal would be expected in the NTCs rounded to the nearest 100. The mean NTC signal intensity and one standard deviation was 426.2±34.2 (n=18) for B.theta and 556.79±366.3 (n=20) for BacHum. Thresholds were defined as the highest NTC droplet signal intensity observed plus two standard deviations for B.theta (500 RFU) and BacHum (2000 RFU) where assay-specific differences in baseline RFU can be attributed to variation in quenching efficiency of different hydrolysis probes (BioRad QX200 Digital Droplet PCR manual) (Supplemental Table 1). Samples were screened by the bioinformatics tool “definetherain” to confirm that individual droplets could be unambiguously categorized as above or below the thresholds (i.e. no “rain events”) (Jones et al. 2014). Samples were considered positive for the marker if they contained three or more positive droplets.

For each sample, 0.12% to 99.0% of droplets generated were scored as positive. Negative and positive controls run on each 96-well plate confirmed the absence of reagent contamination (all NTC registered as below the detection limit), and reproducibility of plate-to-plate PCR quantification conditions (Figure S1). For ddPCR, the dynamic range and limit of detection were determined by the LOD95 method (Stewart et al., 2013, CODEX, 2010, Burd, 2010) where amplification of replicate standards of increasing dilutions (10^8 to 10^1 copies /µL, and 50, 25, 12.5, 6, 3, and 1.5 copies/µL) (Table 1) established the concentration threshold above which 95% of PCR reactions were positive. The limit of quantification was assumed to be equal to the LOD since quantification proceeds in the absence of a standard curve for ddPCR.

2.4 ddPCR and qPCR comparison

We used multiple criteria to compare performance of ddPCR and qPCR for detection and quantification of BacHum and B.theta markers including: depth of quantification, precision, sensitivity, specificity, and marker distribution and concentration in composite and environmental samples. The depth of quantification was defined by the assay limit of detection and precision by the variance among technical replicates of plasmid standards. Assay sensitivity was determined based on the number of target host samples (human stool and sewage samples) testing positive for the assayed marker (B.theta and BacHum), divided by the total number of host samples tested (Kildare et al., 2007). Specificity was calculated as the number of non-target host samples that tested negative for the assayed marker, divided by the total number of non-target samples tested (cats, rabbits, dogs, wild boars, monkeys, chicken and birds) (Kildare et al., 2007). The accuracy of the assays was computed as the ratio of target and non-target host samples identified correctly and the total number of samples tested (Odagiri et al., 2014) and was thus a function of both sensitivity and specificity. The prevalence and

abundance of human markers in environmental and composite fecal samples were compared to determine how the assays performed in complex samples. Composite samples were further analyzed to determine the reproducibility of marker detection based on expected levels in component individual samples, as described previously (Nshimyimana et al. 2017a).

2.5 Statistical analysis

qPCR standard curves (i.e. calibration curves) were generated by linear regression of standard concentrations estimated by dilution of a plasmid stock and crossing-point (Cp) values obtained by measurement of the standard dilutions by qPCR. Sample concentrations were reported as log10 values for qPCR and for ddPCR were log-transformed prior to statistical analysis unless indicated otherwise. The repeatability of quantification by ddPCR and qPCR was compared by calculating the coefficient of variation from sample replicates. Pearson correlation was used to compare human marker concentrations quantified by qPCR and ddPCR in composite, environmental, and serially diluted plasmid DNA samples. ANOVA and Paired T-test of environmental samples were used to compare the performance of qPCR and ddPCR in quantifying levels of human markers in fecally polluted natural water samples. Mean values of human and sewage were compared using ANOVA followed by Tukey's honest significant difference (HSD) post-hoc multiple-comparisons test to determine differences in quantification across assays in human and sewage samples using JMP Pro (SAS Institute Inc., Cary, NC USA).

3. Results

3.1 Analysis of plasmid standards

Quantification of plasmid-borne standard dilutions by ddPCR indicated a dynamic range of 1.5 to 10^4 copies/reaction for B.theta and 5.8 to 10^4 copies/reaction for BacHum (Table 1) where the lower limit of the dynamic range was defined by the limit of detection with 95% confidence. For ddPCR, quantification of standards above 10^4 copies/reaction was not possible due to saturation of droplets with positive signal. Quantification of plasmid-borne standard dilutions by qPCR indicated a dynamic range of 3.0 to 10^8 copies/reaction for B.theta and 1.0 to 10^8 copies/reaction for BacHum (Table 1). All ddPCR and qPCR data generated passed the MIQE guidelines (Bustin et al., 2009, Huggett et al., 2013), where the efficiency of qPCR amplification as determined from analysis of standard curves (Figure S2) ranged from 93% to 98%, falling within the range attributed to good laboratory performance of human feces-associated assays (Shanks et al., 2012, Griffith and Weisberg, 2011) (Figures 2A, 2B and S2).

3.2 Human and animal fecal samples: Sensitivity, specificity and accuracy

The human-associated *Bacteroidales* assays BacHum and B.theta, validated as the best human markers for microbial source tracking in Singapore and Southeast Asia (Nshimyimana et al., 2017a), were used to compare the performance of ddPCR and qPCR implemented on platforms by the same manufacturer (Biorad). A reaction was considered to be positive if the measured concentration was greater than or equal to the assay detection limit for qPCR or ddPCR. In general, the prevalence of BacHum and B.theta markers as determined by ddPCR were the same, or lower, than those determined by qPCR in sewage, and fecal samples from humans, domestic animals, and wild animals (Figure 1). For human stool samples (n = 35) ddPCR indicated 34 were positive for the BacHum marker and 30 were positive for B.theta indicating a sensitivity of 97.1 and 85.7%, respectively. By comparison qPCR indicated higher

marker prevalence with 100% and 94.3% of samples testing positive for BacHum and B.theta, respectively. In sewage samples (n=20), ddPCR indicated all samples were positive for both B.theta and BacHum markers (100% sensitivity), similar to findings from qPCR (Table 2). The modest reduction in sensitivity for human markers in human stool samples by ddPCR compared to qPCR (BacHum: 100% to 97.1% and B.theta: 94.3% to 85.7%) was due to variation in samples quantified near the detection limits of both platforms.

The specificity and accuracy of BacHum and B.theta assays were compared for qPCR and ddPCR platforms by analysis of non-target animal samples. For B.theta, both platforms had a positive cross-reaction for a single cat fecal sample out of 70 animal samples tested (Figure 1, Table 2) indicating similar specificity of the ddPCR and qPCR B.theta assay. However, the accuracy of the B.theta assay was slightly reduced by ddPCR (95%) relative to qPCR (97%) due to the reduced sensitivity of ddPCR for the B.theta marker in human stool samples, as discussed above.

The ddPCR assay improved the specificity and accuracy for detecting the BacHum marker by reducing cross-reaction with non-target animal samples. For ddPCR 7/70 animal samples tested positive for BacHum while 25/70 were positive by qPCR resulting in a specificity of 0.88 by ddPCR compared to 0.58 by qPCR (Tables 2 and 3). Although ddPCR showed a slightly reduced sensitivity for BacHum in human stool samples (100% by qPCR vs. 97% by ddPCR), sensitivity in sewage was 100%, and overall the accuracy of the BacHum assay was significantly improved to 0.94 by ddPCR compared to 0.81 by qPCR due to heightened specificity (Figure 1C, Tables 2 and 3). Since it is possible that differences in assay detection limits could impact the comparisons of sensitivity and specificity, we conducted an *in silico* analysis to examine assay performance as a function of the concentration identified as the assay limit of detection. In order to achieve a specificity >0.88 as obtained by ddPCR the

selected qPCR LOD would have to be increased by over 500-fold, resulting in over 40% reduction in assay sensitivity (Figure 3). This analysis suggests that the heightened specificity of the ddPCR platform for the BacHum marker cannot be solely attributed to the slight reduction (<10%) of ddPCR assay sensitivity relative to qPCR.

3.3 Comparison of human marker prevalence and quantification in composite and environmental samples

We examined the relationship between concentrations of human markers by qPCR and ddPCR using human stool, sewage, composite, environmental samples, and standards. Overall, concentrations in samples determined by qPCR were significantly higher than those measured by ddPCR by a factor of 18.7 ± 10.0 and 2.6 ± 2.8 for BacHum and B.theta, respectively, and independent of sample type (Table 4, S2). In contrast dilutions of B.theta and BacHum plasmid standards over the interval of 10 to 10^4 copies per reaction were measured at nearly the same level by qPCR and ddPCR (qPCR to ddPCR ratios were 1.1 ± 0.2 for B.theta and 1.3±1.2 for BacHum; Tables 1 and 4). Further analysis of the diluted plasmid standards indicated good agreement of qPCR measurement of BacHum and B.theta and ddPCR measurement of B.theta with the concentrations predicted based on the dilution series (slope near or equal to 1.0 and intercept near zero for measured versus expected values; $R^2 \geq 0.99$) (Figure 2). Significant differences between ddPCR and qPCR based measurements of BacHum in samples, but not standards, suggest factors related to the biological and environmental context of the marker genes may influence quantification results.

3.3.1 Environmental samples

The concentrations of human markers (B.theta and BacHum) in twenty catchment water samples were quantified by qPCR and ddPCR (Figure 4A and B). By ddPCR 8 of 20 samples

were positive for the B.theta marker and 9 of 20 samples positive for the BacHum marker, while by qPCR all samples (20/20) were positive for both markers where 13 of 20 and 8 of 20 samples had quantifiable levels (>LOQ) of BacHum and B.theta, respectively. For all samples, qPCR indicated significantly higher concentrations of human markers than ddPCR (Paired T. test BacHum: $p < 0.0001$ and B.theta: $p>0.0012$) by an average of 11.1-fold for BacHum and 8.1-fold for B.theta (Table 4). The measured concentrations of marker in catchment water samples by qPCR and ddPCR strongly correlated for B.theta but not for BacHum (B.theta: $R = 0.72$, $p = 0.04$, and BacHum: $R = 0.42$, $p = 0.25$) (B.theta: slope = 0.88, $R^2 = 0.53$, intercept = 0.95, and BacHum: slope = 1.44, $R^2=0.18$, intercept = 0.54) (Figure 4A and 4B).

3.3.2 Composite samples

The prevalence of the BacHum and B.theta markers across 35 composite samples comprised of DNA from human feces plus a variable mixture of DNA from sewage and animal feces was similar for ddPCR and qPCR, with several exceptions. Five composite samples showed divergent results for presence/absence of the B.theta marker; two samples were ddPCR-positive/qPCR-negative for B.theta while three samples were ddPCR-negative/qPCR-positive. Similarly, four composite samples that were ddPCR-negative for the BacHum marker were positive by qPCR. Since all composite samples were comprised of DNA previously analyzed individually by ddPCR and qPCR, the expected marker occurrence in sample mixtures was compared to observations from each platform to assess the reproducibility of findings in complex mixtures (Table 2). Analysis of composite samples by ddPCR yielded the expected marker incidence in 34 of 35 samples (97%) for BacHum and 31 of 35 samples for B.theta (89%). QPCR analyses were similar to ddPCR for BacHum with all 35 composite samples reflecting expectations (100%) and more variable for B.theta with 26 composite samples reflecting expectations (74%). Concentrations of human markers (BacHum and

B.theta) in composite fecal samples determined by qPCR were significantly higher than by ddPCR by a factor of 26.6-fold and 3.5-fold, respectively (Table 4), and for each marker, concentrations determined by the two PCR technologies were strongly correlated (B.theta: slope = 0.98, R^2 = 0.64, intercept = 0.47 and BacHum: slope = 0.75, R^2 = 0.75, intercept = 2.11 and B.theta: R = 0.80, $p < 0.0001$ and BacHum: R = 0.85, $p < 0.0001$) (Figure 5A and 5B).

4. Discussion

The penetration of ddPCR as a platform for quantitative PCR holds promise to improve genetic marker detection and quantification in complex environmental samples. In this study we sought to address first, whether implementation of ddPCR according to the manufacturer's recommendations for droplet emulsion chemistry supported good performance for assays surveying the distribution of the human-associated *Bacteroidales* markers B.theta and BacHum in complex fecal and environmental samples. Secondly, we asked whether the assays adapted for ddPCR improved the detection and quantification of BacHum and B.theta relative to qPCR using conditions previously established in multi-laboratory studies and considering depth of quantification, performance in complex samples, measurement precision, dynamic range, reliance on external standards, assay sensitivity, and specificity (Table 5).

4.1 Expected versus observed outcomes of assay performance

The qPCR method has demonstrated inconsistences and errors in the detection of human fecal pollution at low concentrations in environmental samples (Grgicak et al., 2010, Cao et al., 2012, Harwood et al., 2014). Recent work has shown ddPCR to have a high sensitivity and specificity, estimating target numbers without the use of a standard curve (Hindson et al., 2011, Hindson et al., 2013, Hayden et al., 2013, Yang et al., 2014, Cao et al., 2015, Te et al., 2015). Therefore, our initial expectation was that ddPCR would emerge as the superior platform for microbial source tracking for both assays considered. Our findings for the most part support this initial hypothesis with ddPCR showing improved specificity and accuracy relative to qPCR for detection of the BacHum marker with high sensitivity and reproducibility (Tables 3 and 5, Figure 3), and showing improved reproducibility for detection of B.theta marker in fecal composite samples despite modestly reduced sensitivity relative to qPCR. However, in several criteria qPCR, as implemented in the current study, was superior to

ddPCR including the precision of replicate measurements, the ability to quantify >10^4 copies of template, and increased sensitivity for the human marker in fecal and environmental samples (Table 5). Despite high agreement of quantification results for plasmid standards for both BacHum and B.theta markers and for the B.theta marker in all sample types (Table 5), unexpectedly high variation between qPCR and ddPCR measurements of BacHum marker in complex samples (ave. 18.7-fold higher by qPCR) raised significant questions about the reliability of BacHum quantification and identification of the best-performing platform.

4.2 Assay performance across platforms and markers

Comparison of assay sensitivity, specificity and marker concentrations revealed high agreement between qPCR and ddPCR platforms for the B.theta assay. In contrast, significant differences between platforms was observed for the BacHum marker where qPCR results indicated higher sensitivity, lower specificity, and 18.7-fold higher overall concentrations of BacHum relative to ddPCR in fecal and environmental samples. Notably, variable assay performance was not observed during quantification of plasmid standards, suggesting that assay-specific differences in performance may be related to sample complexity. Variable performance of B.theta and BacHum assays on the two platforms may be explained by the nature of the different platforms used (qPCR vs. ddPCR), by the conditions used in each assay, or by the properties of each assay's DNA target.

4.2.1 Gene Copy Number.

Assay-specific variation in marker quantification on ddPCR and qPCR platforms may be expected based on the way each platform interacts with multiple-copy genes that are genetically-linked (Wang et al., 2016). The B.theta assay target is expected to occur as a single-copy in the genome (Yampara-Iquise et al., 2008) while the 16S rRNA gene targeted by the BacHum assay may be multi-copy in certain members of the *Bacteroidales* (up to 6

458 operons/genome, (Klappenbach et al., 2000)) although whether these operons occur with close
459 proximity in the genome cannot be determined since BacHum primers target the taxonomic
460 order-level, and thus an unknown diversity of genome-types. While qPCR is expected to
461 independently quantify multi-copy genes co-occurring on the same DNA fragment, the droplet-
462 based analysis of ddPCR would be expected to score neighboring multi-copy genes together as
463 a single count, underestimating the true copy number and potentially affecting determination of
464 presence/absence for low-abundance targets. Thus, for multi-copy genes that co-occur in the
465 same gene neighborhood, ddPCR may provide lower concentration estimates than qPCR
466 (Wang et al., 2016) and lower apparent sensitivity and cross-reactivity for targets near the limit
467 of detection. As the BacHum marker is associated with a multi-copy rRNA operon,
468 undercounting of separate ribosomal targets that occur on the same DNA fragment may thus
469 contribute to the reduced estimates for BacHum marker concentration by ddPCR relative to
470 qPCR in complex samples. Plasmid standards would not be influenced by this assay-specific
471 difference as plasmids are purified in a size-selective manner to contain a single target gene
472 copy.

473 4.2.2 PCR reagent composition.

474 Master mix composition may also contribute to differences in assay performance on
475 qPCR and ddPCR platforms. We also note that PCR inhibition is unlikely to play a major role
476 in our study as all templates previously tested negative for significant PCR inhibition
477 (Nshimyimana et al. 2017a). Assay conditions for ddPCR were selected to be compatible with
478 the emulsion PCR platform, per the manufacturer's recommendations. For the B.theta assay,
479 which had identically high specificity across ddPCR and qPCR platforms, identical
480 concentrations of primers and probes were used for both platforms. In contrast, for BacHum the
481 primer and probe concentrations recommended for ddPCR were higher than those previously

optimized for the qPCR assay (Kildare et al., 2007). Typically, increased concentrations of template DNA or oligonucleotides are associated with increased probability of (mis)hybridization with reduced specificity and increased sensitivity of PCR assays (Innis and Gelfand, 1990, Wang et al, 2014); however, we observed higher specificity for the BacHum ddPCR assay despite higher concentrations of primer and probes. We thus attribute this heightened specificity to the ddPCR platform, and not to the elevated primer and probe concentrations, which would be predicted to have the opposite effect.

4.2.3 Abundance of closely related non-target sequences for ribosomal RNA versus protein-coding marker genes.

Co-occurring non-target sequences may inflate estimates of target abundance when the fidelity of primer and probe hybridization is compromised. This effect may be assay-specific depending on the extent of closely related genetic diversity in primer and probe binding sites. The BacHum primers target a phylogenetically variable region of the 16S rRNA gene and closely related non-target organisms are expected to co-occur in complex samples. In contrast, the B.theta assay targets a conserved protein-coding gene, where less fine-scale phylogenetic diversity may be expected at the primer and probe binding sites. Thus, the B.theta assay may be less vulnerable to small changes in PCR conditions that affect the fidelity of primer and probe hybridization, while such shifts in fidelity may enable amplification of non-exact matches for BacHum. It is possible that the qPCR platform and reagents in this study yielded less stringent binding of primers and probes than the ddPCR platform. Reduced specificity of the BacHum qPCR assay in animal fecal samples relative to ddPCR supports this notion. If true, reduced specificity of BacHum primer and probe binding during qPCR may explain the large variation between qPCR and ddPCR measurements observed in this study in complex samples. Plasmid standards would not be susceptible to this assay-specific difference since plasmid-borne

506 standards reflect a single target DNA sequence and would not have closely related diversity at
507 the target sequence.

508 **4.3 Tradeoffs between assay sensitivity and specificity**

509 While further work is necessary to validate the above models to explain the different
510 behaviors of the two assays on the ddPCR and qPCR platforms, taken together gene copy
511 number and expected abundance of closely related non-target sequences, may help to explain
512 the high reproducibility of plasmid standard quantification and B.theta specificity estimates
513 across qPCR and ddPCR platforms and the elevated BacHum marker levels measured by qPCR
514 in complex samples.

515 Consideration of results from this study in light of qPCR results obtained from a
516 previous study conducted by our group reveals a potential tradeoff between specificity and
517 sensitivity for the BacHum assay that is not evident for the B.theta assay. In Nshimyimana et al
518 2017, qPCR analyses indicated that BacHum and B.theta were the best performing markers for
519 MST based on observed specificities (91% and 98%, respectively) and sensitivities (65% and
520 69%, respectively) for human fecal material (Nshimyimana et al 2017). In the current study,
521 which has employed both qPCR and ddPCR, the sensitivity of the BacHum and B.theta assays
522 were markedly improved for both platforms, with unchanged high specificity for the B.theta
523 assay. In contrast, the specificity of the BacHum assay was significantly reduced by qPCR
524 implemented in the current work although specificity remained similar when determined by
525 ddPCR. qPCR analyses from the two studies employed identical thermal cycling parameters
526 and concentrations of primers and probes, with nearly-identical profiles of human and animal
527 fecal materials (modified in this study by a standardized template dilution across samples and
528 excluding Myna Birds due to prior non-detection of Bacteroidales DNA). These qPCR analyses
529 were conducted in separate laboratories, using different master mix reagents, on qPCR

530 machines from different manufacturers, and with a lower baseline threshold observed in the
531 current work. Lower limits of marker detection achieved in this study by qPCR, may explain
532 part of the increased sensitivity for both markers, however would not explain the decreased
533 specificity of the qPCR assay for BacHum while B.theta specificity remained high.

534 An in silico analysis of increased limit of detection set point for presence/absence
535 determination in this study (Figure 3) did not restore BacHum assay specificity to levels
536 observed previously by qPCR (Nshimyimana et al 2017) or via ddPCR (this study), indicating
537 that variations in BacHum assay specificity across qPCR systems and between qPCR and
538 ddPCR was likely influenced by additional unknown factors, which could potentially be related
539 to the nature of the DNA targets as discussed in the previous section. Notably, in this study the
540 ddPCR platform was able to simultaneously deliver both high sensitivity (97%) and specificity
541 (88%) for the BacHum marker suggesting reduced impacts from potential trade-offs between
542 sensitivity and specificity.

543 **4.4 Comparison to earlier studies**

544 Previous studies employing ddPCR and qPCR have observed similar results as reported
545 here for limit of detection, dynamic range, assay precision, and variability of qPCR and
546 ddPCR-based measurements of the same targets. The technical reproducibility of B.theta and
547 BacHum marker concentrations in replicates as measured by qPCR and ddPCR and the limits
548 of detection for ddPCR are within the range of earlier studies for different assays (Morisset et
549 al., 2013, Whale et al., 2013, Cao et al., 2015). Similarly, qPCR demonstrated a wider linear
550 dynamic range than ddPCR for serially diluted standards in two recent studies targeting
551 different microbial marker sequences with application to water quality monitoring in
552 California, USA (Cao et al., 2015) and Singapore (Te et al., 2015).

553 Observed trends differ for similar comparisons of qPCR and ddPCR-based
554 measurements of the same targets. A tropical study quantifying *Microcystis* demonstrated that
555 qPCR measurements were 1.3-fold to 6.8-fold (average 2.8-fold) higher than ddPCR (Te et al.,
556 2015). In contrast, in two different studies of gene quantification in clinical samples
557 concentrations measured by qPCR tended to be 7-fold (up to 30-fold) lower (Hindson et al.,
558 2013) than those determined by ddPCR (Taylor et al., 2015). Such studies, like ours, were
559 based on qPCR assays that met the MIQE (2009) standards, raising the distinct possibility that
560 significant variability can exist between qPCR and ddPCR platforms. Further work is needed to
561 understand how assay-specific factors, such as multi-copy gene linkage and properties of the
562 DNA targets, including closely-related complexity, may lead to differences in platform
563 performance.

564 Overall the performance of ddPCR and qPCR validates the utility of either platform for
565 MST using B.theta. For the BacHum marker, the ddPCR platform was validated while variation
566 across qPCR platforms suggest trade-offs between assay sensitivity and specificity that should
567 be taken into consideration for multi-laboratory studies. Given the importance of source
568 identification at low concentrations of target in environmental samples and uncertainties that
569 may be associated with qPCR standard curves, the ddPCR performance was more consistent in
570 this study and thus would be preferable to qPCR for MST (Table 5). However, the application
571 of ddPCR for MST should be based on consideration of the performance of the selected genetic
572 marker, expected levels of target in water samples to be processed, and other factors such as
573 cost-effectiveness**.**

574

575 **5.5 Conclusions**

- Digital droplet PCR implemented according to the manufacturer's recommendations for primer and probe concentrations is a suitable platform for microbial source tracking using the human-*Bacteroidales* markers BacHum and B.theta.
- Performance similarity between qPCR and ddPCR platforms for sensitivity, specificity, and detecting human markers in composite samples indicate both methods can be used with similar confidence for presence/absence determination of the B.theta marker.
- Similar or higher reproducibility of ddPCR-based marker detection with enhanced specificity for BacHum and quantification independent of a standard curve, prone to systematic errors, make ddPCR attractive for MST.

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599 **Appendix A. Supplementary data.**

600 Supplementary data related to this article have been submitted in a different file.

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Table1. Quantification of plasmid stock solution dilutions containing targeted markers BacHum or B.theta by qPCR and ddPCR methods

Expected concentration[1]		**qPCR (log copies/reaction)**						**ddPCR (log copies/reaction)**					
Copies/ reaction	**log copies/ reaction**	**BacHum**	**SD**	**% CV**	**B.theta**	**SD**	**% CV**	**BacHum**	**SD**	**% CV**	**B.theta**	**SD**	**% CV**
10^8	8.0	7.4	0.01	0.22	7.7	0.08	1.12	TNTC	ND	ND	TNTC	ND	ND
10^7	7.0	7.1	0.16	2.35	7.6	0.17	2.29	TNTC	ND	ND	TNTC	ND	ND
10^6	6.0	6.2	0.02	0.33	6.0	0.04	0.59	TNTC	ND	ND	TNTC	ND	ND
10^5	5.0	5.3	0.02	0.45	4.8	0.03	0.64	TNTC	ND	ND	TNTC	ND	ND
10^4	4.0	4.1	0.09	2.33	3.9	0.02	0.56	3.6	0.05	1.49	4.0	0.04	1.09
10^3	3.0	2.9	0.05	1.90	2.8	0.04	1.32	2.8	0.04	1.47	2.6	0.02	0.65
100	2.0	1.8	0.06	3.57	1.9	0.00	0.08	2.0	0.07	3.18	2.0	0.00	0.26
50	1.7	ND	ND	ND	ND	ND	ND	1.8	0.02	1.20	1.6	0.03	1.65
25	1.4	ND	ND	ND	ND	ND	ND	1.5	0.03	2.22	1.4	0.01	0.76
12.5	1.1	ND	ND	ND	ND	ND	ND	1.2	0.02	1.66	1.1	0.02	2.23
10	1	0.76	0.02	4.78	1.2	0.03	2.82	1.1	0.06	5.72	0.9	0.02	2.10
5.8	0.7	ND	ND	ND	ND	ND	ND	0.7***	0.03	3.53	ND	ND	ND
3.5	0.5	ND	ND	ND	ND	ND	ND	*0.77*	*0.40*	*54.4*	0.4*	0.01	2.40
1.5	0.2	ND	ND	ND	ND	ND	ND	*0*	*0*	*0*	0.2**	0.06	3.50

Notes: [1]Copy numbers in standards were calculated based on plasmid molecular weight, measured concentration in stock solution, and dilution.

Abbreviations: CV: coefficient of variation, ND: Not determined; TNTC: positive droplets were Too numerous to count;

All averages represent triplicate samples with asterisks denoting exceptions based on duplicate (*), n=6 (**), and n=9 (***) samples.

Italic font represent values obtained below the detection limits determined at 95% confidence: qPCR BacHum = 1.0 copies/reaction; qPCR B.theta = 3.0 copies/reaction; ddPCR BacHum = 5.8 copies/reaction; ddPCR B.theta = 1.5 copies/reaction.

822 **Table 2**. Performance of human-associated *Bacteroidales* assays as revealed by qPCR and
823 ddPCR quantification of human fecal samples and domesticated and wild animal fecal samples,
824 and environmental and composite samples.

Sample category	Percentage of positive samples				n
	qPCR		ddPCR		
	BacHum	B. theta	BacHum	B. theta	
Humans					
Sewage	95	100	100	100	20
Human Stool	100	94.3	97.1	85.7	35
Domesticated Animals					
Rabbits	60	0	50	0	10
Dogs	10	0	0	0	10
Cats	100	10	0	10	10
Chickens	80	0	20	0	10
Wild Animals					
Monkeys	0	0	0	0	10
Wild boards	0	0	0	0	10
Myna birds	0	0	0	0	10
Environmental (water)	100	100	45	40	20
Percentage of agreement (Observed vs. Expected detection)					**n**
Composite Fecal	100	74.3	97.1	88.6	35

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843

844 **Table 3**. Human assay performance metrics calculated using BacHum and B. theta qPCR and
845 ddPCR concentrations

Assay performance metric[a]	Sample Type	qPCR		ddPCR	
		BacHum	**B.theta**	**BacHum**	**B.theta**
Sensitivity	Sewage (n=20)	0.95	1.00	1.00	1.00
	Human stool (n=35)	1.00	0.94	0.97	0.85
Specificity	Animals (n=60)[b]	0.58	0.98	0.88	0.98
Accuracy	Sewage, human stool and animals (n=105)	0.77	0.97	0.94	0.95

846

847 **Notes:** [a] Sensitivity = TP/(TP+FN); Specificity = TN/(TN+FP); Accuracy =
848 (TP+TN)/(TP+TN+FP+FN) as described in Kildare et al 2007 and Odagiri et al 2014. (TP: true
849 positive; TN: true negative; FP: false positive; FN: false negative)

850 [b] Mynah Birds (n = 10) were excluded from specificity calculation as their feces tested negative
851 for the Bacteroidales universal marker (BacUni) in Nshimyimana et al 2017 and they were
852 confirmed to be negative for B. theta and BacHum by qPCR and ddPCR in this study.

853

Table 4. Comparison of qPCR and ddPCR based on measurement of human-associated B. theta and BacHum markers across sample types to standards measured by Qubit fluorometry at high concentration followed by dilution. Average and median reported are BacHum & B.theta marker concentrations determined by qPCR divided by the concentration determined by ddPCR.

Type of samples	Average (SD)	Median (Range)
BacHum		
Human (n = 35)	26.8 (11.6)	24.7 (1.3-44.1)
Sewage (n = 20)	14.9 (5.1)	16.4 (4.8-21.1)
Composite (n = 35)	26.6 (9.4)	28.9 (0.6-38.7)
Environmental (n = 20)	11.6 (3.6)	12.3 (2.9-18.5)
Standards[b] (n = 4)	1.3 (1.2)	1.0 (0.5-3.2)
All Data (n = 114)	18.7 (10.0)	18.5 (0.1-38.7)
B.theta		
Human (n = 35)	3.3 (2.3)	2.7 (0.4-10.4)
Sewage (n = 20)	2.8 (1.6)	3.7 (0.8-7.6)
Composite (n = 35)	3.5 (1.8)	3.7 (0.0-6.4)
Environmental (n = 20)	8.1 (5.1)	11.1 (1.8-15.0)
Standards[b] (n = 4)	1.1 (0.2)	1.0 (0.9-1.3)
All Data (n = 114)	2.6 (2.8)	3.1 (0.0-14.6)

[a] Marker concentrations in samples were measured in copies/reaction. Average, standard deviation, median, max and min were determined for ratios of qPCR- to ddPCR-measured concentrations for each sample within each sample type.

[b] Plasmid dilutions at 10 to 10^4 copies/reaction were measured by ddPCR.

860 **Table 5.** Comparison of ddPCR and qPCR for microbial source tracking using Human-
861 associated *Bacteroidales* markers BacHum and B.theta

Comparison of ddPCR and qPCR	qPCR	ddPCR	Superior platform
• Precision of replicate standard testing	CV (0.08 to 4.8) %	CV (0.26 to 5.7) %	qPCR
• Upper limit of detection (copies per reaction)	10^8	10^4	qPCR
• Limit of detection (LOD) for BacHum and B.theta, respectively (copies per reaction)	1.0 and 3.0	5.8 and 1.5	qPCR
• Limit of quantification (LOQ) for BacHum and B.theta, respectively (copies per reaction)	11.0 and 12.0	5.8 and 1.5	ddPCR
• LOD base reproducibility of presence/absence results in fecal composite samples for BacHum and B. theta, respectively	100% and 77%	97% and 89%	qPCR & ddPCR
• Prevalence of markers in environmental samples	100%	40-45%	qPCR
• Specificity for BacHum and B.theta, respectively	58% and 98%	88% and 98%	ddPCR
• Sensitivity for BacHum and B.theta in human feces, respectively	100% to 94%	97% to 85%	qPCR
• Quantification dependent on error-prone standard curve	Dependent	Independent	ddPCR

862

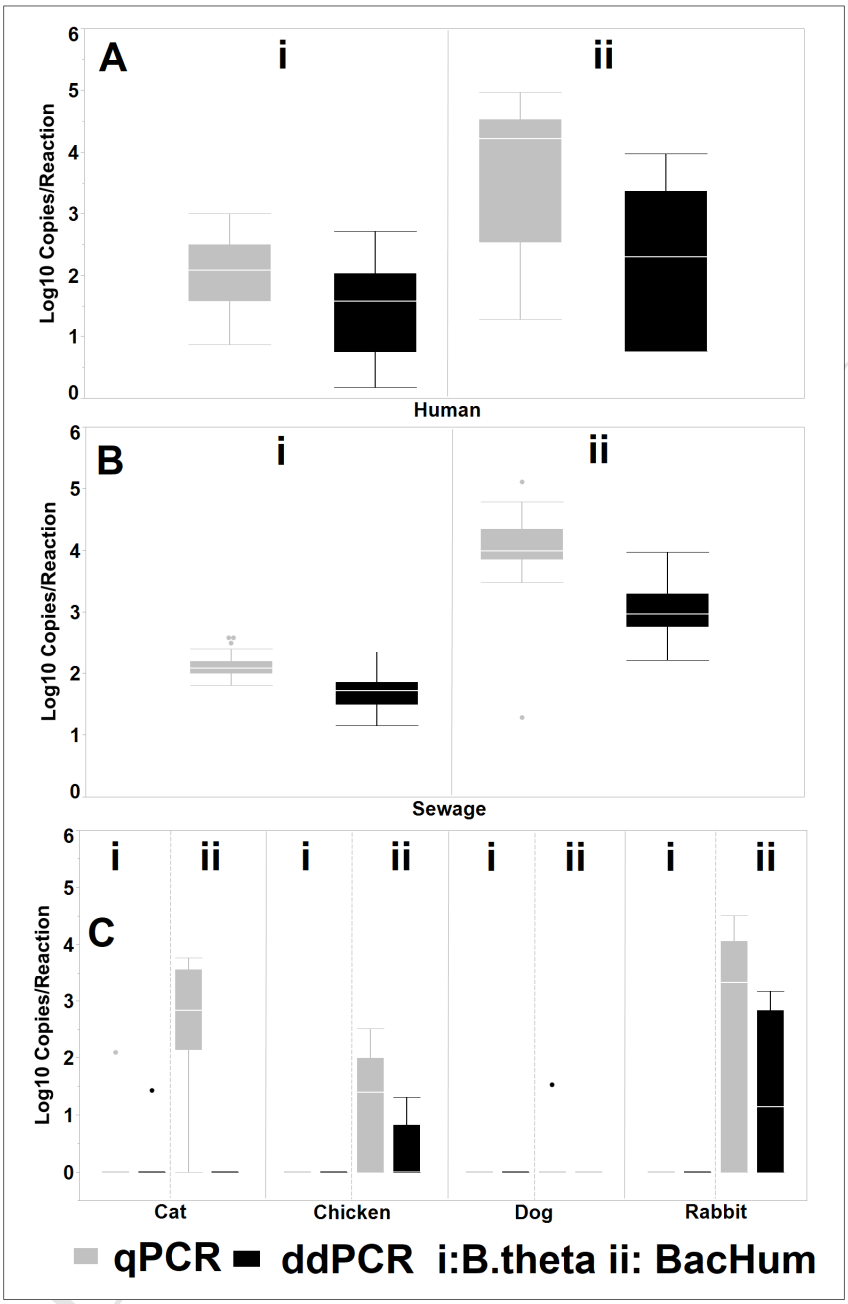


Figure 1. Distribution of concentrations of human-associated *Bacteroidales* markers B.theta (bt) and BacHum (bh) in human stool (Figure 1A), sewage (Figure 1B), and domesticated animal fecal samples (Figure 1C) as quantified using ddPCR (black) and qPCR (dark grey). Box and whiskers plots depict median and quartile distributions with outliers plotted as single points. All individual assay limits of detection were below 1 log10 Copies/Reaction.

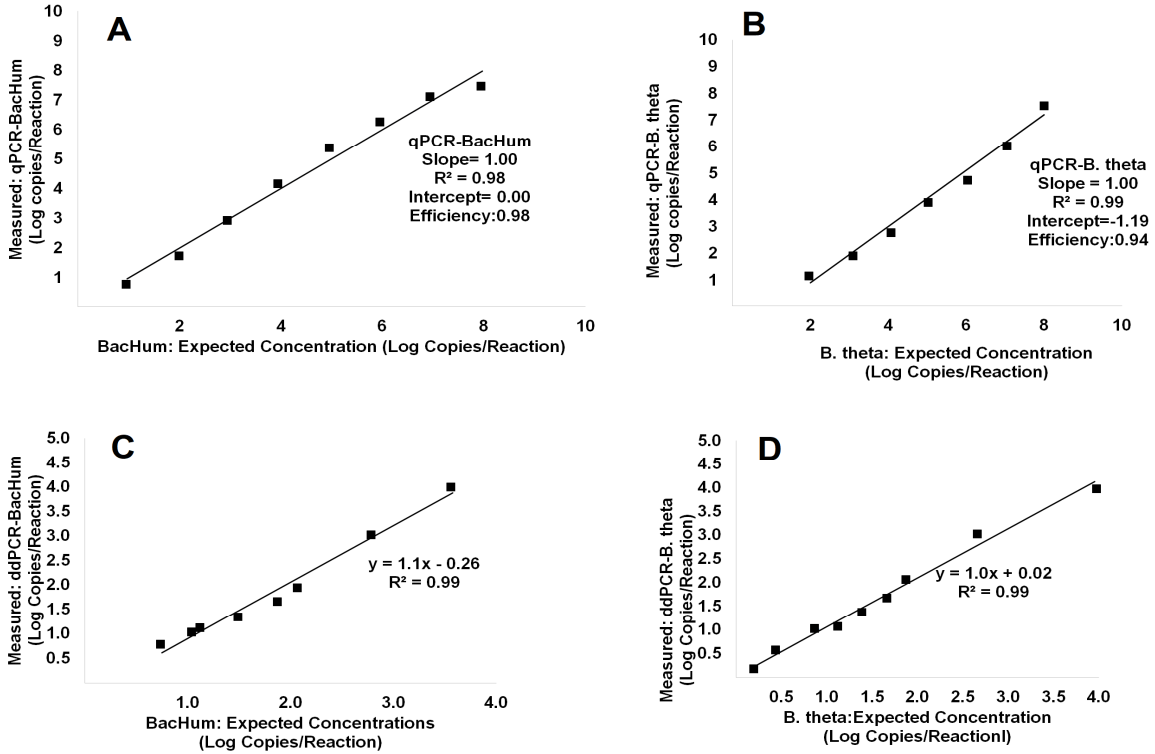
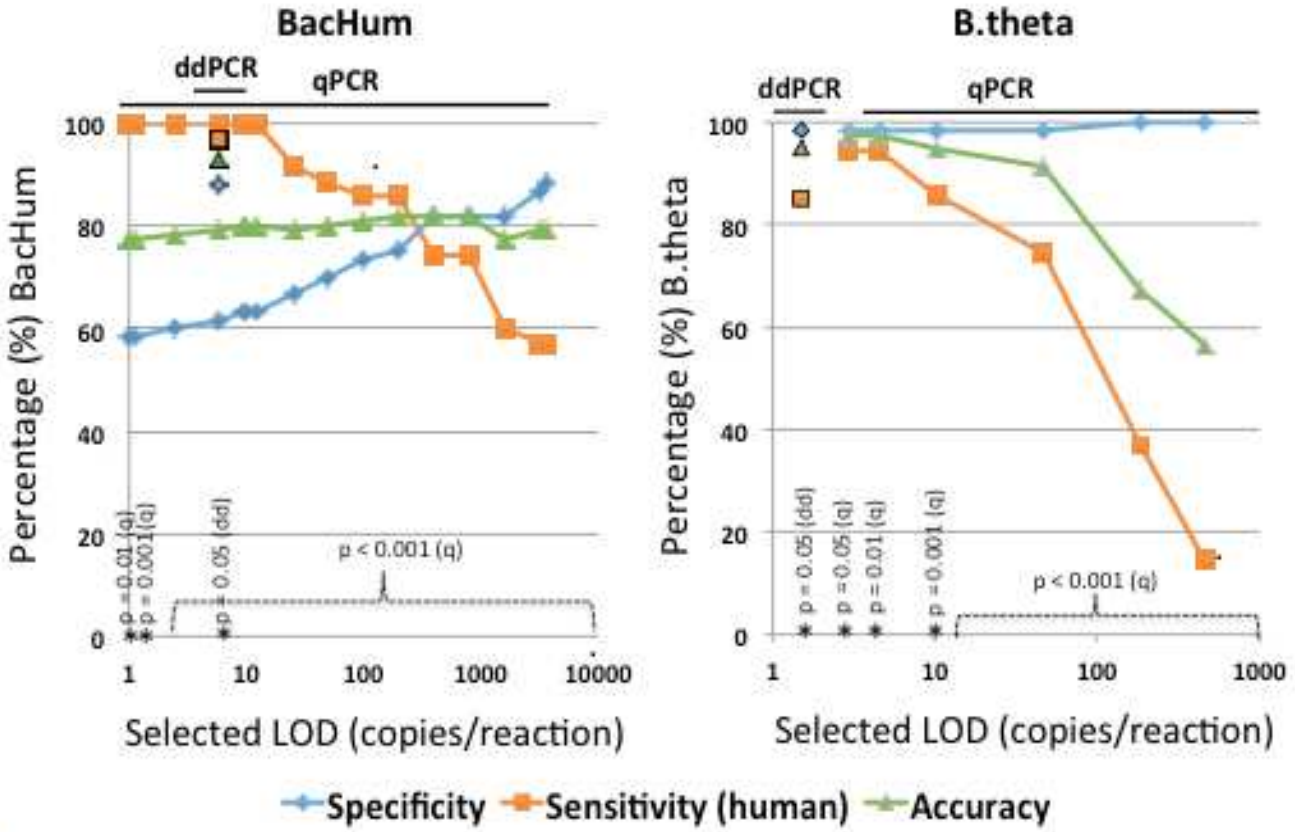
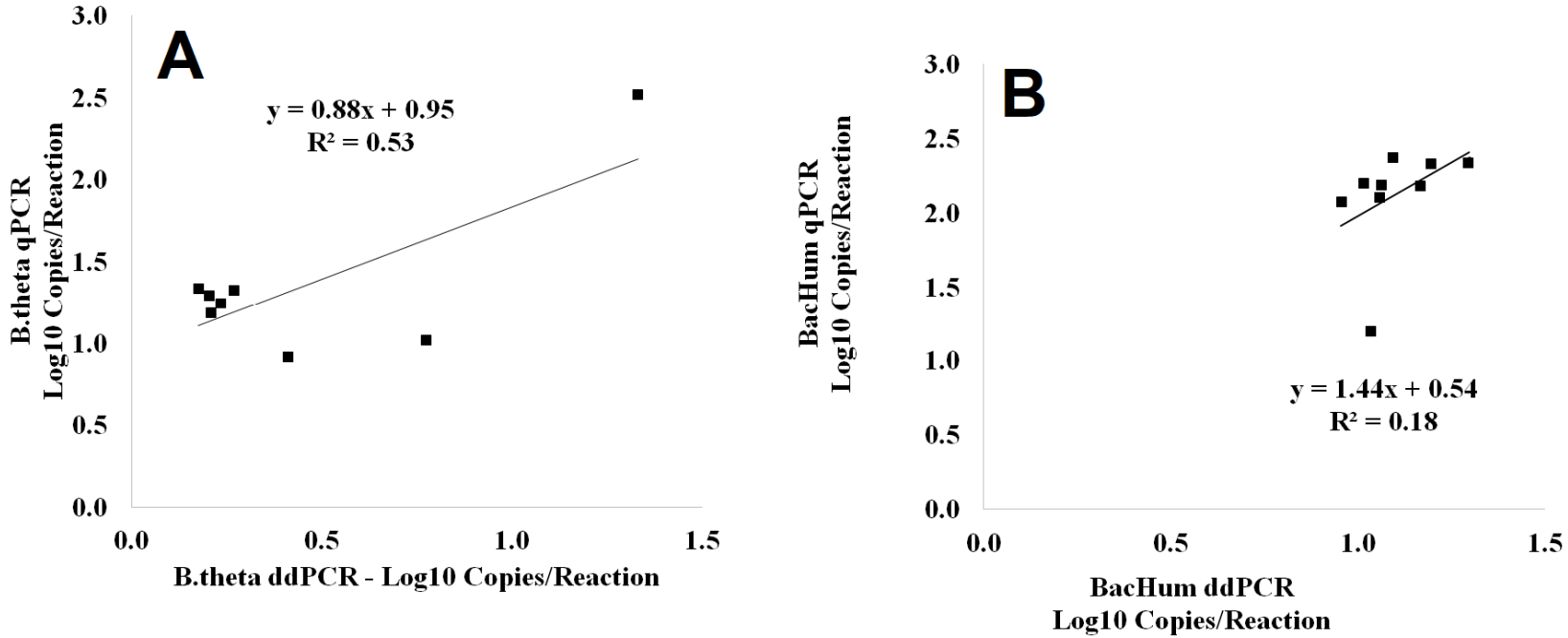


Figure 2. Measured concentrations of positive controls of human-*Bacteroidales* markers (B.theta and BacHum) quantified using qPCR and ddPCR compared to expected concentrations based on dilution of plasmid standards where DNA concentrations of the lowest dilutions were measured by Qubit Fluorometry. A slope of 1 and $R^2 \geq 0.99$ demonstrate a good fit between measured and expected concentrations. QPCR efficiencies of BacHum and B.theta assays are demonstrated (2A and 2B) (Efficiency=$10^{-1/slope}$-1). X-axis is the expected concentrations and Y-axis is the measure concentrations by qPCR (2B and 2D) or by ddPCR (2A and 2C). Measured and expected concentrations of BacHum (0 – 10^8 copies/reaction) by qPCR (2A). Measured and expected concentrations of B.theta (0 – 10^8 copies/reaction) by qPCR (2B). Measured and expected concentrations of BacHum (0 – 10^4 copies/reaction) by ddPCR (2C). Measured and expected concentrations of B.theta (0 – 10^4 copies/reaction) by ddPCR (2D).



880

881 **Figure 3.** *In silico* analysis of variation of qPCR assay performance as a function of the concentration identified as the assay limit of
882 detection. Higher thresholds for limits of detection are associated with decreased sensitivity and slight to marginally increased
883 sensitivity for B. theta and BacHum qPCR assays, respectively. DdPCR results indicated for comparison (symbols with black
884 border). Asterisks on the x-axis denote limits of detection at the indicated confidence threshold for ddPCR (dd) and qPCR (q).



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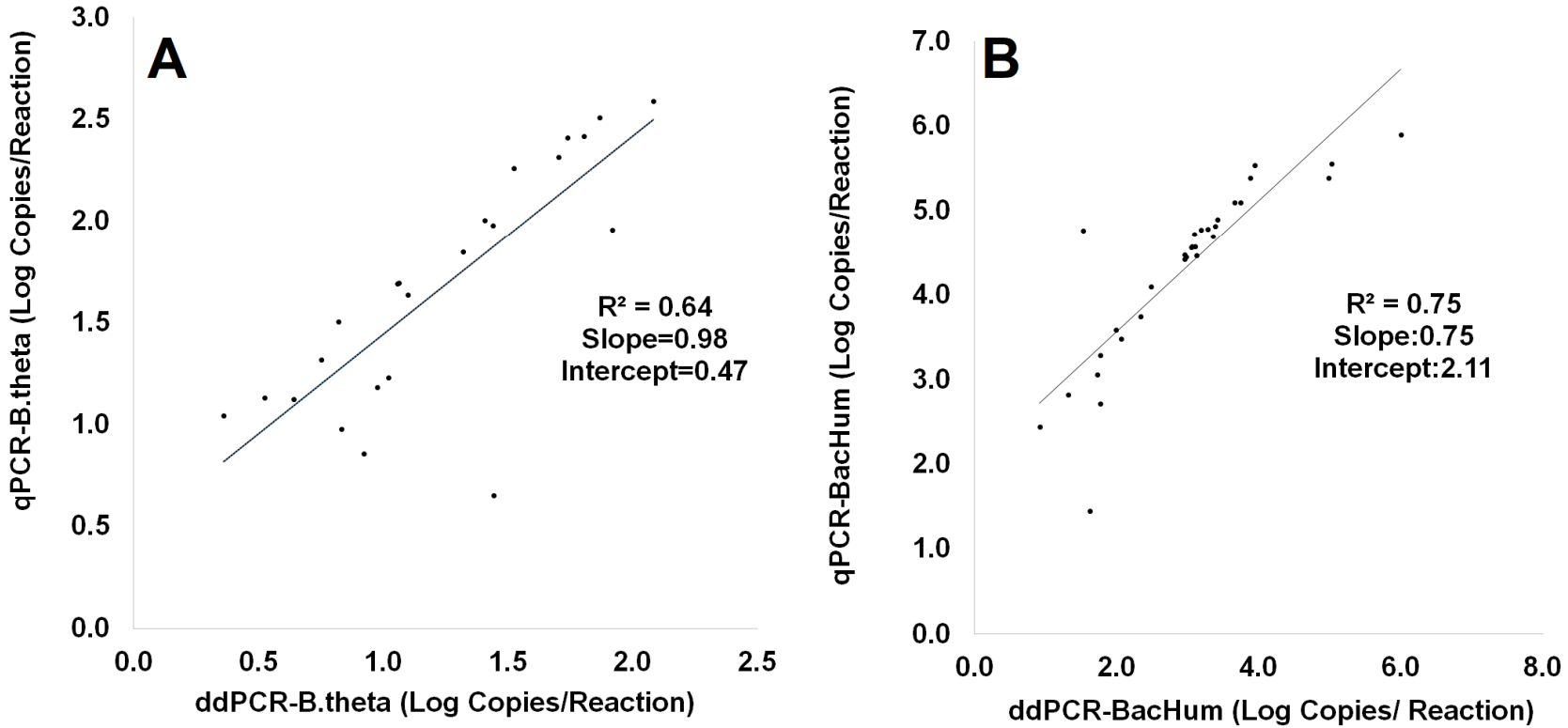
886 **Figure 4.** Comparison of concentrations of human-*Bacteroidales* markers (BacHum and B.theta) in environmental samples.
887 Comparison of concentrations of B.theta quantified by ddPCR and qPCR in environmental samples (4A). Comparison of
888 concentrations of BacHum quantified by ddPCR and qPCR in environmental samples (4B). All graphs include slope and R^2 values.
889 Environmental samples are expressed as log Copies/Reaction of sampled water volume and only concentrations above the limit of
890 detection (LOD) plotted.

891



892

893 **Figure 5.** Comparison of concentrations of human-*Bacteroidales* markers in fecal composite samples. 5A, B.theta quantified by
894 qPCR and ddPCR; 5B, BacHum quantified by qPCR and ddPCR. Marker concentrations plotted as log copies/ng DNA. Graphs
895 include slope and R^2 values. All plotted values are above the limit of detection.

Highlights:

1. Digital droplet (dd) PCR was validated for *Bacteroidales*-based microbial source tracking
2. Sensitivity of quantitative (q) PCR for *Bacteroidales* human markers in feces was superior to ddPCR
3. Assay specificity and reproducibility in feces by ddPCR were greater than or nearly equal to those by qPCR
4. qPCR and ddPCR platform performance may vary with assay

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: