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Comparison of RT-qPCR and RT-dPCR Platforms for the Trace Detection of SARS-CoV-2 RNA in Wastewater

Warish Ahmed,* Wendy J. M. Smith, Suzanne Metcalfe, Greg Jackson, Phil M. Choi, Mary Morrison, Daniel Field, Pradip Gyawali, Aaron Bivins, Kyle Bibby, and Stuart L. Simpson

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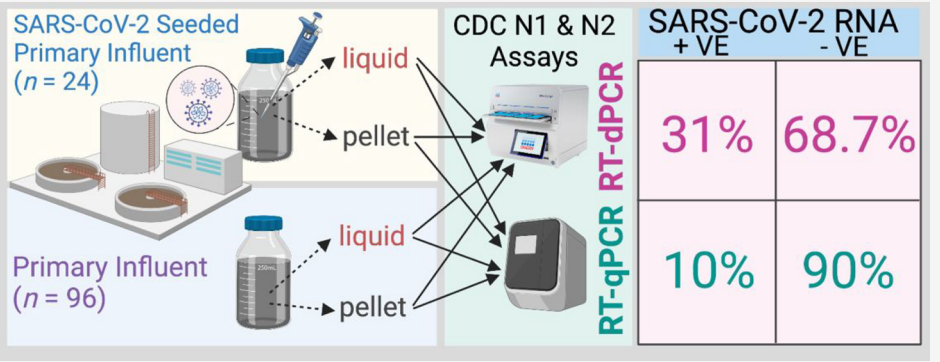

ABSTRACT: We compared reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and RT digital PCR (RT-dPCR) platforms for the trace detection of SARS-CoV-2 RNA in low-prevalence COVID-19 locations in Queensland, Australia, using CDC N1 and CDC N2 assays. The assay limit of detection (ALOD), PCR inhibition rates, and performance characteristics of each assay, along with the positivity rates with the RT-qPCR and RT-dPCR platforms, were evaluated by seeding known concentrations of exogenous SARS-CoV-2 in wastewater. The ALODs using RT-dPCR were approximately 2−5 times lower than those using RT-qPCR. During sample processing, the endogenous (n = 96) and exogenous (n = 24) SARS-CoV-2 wastewater samples were separated, and RNA was extracted from both wastewater eluates and pellets (solids). The RT-dPCR platform demonstrated a detection rate significantly greater than that of RT-qPCR for the CDC N1 and CDC N2 assays in the eluate (N1, p = 0.0029; N2, p = 0.0003) and pellet (N1, p = 0.0015; N2, p = 0.0067) samples. The positivity results also indicated that for the analysis of SARS-CoV-2 RNA in wastewater, including the eluate and pellet samples may further increase the detection sensitivity using RT-dPCR.

KEYWORDS: *COVID-19, SARS-CoV-2, wastewater surveillance, RT-qPCR, dPCR*

INTRODUCTION

The emergence and spread of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) have highlighted the utility of wastewater surveillance for early identification of infection in communities and guidance of public health measures to reduce transmission risks.[1−5] Wastewater surveillance of SARS-CoV-2 RNA has been employed in at least 55 countries to monitor the presence and support management of COVID-19 in various communities.[6−17] This approach uses a series of complex environmental microbiology procedures ranging from wastewater sampling to the detection and quantification of viral RNA primarily via polymerase chain reaction (PCR)-based or loop-mediated isothermal amplification (LAMP) assays.[6,8,18] These procedures require careful optimization to maximize their sensitivity for detecting traces of SARS-CoV-2 in wastewater, especially in the absence of standard methods.[19−21] Trace detection, the reliable detection of minuscule amounts of RNA in wastewater, is crucial for regions with few COVID-19 cases and for jurisdictions in which elimination is a management strategy.

Reverse transcription-quantitative PCR (RT-qPCR) has been employed as a gold standard method for detecting and quantifying SARS-CoV-2 RNA in both clinical and environmental samples.[6,21,22] However, several factors can result in quantification bias, as RT-qPCR assays rely on calibration (i.e., standard) curves constructed using a dilution series of the defined number of target molecules (i.e., plasmid DNA, PCR

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amplicons, synthetic nucleic acid, genomic DNA, and cDNA), typically having five or six dilution points (10-fold each) with at least three replicates per point.[19] Variations in protocols, reagents, sample quality, instruments (e.g., ultraviolet spectrophotometers, PCR platforms, and sample homogenizers), data analysis, software, and interpretation within and across laboratories can lead to the inaccurate and unreliable quantification of SARS-CoV-2 RNA in wastewater.[21,22] In addition, RT-qPCR amplification may be affected by wastewater samples that often contain inhibitors, such as pharmaceuticals, personal care products, household detergents, industrial effluents, and metals.[24,25] These compounds can influence the efficiency of RT-qPCR amplification and the subsequent detection and quantification.[26]

Some of the limitations of RT-qPCR may be overcome using digital PCR (dPCR). The dPCR platform provides absolute quantification through limiting dilution and most-probable number techniques premised on Poisson statistics rather than a calibration curve.[27,28] This leads to the improved repeatability and accuracy of quantified target molecules. Compared to qPCR/RT-qPCR in many applications, dPCR has achieved greater analytical sensitivity for detecting various target molecules in both environmental and clinical samples.[29,30] dPCR is also more resilient to inhibition than qPCR as observed for clinical samples, including feces, and environmental samples, including soil, water, and wastewater.[26,31−33] As a result, the dPCR platform could be ideally suited for the sensitive detection of SARS-CoV-2 RNA in complex wastewater matrices.[34−38] The results from testing of clinical samples for SARS-CoV-2 RNA indicate RT-dPCR can be more accurate in diagnosing COVID-19 than RT-qPCR, and such results are promising for the application of dPCR in wastewater surveillance.[39−43]

Compared to the numerous studies using RT-qPCR, a limited number of studies have reported the application of RT-dPCR platforms for the detection of SARS-CoV-2 RNA in wastewater.[38,44−49] Ciesielski et al.[45] demonstrated that RT droplet digital PCR (ddPCR, Bio-Rad) was not influenced by potential PCR inhibitors in wastewater matrices and frequently demonstrated greater analytical sensitivity, yielding fewer false-negative results compared to RT-qPCR. Boogaerts et al.[47] compared RT-qPCR and RT-dPCR (QIAcuity, Qiagen) for the detection of SARS-CoV-2 RNA in eight untreated wastewater samples in Belgium and reported that the results were comparable. Conversely, D'Aoust et al.[46] reported poorer performance of RT-ddPCR (Bio-Rad) than of RT-qPCR when used for the detection of SARS-CoV-2 RNA in influent post grit and solids and primary clarified sludge samples.

In this study, we compared RT-qPCR and RT-dPCR for the trace detection of SARS-CoV-2 RNA in 96 wastewater samples from low-prevalence COVID-19 locations in Queensland, Australia. Additionally, a subset (n = 24) of wastewater samples that had previously tested negative for SARS-CoV-2 RNA were seeded with known concentrations of SARS-CoV-2 to determine RT-qPCR and RT-dPCR detection sensitivity. We also identify and discuss advantages and disadvantages that RT-dPCR may offer over RT-qPCR for trace detection of SARS-CoV-2 RNA in wastewater.

MATERIALS AND METHODS

Wastewater Samples. Wastewater samples used in this study were from the Queensland's wastewater surveillance program for SARS-CoV-2 (https://www.qld.gov.au/health/conditions/health-alerts/coronavirus-covid-19/current-status/wastewater). In total, 96 untreated wastewater samples that had been collected between June 7, 2021, and June 14, 2021, from 45 wastewater treatment plants (WWTPs) across Queensland, Australia, were selected. Automated samplers operating in time proportional mode (every 15 min for 24 h) were used to collect 500 mL to 1 L composite wastewater samples.[4]

SARS-CoV-2 Concentration and Extraction. Before primary concentration, each wastewater sample was seeded with a known number of murine hepatitis virus (MHV) as an RNA extraction positive control. SARS-CoV-2 was concentrated from 50 mL of the wastewater samples using a rapid concentrator pipet instrument (Concentrating Pipette Select, CP Select, InnovaPrep, Drexel, MO) designed for concentrating bacteria, protozoa, and viruses from water matrices simultaneously.[50,51] Prior to the concentration step, the wastewater sample was first centrifuged at 3000g for 5 min to pellet the suspended solids (Avanti J-15R Centrifuge, Beckman Coulter). The supernatant (∼49 mL) was then concentrated with the CP Select using an unirradiated 0.05 μm PS hollow fiber filter concentrating pipet tip (catalog no. CC08004-200), following the optimized wastewater application settings provided in the instrument instructions. Following concentration, the pipet tip was eluted twice using CP elution fluid (HC08001) containing 0.075% Tween 20 and 25 mM Tris (catalog no. HC08001). The eluate (0.7−0.8 mL) was collected in sterile 15 mL polypropylene tubes. Four concentration method negative controls were included to identify potential contamination during sample concentration using the CP Select. Immediately after concentration, RNA was extracted from an aliquot of 140 μL of the eluate using the QIAamp Viral RNA Mini Kit (catalog no. 52905, Qiagen) with a minor modification. In the final step, a 100 μL volume of buffer AVE was used to elute the RNA instead of 60 μL to have sufficient RNA samples for downstream analysis. RNA (100 μL of eluate) was also extracted directly from the 50−140 mg wet mass pellet (separated during the initial centrifugation step) using the RNeasy Power Microbiome Kit (Qiagen) according to the manufacturer's instructions. The pellet was included because our previous study reported the partitioning of SARS-CoV-2 in the eluate and pellets from wastewater.[50] Four RNA extraction negative controls were included to identify potential contamination during RNA extraction. All RNA samples were stored at −20 °C for 24 h and subjected to RT-qPCR and RT-dPCR analysis at the same time to minimize nucleic acid degradation.

RT-qPCR Analysis. Previously-published RT-qPCR assays targeting the N gene of SARS-CoV-2 (CDC N1 and CDC N2) were used for SARS-CoV-2 RNA detection and quantification.[52] The primer and probe sequences, along with cycling parameters for all of the RT-qPCR assays used, including MHV, are shown in Supplementary Table ST1. RNA extracted from γ-irradiated SARS-CoV-2 hCoV 19/Australia/VIC01/2020 using a QIAamp Viral RNA mini kit was quantified with RT-dPCR and used as the RT-qPCR standard for both CDC N1 and N2 assays. CDC N1 and CDC N2 standard dilutions ranged from 1 × 10^6 to 1 gene copy (GC)/μL of template RNA. For MHV, gBlock gene fragments were purchased from Integrated DNA Technologies (Integrated DNA Technology, Coralville, IA) and used as a positive control. MHV and SARS-CoV-2 N gene assays were performed in 20 μL reaction mixtures using TaqMan Fast Virus 1-Step Master Mix (Applied

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Biosystems). The MHV RT-qPCR mixture contained 5 μL of Supermix, 300 nM forward primer, 300 nM reverse primer, 400 nM probe, and 5 μL of template RNA. The CDC N1 and N2 RT-qPCR mixtures contained 5 μL of Supermix, 2019-nCoV Kit (500 nM forward primer, 500 nM reverse primer, and 125 nM probe) (catalog no. 10006606), and 2.5 μL of template RNA. Four RT-qPCR replicates were used for each sample. The RT-qPCR assays were performed using a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, Richmond, CA), using automatic settings for the threshold and baseline. The experiments were performed using triplicate PCR negative and positive (γ-irradiated SARS-CoV-2 RNA) controls. To minimize RT-qPCR contamination, RNA extraction and RT-qPCR setup were performed in separate laboratories.

RT-dPCR Analysis. The CDC N1 and N2 assays were optimized for primer and probe concentrations and annealing temperature and time. Using the optimized conditions, CDC N1 and N2 assays were performed in 40 μL reaction mixtures using the QIAcuity One-Step Viral RT-PCR Kit (catalog no. 1123145, Qiagen) and 26000 24-well Nanoplates (catalog no. 250001, Qiagen). The 26000 QIAcuity 24-well Nanoplates are microfluidic dPCR plates that enable 24 samples to be run with up to 26000 partitions/well. The PCR occurs in each partition, and the partition volume is 0.91 nL. The CDC N1 and N2 RT-dPCR mixture contained 10 μL of master mix, 800 nM forward primer, 800 nM reverse primer, 200 nM probe, 0.4 μL of 100× Multiplex Reverse Transcription Mix, 19.2 μL of DNase and RNase free water, and 5 μL of template RNA. Two RT-dPCR replicates were used for each sample. The 40 μL RT-dPCRs were prepared in a 96-well preplate and then transferred into the 26000 24-well Nanoplates. The nanoplate was then loaded onto the QIAcuity dPCR 5-plex platform (Qiagen) and subject to an automated workflow that included (i) a priming and rolling step to generate and isolate the chamber partitions, (ii) an amplification step using the thermal cycling protocol (50 °C for 40 min for reverse transcription, 95 °C for 2 min for enzyme activation, 95 °C for 5 s for denaturation, and 60 °C for 90 s for annealing/extension for 45 cycles), and (iii) a final imaging step made by reading in the FAM channel. The experiments were performed using duplicate RT-dPCR negative and positive (γ-irradiated SARS-CoV-2 RNA) controls. To minimize RT-dPCR contamination, RNA extraction and RT-dPCR setup were performed in separate laboratories. Data were analyzed using the QIAcuity Suite Software version 1.1.3 193 (Qiagen), and quantities expressed as GC per microliter of reaction mixture. The RT-dPCR assays were performed using automatic settings for the threshold and baseline.

RT-qPCR and RT-dPCR ALODs. To determine CDC N1 and N2 RT-qPCR and dPCR assay limits of detection (ALODs), γ-irradiated SARS-CoV-2 was diluted and analyzed using RT-qPCR and RT-dPCR. At each dilution, 15 replicates were analyzed. The ALOD was defined by fitting a cumulative Gaussian distribution to the observed proportion of positive technical replicates along the dilution series. The 95% ALOD was estimated as the 95th percentile of the resulting normal distribution.[48]

SARS-CoV-2 Seeding Experiments. Because the wastewater samples were collected from low-prevalence COVID-19 regions, a subset of samples (n = 24) confirmed negative for SARS-CoV-2 by RT-qPCR/dPCR during initial analysis were seeded with γ-irradiated SARS-CoV-2. γ-Irradiated SARS-CoV-2 hCoV-19/Australia/VIC01/2020 was provided by the Australian Centre for Disease Preparedness, CSIRO. γ-Radiation was administered with a 50 kiloGy or 5 Mrad dose using an MDS Nordion Irradiator to mitigate the risk of infection associated with handling infectious SARS-CoV-2 in a biosecurity containment level 2 (BC2) laboratory, where the study was conducted. Among the 24 wastewater samples, 12 samples (WW 1−WW 12) were seeded with $2.83 \times 10^4 \pm 3.80 \times 10^3$ GC/50 mL and the remaining 12 samples (WW 13−WW 24) were seeded with $1.67 \times 10^5 \pm 9.71 \times 10^3$ GC/50 mL. After seeding, primary concentration, RNA extraction, and RT-qPCR/dPCR detections were performed according to the methods described above.

PCR Inhibition Test Assessment. The presence of PCR inhibition was assessed for a subset (n = 20) of the 96 RNA extracts (20.8%) that yielded negative results (no detection) during initial testing by RT-qPCR. These extracts were seeded with a known number of γ-irradiated SARS-CoV-2 for RT-qPCR ($1.0-1.2 \times 10^3$ GC/reaction) and for RT-dPCR ($2.0-2.4 \times 10^3$ GC/reaction). The concentration of the γ-irradiated SARS-CoV-2 stock was determined using dPCR as described above. The SARS-CoV-2 CDC N1 assay was used to assess PCR inhibition by comparing Cq values (for RT-qPCR) and absolute concentrations (for RT-dPCR) of seeded extracts with reference values from DNase and RNase free water seeded with the same numbers of SARS-CoV-2. CDC N1 RT-qPCR and RT-dPCR assays were performed as described previously. All samples were analyzed alongside three RT-qPCR/RT-dPCR negative controls. For RT-qPCR, if the Cq value of the RNA sample was >2 Cq values compared to the reference Cq value for distilled water, the sample was considered to have PCR inhibitors.[53,54] For RT-dPCR, if the concentrations of SARS-CoV-2 RNA in seeded wastewater samples were 4-fold lower than the benchmark concentrations, the sample was considered inhibited.

Data Analysis. For RT-qPCR, samples were considered positive (SARS-CoV-2 detected) if amplification was observed in at least one of the four replicates. Samples were considered quantifiable by RT-qPCR if amplification was observed for all four replicates and the mean concentration was above the ALOD. Samples were considered negative (<ALOD) when no amplification was not observed for all four replicates. For RT-dPCR, samples were considered positive if there was at least one positive partition following the merging of two replicate wells. Samples were considered quantifiable by RT-dPCR if the concentrations were above the ALOD, and the average number of partitions was >10000.[45] Samples were considered negative (<ALOD) when no amplification was observed in any of the partitions.

Supplementary Figure SF2 displays two positive amplifications (green circles), while panel H3 shows an NTC (no amplification). Differences in the SARS-CoV-2 RNA positivity rate for the pellet, supernatant, and combined fractions determined by RT-qPCR and RT-dPCR were assessed using Fisher's exact test.[55] Minimum Information for the Publication of Quantitative Real-Time PCR Experiments (MIQE) and digital MIQE checklists are provided for all RT-qPCR and RT-dPCR experimental details in Supplementary Tables ST2 and ST3, respectively.[19,58]

RESULTS

RT-qPCR and RT-dPCR Assay Performance and Quality Assurance/Quality Control (QA/QC). The RT-qPCR standard curves for CDC N1 and CDC N2 had a

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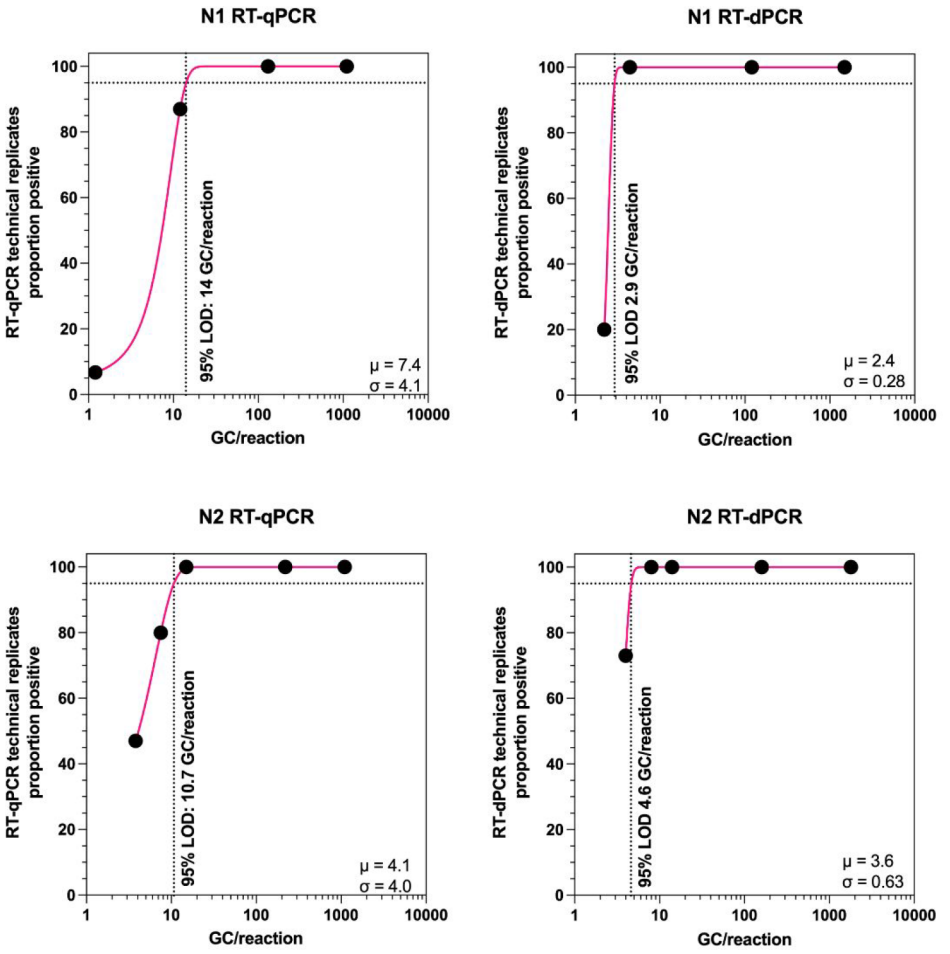

Figure 1. Assay limits of detection for RT-qPCR and RT-dPCR for CDC N1 and N2.

dynamic linear range of quantification from 2.5×10^6 to 2.5 GC/reaction (1×10^6 to 1 GC/μL). The log-linear slopes of the standard curves were −3.382 (CDC N1) and −3.391 (CDC N2). The amplification efficiencies and y-intercepts were 97.5% and 97.2% and 38.89 and 41.50 for CDC N1 and CDC N2 assays, respectively. The correlation coefficient (r^2) ranged from 0.994 to 0.990 for CDC N1 and N2 assays. The RT-qPCR ALODs were 14 and 11 GC/reaction for CDC N1 and CDC N2, respectively (Figure 1). All methods, extraction, and RT-qPCR/RT-dPCR negative controls were negative. All positive controls or standard curves were amplified in each PCR run. Furthermore, MHV amplification was observed in each RNA sample, suggesting successful RNA extraction. For CDC N1 RT-dPCR, the number of partitions ranged from 11002 to 25463 with a mean ± standard deviation (SD) of 21855 ± 3218. For CDC N2 RT-dPCR, these were 10436−25465 (range) and 21098 ± 3918, respectively. The mean partitions using CDC N1 and N2 RT-dPCR are shown in Supplementary Figure SF1. The RT-dPCR ALODs were 2.9 and 4.6 GC/reaction for CDC N1 and CDC N2, respectively (Figure 1).

RT-qPCR inhibition was identified in one (sample WW 7) of 20 wastewater eluate RNA samples based on the seeded GC of SARS-CoV-2. However, none of the 20 pellet RNA samples (all within 2 Cq values of the reference Cq value) exhibited PCR inhibition (Table 1). No RT-dPCR inhibition was identified in the tested RNA samples, with concentrations of SARS-CoV-2 RNA in eluate and pellet samples being not less than 25% of the reference values.

Prevalence and Concentrations of SARS-CoV-2 in Wastewater Samples. Among the 96 wastewater eluate RNA samples, two (2.08%) were detected as positive by CDC N1 RT-qPCR while 14 (14.6%) were detected as positive by RT-dPCR. Similarly, for wastewater pellet RNA samples, three (3.12%) were detected as positive by CDC N1 RT-qPCR and 17 (17.7%) were detected as positive by RT-dPCR (Table 2). None of the eluate and pellet RNA samples were detected as positive by CDC N2 RT-qPCR, while 12 (12.5%) eluate and 8 (8.33%) pellet RNA samples were detected as positive by the CDC N2 RT-dPCR assay. Five (5.20%) unique positive detections were observed using the CDC N1 RT-qPCR assay when combining wastewater eluate and pellet RNA samples, while 26 (27.1%) samples were detected as positive using CDC N1 RT-dPCR. None of the samples were detected as positive using CDC N2 RT-qPCR for combined eluate and pellet RNA samples, while 18 (18.8%) were detected as positive by CDC N2 RT-dPCR. None of the RT-qPCR-detected samples were quantifiable. Concentrations of SARS-CoV-2 RNA in eluate samples determined by CDC N1 RT-dPCR ranged from nonquantifiable to 6.20 GC/reaction. Similarly, the concentrations in pellet samples ranged from nonquantifiable to 21.7 GC/reaction. Using the CDC N2 RT-dPCR assay, concentrations of SARS-CoV-2 RNA in eluate samples ranged from nonquantifiable to 6.48 GC/reaction. SARS-CoV-2 RNA in pellet samples could not be quantified

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Table 1. Mean Quantification Cycle (Cq) Values (for RT-qPCR) and GC Numbers (for RT-dPCR) of SARS-CoV-2 RNA Seeded in Sterile Water (reference values) and Nucleic Acid Extracted from the Eluate and Pellet from Untreated Wastewater

	RT-qPCR Cq value/reaction		RT-dPCR GC/reaction	
wastewater sample	eluate	pellet	eluate	pellet
WW 1	26.9	27.9	1508	1752
WW 2	28.2	27.4	1592	1752
WW 3	27.2	27.5	532	1944
WW 4	27.9	27.6	1012	1776
WW 5	27.3	27.0	1652	1476
WW 6	27.4	27.4	1536	2016
WW 7	31.8	27.1	1432	1888
WW 8	27.2	27.3	1116	1644
WW 9	27.3	27.0	1520	1716
WW 10	27.3	27.3	1612	1328
WW 11	28.4	28.1	1544	1404
WW 12	27.6	27.5	1544	1500
WW 13	27.2	27.9	1364	1496
WW 14	27.5	27.2	1536	2084
WW 15	27.7	27.0	840	1936
WW 16	27.3	26.8	1116	2180
WW 17	27.9	26.9	1532	2032
WW 18	27.6	26.7	1616	1896
WW 19	26.9	27.4	1416	1932
WW 20	26.9	27.4	1704	1476
reference values	26.8	26.8	1508	2220

Table 2. Prevalence of SARS-CoV-2 RNA (CDC N1 and CDC N2 assays) in the Eluate and Pellet from Wastewater Samples Determined Using RT-qPCR and RT-dPCR

	no. of positive samples (%)			
	CDC N1		CDC N2	
wastewater samples (no. of samples tested)	RT-qPCR	RT-dPCR	RT-qPCR	RT-dPCR
wastewater eluate (n = 96)	2 (2.08)	14 (14.6)	0 (0)	12 (12.5)
wastewater pellet (n = 96)	3 (3.12)	17 (17.7)	0 (0)	8 (8.33)
wastewater eluate and pellet (n = 96)	5 (5.20)	26 (27.1)	0 (0)	18 (18.8)

using the CDC N2 RT-dPCR assay. For these 96 wastewater samples, RT-dPCR demonstrated a significant increase in the positivity rate compared to that with RT-qPCR for the CDC N1 assay in both the eluate (p = 0.0029) and pellet (p = 0.0015) samples. For the CDC N2 assay, RT-dPCR positivity was also significantly increased for the eluate (p = 0.0003) and pellet (p = 0.0067) samples.

Prevalence and Concentrations of SARS-CoV-2 in Seeded Wastewater Samples. For wastewater samples seeded with 2.83×10^4 GC (~707 GC/reaction; assuming 100% recovery) γ-irradiated SARS-CoV-2, one of 12 eluate and zero of 12 pellet samples were CDC N1 RT-qPCR positive. In contrast, four of 12 eluate and five of 12 pellet samples were detected as positive by the CDC N1 RT-dPCR assay (Table 3). The CDC N2 RT-qPCR assay was less sensitive with all eluate and pellet samples being negative. However, only three of 12 eluate and one of 12 pellet samples were positive by CDC N2 RT-dPCR. When wastewater samples were seeded with 1.67×10^5 GC (~7070 GC/reaction; assuming 100% recovery) SARS-CoV-2, 11 of 12 eluate and one of 12 pellet samples were detected as positive by the CDC N1 RT-qPCR assay. The concentrations of SARS-CoV-2 in these samples were not quantifiable (amplification was not observed for all replicates, and the mean concentration was less than the ALOD) by RT-qPCR. In contrast, all eluates (12 of 12) and three of 12 pellet samples were detected using the CDC N1 RT-dPCR assay. The concentrations of SARS-CoV-2 in these samples ranged from nonquantifiable (concentrations were less than the ALOD, and the average numbers of partitions was <10000) to 6.48 GC/reaction. Again, the CDC N2 RT-qPCR assay was less sensitive; eight of 12 eluate and zero of 12 pellet samples were positive. The concentrations of SARS-CoV-2 in these samples were not quantifiable. Using CDC N2 RT-dPCR, nine of 12 eluate and two of 12 pellet samples were positive. The concentrations of SARS-CoV-2 in these samples ranged from nonquantifiable to 5.52 GC/reaction.

Combining the eluate and pellet results (Table 3) for each unique wastewater sample seeded with 2.83×10^4 SARS-CoV-2 did not increase the sensitivity of the CDC N1 (1/12 positive) and N2 (0/12 positive) RT-qPCR assays but did increase the sensitivity for the CDC N1 (eight of 12 positive when combining results for eluate and pellet samples) and CDC N2 (four of 12 when combining results for eluate and pellet samples) RT-dPCR assays. When 1.67×10^5 (~7070 GC/reaction; assuming 100% recovery) SARS-CoV-2 were seeded in wastewater samples, combining the eluate and pellet results did not increase the sensitivity for CDC N1 and N2 RT-qPCR or RT-dPCR assays. For wastewater samples seeded with 2.83×10^4 GC SARS-CoV-2, the positive detection rate by RT-dPCR was only significantly increased compared to that of RT-qPCR using the CDC N1 assay for the pellet (p = 0.0373) and the combination of the eluate and pellet (p = 0.0016). When wastewater samples were seeded with 1.67×10^5 GC SARS-CoV-2, there was no significant difference in positive detection by RT-dPCR and RT-qPCR for either CDC N1 or N2 assays.

DISCUSSION

Many research laboratories with varying degrees of expertise and significantly differing methodologies are conducting SARS-CoV-2 wastewater surveillance investigations. Such monitoring efforts require both efficient and sensitive detection methods. However, many of these protocols or workflows may not be optimized for the detection of low levels of SARS-CoV-2 RNA, which could lead to false-negative errors.[21] This is particularly important in regions with a low prevalence of COVID-19 or for early warning of the emergence of variants of concern, such as the Delta variant, where accurate and reliable early warning to public health units may be crucial in the absence of clinical tests. While research is being conducted on the most effective sampling protocols and recovery of SARS-CoV-2 from wastewater,[22,48,50,56] less attention has been given to the detection and quantification platforms regarding sensitivity and accuracy.[45]

A few studies have compared the performance of RT-qPCR and RT-dPCR for the detection and quantification of SARS-CoV-2 in wastewater and solids separated from wastewater, with conflicting findings regarding sensitivity.[45−47] In the study presented here, we compared RT-qPCR (Bio-Rad CFX96) and RT-dPCR (QIAcuity) platforms for the sensitive detection of low levels of SARS-CoV-2 RNA (both endogenous and exogenous) in wastewater with emphasis on the comparability

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Table 3. Detection of SARS-CoV-2 RNA (CDC N1 and CDC N2 assays) in the Eluate and Pellet from Wastewater Samples Seeded with SARS-CoV-2 RNA Determined Using RT-qPCR and RT-dPCR[a]

	sample positive (GC/reaction)							
	CDC N1				CDC N2			
	RT-qPCR		RT-dPCR		RT-qPCR		RT-dPCR	
seeded wastewater sample	eluate	pellet	eluate	pellet	eluate	pellet	eluate	pellet
WW 1[b]	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD
WW 2[b]	<ALOD	<ALOD	<ALOD	+	<ALOD	<ALOD	<ALOD	<ALOD
WW 3[b]	<ALOD	<ALOD	+	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD
WW 4[b]	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD
WW 5[b]	<ALOD	<ALOD	<ALOD	+	<ALOD	<ALOD	+	<ALOD
WW 6[b]	+	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD	+
WW 7[b]	<ALOD	<ALOD	<ALOD	+	<ALOD	<ALOD	+	<ALOD
WW 8[b]	<ALOD	<ALOD	<ALOD	+	<ALOD	<ALOD	<ALOD	<ALOD
WW 9[b]	<ALOD	<ALOD	+	+	<ALOD	<ALOD	<ALOD	<ALOD
WW 10[b]	<ALOD	<ALOD	+	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD
WW 11[b]	<ALOD	<ALOD	+	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD
WW 12[b]	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD	+	<ALOD
WW 13[c]	+	<ALOD	+ (5.52)	<ALOD	<ALOD	<ALOD	+	<ALOD
WW 14[c]	+	<ALOD	+	<ALOD	+	<ALOD	<ALOD	<ALOD
WW 15[c]	+	<ALOD	+ (4.40)	<ALOD	+	<ALOD	+	<ALOD
WW 16[c]	+	+	+	<ALOD	+	<ALOD	+	<ALOD
WW 17[c]	+	<ALOD	+	<ALOD	+	<ALOD	+ (6.48)	+
WW 18[c]	+	<ALOD	+	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD
WW 19[c]	+	<ALOD	+	<ALOD	<ALOD	<ALOD	+	+
WW 20[c]	+	<ALOD	+	+	+	<ALOD	+	<ALOD
WW 21[c]	+	<ALOD	+ (4.48)	<ALOD	+	<ALOD	+	<ALOD
WW 22[c]	+	<ALOD	+	+	+	<ALOD	+	<ALOD
WW 23[c]	+	<ALOD	+	+	+	<ALOD	+	<ALOD
WW 24[c]	<ALOD	<ALOD	+	<ALOD	<ALOD	<ALOD	<ALOD	<ALOD

[a]Legend: <ALOD, below the assay limit of detection; +, detected as positive. [b]With $2.83 \times 10^4 \pm 3.80 \times 10^3$ GC/50 mL. [c]With $1.67 \times 10^5 \pm 9.71 \times 10^3$ GC/50 mL.

of the results between platforms. In the study design, careful attention was given to not introduce variations other than the PCR platforms themselves, and the specific reagents for dPCR and optimized cycling parameters for each platform. Both CDC N1 and N2 assays were empirically optimized on each platform prior to comparison. Therefore, the virus concentration method, the RNA extraction protocol, and the ratio of the PCR volume (1:2; 20 μL of RT-qPCR:40 μL of RT-dPCR) to the RNA template volume (1:2; 2.5 μL/reaction for RT-qPCR:5 μL/reaction for RT-dPCR) were identical for each platform. RT-PCR analysis was conducted on both platforms at the same time to avoid freezing and thawing and other potential loss of target molecules. All RT-qPCR and RT-dPCR analyses were conducted by one analyst to avoid variations and minimize potential pipetting errors. For RT-dPCR, a recently launched platform, QIAcuity based on nanoplates was used. The sample preparation steps for RT-dPCR were similar to those for RT-qPCR and included preparing master mix that combined probes, primers, and RNA samples and then was transferred to 96-well plates (for qPCR) and a 24-well nanoplate for dPCR. Partitioning, thermal cycling, and imaging all took place on the QIAcuity dPCR platform.

The analytical sensitivities of RT-dPCR and RT-qPCR were compared by analyzing serial dilutions of γ-irradiated SARS-CoV-2. For RT-dPCR, the empirically determined 95% ALODs for the CDC N1 and N2 assays were 2.9 and 4.6 GC/reaction, respectively, while the RT-qPCR ALODs were 14 and 10 GC/reaction, respectively. These results indicated that RT-dPCR is approximately 2−5 times more analytically sensitive than RT-qPCR with estimated ALODs near the theoretical limit of three GC.[19] This is consistent with the results of Ciesieleki et al.,[45] who found the 60% ALOD for the CDC N2 assay to be lower when using RT-ddPCR than RT-qPCR, although the template volumetric ratios in the reactions were slightly different [RT-qPCR 25% (v/v):RT-ddPCR 20% (v/v)]. On the contrary, D'Aoust et al.[46] found that ALODs for the CDC N1 and N2 assays were greater using RT-ddPCR than using RT-qPCR (5 GC/reaction vs 2 GC/reaction) using in vitro-transcribed RNA. In the study presented here, we found the CDC N1 assay to be more sensitive than the CDC N2 assay on the RT-dPCR platform using γ-irradiated SARS-CoV-2, while Gonzalez et al.[44] found the CDC N2 assay to be more sensitive on the RT-ddPCR platform using plasmid control material. Again, direct comparisons between studies are difficult due to varying definitions, control materials, and procedures for determining the ALOD.[23] Nonetheless, empirically determined ALODs from the work presented here and several studies indicate RT-dPCR is consistently able to achieve ALODs near the theoretical limit of detection.

We determined the presence of potential PCR inhibitors in a subset of RNA wastewater eluate and pellet samples. Our results based on the benchmark Cq values (for RT-qPCR) or the absolute concentration (for RT-dPCR) indicated that PCR inhibition was largely absent in the wastewater RNA samples except for one wastewater eluate sample in which RT-qPCR

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results indicated inhibition. However, the RT-dPCR results did not show any such effects. It is hypothesized that the partitioning of the bulk PCR mix (i.e., 40 μL) into 26000 partitions makes dPCR more resilient to inhibition.[32] The results of this study indicated the extracted RNA samples were mostly not affected by inhibition during RT-qPCR or RT-dPCR analyses. D'Aoust et al.[46] reported that for analysis of primary solids, RT-ddPCR was more inhibited than RT-qPCR; however, the ratios of template to reaction volume were different between the two platforms [RT-qPCR template, 15% (v/v); RT-ddPCR template, 25% (v/v)], which makes direct comparisons difficult. Conversely, no inhibition was observed for RT-ddPCR using an inhibition control in the analysis of primary influent and wastewater solids during two studies in Indiana, USA.[48,49] The inconsistent comparisons between RT-qPCR and RT-ddPCR inhibition highlight the difficulty of comparing studies that use different concentration and extraction methods. However, most of the studies indicated that RT-dPCR can be resilient to inhibition when wastewater is tested for SARS-CoV-2 RNA.[46,48,49] The absence of potential inhibitors in wastewater samples from this study should be interpreted with care as only 50 mL of wastewater samples was processed. Concentrating larger volumes (>100 mL) of wastewater may make the effects of PCR inhibitors more pronounced.

For the detection of endogenous SARS-CoV-2 RNA in wastewater samples, both the eluate and the pellet, RT-dPCR achieved a detection rate greater than that of RT-qPCR using the CDC N1 and N2 assays. These results corroborate the greater analytical sensitivity of RT-dPCR suggested by the ALOD determination in this study. We also noted that CDC N1 RT-qPCR yielded more positive results than CDC N2 RT-qPCR, which is consistent with the analytical sensitivity observed during the assay development.[57] However, CDC N2 RT-dPCR detection rates were slightly lower than CDC N1 RT-dPCR detection rates, suggesting a similar detection sensitivity of RT-dPCR for ambient wastewater samples. Differences in the RT step (10 min for RT-qPCR vs 40 min for RT-dPCR), cycling parameters and master mix may also contribute to the increased sensitivity of RT-dPCR. It has been reported previously that the master mix composition may contribute to qPCR and dPCR assay performance.[58] While it would be ideal to use the same reagents and cycling parameters for comparing RT-dPCR and RT-qPCR, QIAcuity requires a specific master mix with a reference dye for the reliable detection of proper partition filling in the Nanoplates leading to a longer (i.e., 40 min) RT step than RT-qPCR master mix (i.e., 10 min) used in this study.

Overall, the concentrations of SARS-CoV-2 measured using RT-dPCR were low, while none of the samples could be quantified using RT-qPCR. This is expected given that the wastewater samples were collected from areas with no observed community transmission other than COVID-19-infected individuals in quarantine hotels and hospitals. A subset of 96 wastewater samples tested in this study were previously analyzed with CDC N1 and CDC N2 assays as part of routine surveillance in Queensland, Australia. However, the concentration method (adsorption−extraction) and RNA template (5 μL/reaction) volume were different from those employed in the study presented here (CP method and 2.5 μL/reaction). We compared the RT-qPCR results from the previous analysis with RT-qPCR and RT-dPCR results obtained in the study presented here to consider the possibility that low copy detection of SARS-CoV-2 RNA using dPCR is not an artifact from something such as sensor dust. In addition, the dPCR platform was serviced before this study. A good agreement was found between previously generated RT-qPCR and current RT-dPCR data. All RT-dPCR negative controls were negative in each plate, which suggests that RT-dPCR detections of SARS-CoV-2 RNA were not artifacts.

We also seeded known concentrations of γ-irradiated SARS-CoV-2 in wastewater samples that had previously tested negative via RT-qPCR/RT-dPCR to further examine the detection sensitivity of both platforms in the presence of matrix interference. For the low-concentration seeding scenario, the RT-dPCR N1 assay yielded more positive detections for both wastewater eluate and pellet samples compared to the RT-qPCR N1 assay. Similarly, the RT-dPCR N2 assay also yielded two positive results for one eluate and pellet sample, but RT-qPCR did not produce any positive results. Another approach for increasing dPCR analytical sensitivity is to combine several wells into a single well (i.e., hyper-well functionality), which will also be useful for measuring dilute target molecules such as SARS-CoV-2 in wastewater. For high-concentration seeding scenarios, RT-dPCR N1 and N2 assays yielded similar positive detections for wastewater eluate samples compared to RT-qPCR N1 and N2 assays. dPCR detection rates for pellet samples were also greater than RT-qPCR rates. Our seeding experiment results agree with ambient wastewater sample results, suggesting dPCR is more sensitive when the concentration of SARS-CoV-2 RNA is low. At greater concentrations, it is highly likely both platforms will yield similar positive detections. For exogenous SARS-CoV-2 seeded into wastewater, we observed that SARS-CoV-2 RNA was found in both eluate and pellet samples. When the detection rates in eluate and pellet samples were combined, the results were better than individual eluate and pellet detections alone, suggesting that separating pellets from wastewater could result in reduced sensitivity. This is particularly important for trace detection of SARS-CoV-2 for public health purposes.

Although RT-dPCR was more sensitive for the detection of SARS-CoV-2 in wastewater samples in the study presented here, there are some disadvantages compared to RT-qPCR. Typically, dPCR platforms and the associated consumables are more expensive than RT-qPCR. Additionally, because of their dependence on Poisson statistics, the quantifiable range for dPCR is much narrower (≤26000 GC/reaction in the study presented here) compared to that of RT-qPCR and may require dilution for target molecules with concentrations of >26000 GC/reaction. Furthermore, RT-dPCR experiments typically require more time (∼3.5 h) than do RT-qPCR experiments (∼1.5 h) and include a manual setup compared to RT-qPCR, which can be set up using a liquid handler. For the QIAcuity platform, the number of samples that were processed per nanoplate (24 samples/well) is only one-quarter of the 96 well plates (96 samples/well), which can greatly limit throughput for large-scale wastewater monitoring programs. However, this limitation may be partially overcome by duplexing or multiplexing SARS-CoV-2 assays, whereas currently two singleplex assays (CDC N1 and CDC N2) are being used for routine wastewater surveillance of SARS-CoV-2. ddPCR measurements of duplex *Enterococcus* spp. and the human fecal *Bacteroides* HF183 marker gene were nearly identical to singleplex measurements.[33] Multiplexing could improve RT-dPCR throughput and may lower per analyte costs. Despite these limitations, the results of the study

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presented here indicate that RT-dPCR offers significantly improved analytical sensitivity over RT-qPCR for the workflow used. Such improved sensitivity will likely be vital for wastewater surveillance in settings with little or no COVID-19 cases in the community.

CONCLUSIONS

RT-dPCR implemented in this study is a suitable platform for the detection of low levels of SARS-CoV-2 RNA in wastewater samples.

The ALODs of both RT-qPCR and RT-dPCR were within the same order of magnitude for γ-irradiated SARS-CoV-2; however, in the presence of a matrix (wastewater), RT-dPCR detection rates outperformed RT-qPCR.

Because SARS-CoV-2 RNA was detected in wastewater eluate and pellet samples, SARS-CoV-2 should be concentrated from both liquid and solid phases of wastewater to increase the detection sensitivity.

Future research is needed to study the duplexing or multiplexing potential of the QIAcuity platform to save cost and improve throughput.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.1c00387.

Supplementary Tables ST1−ST3 and Supplementary Figures SF1 and SF2 (PDF)

AUTHOR INFORMATION

Corresponding Author

Warish Ahmed − *CSIRO Land and Water, Ecosciences Precinct, Dutton Park, QLD 4102, Australia;* orcid.org/0000-0002-1351-8963; Phone: +617 3833 5582; Email: Warish.Ahmed@csiro.au

Authors

Wendy J. M. Smith − *CSIRO Land and Water, Ecosciences Precinct, Dutton Park, QLD 4102, Australia*

Suzanne Metcalfe − *CSIRO Land and Water, Ecosciences Precinct, Dutton Park, QLD 4102, Australia*

Greg Jackson − *Water Unit, Health Protection Branch, Prevention Division, Queensland Health, Brisbane, QLD 4001, Australia*

Phil M. Choi − *Water Unit, Health Protection Branch, Prevention Division, Queensland Health, Brisbane, QLD 4001, Australia*

Mary Morrison − *Water Unit, Health Protection Branch, Prevention Division, Queensland Health, Brisbane, QLD 4001, Australia*

Daniel Field − *Water Unit, Health Protection Branch, Prevention Division, Queensland Health, Brisbane, QLD 4001, Australia*

Pradip Gyawali − *Institute of Environmental Science and Research Ltd. (ESR), Porirua 5240, New Zealand*

Aaron Bivins − *Department of Civil and Environmental Engineering and Earth Sciences, University of Notre Dame, Notre Dame, Indiana 46556, United States*

Kyle Bibby − *Department of Civil and Environmental Engineering and Earth Sciences, University of Notre Dame, Notre Dame, Indiana 46556, United States;* orcid.org/0000-0003-3142-6090

Stuart L. Simpson − *CSIRO Land and Water, Lucas Heights, NSW 2234, Australia*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsestwater.1c00387

Notes

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