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Coronavirus Pandemic

Retrospective quantitative detection of SARS-CoV-2 by digital PCR showing high accuracy for low viral load specimens

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Abstract

Introduction: Accurate detection of severe acute respiratory syndrome coronavirus 2 is critical for diagnosis and disease status evaluation of Coronavirus disease 2019. We retrospectively evaluated the infection status and viral load of severe acute respiratory syndrome coronavirus 2 in Nantong city, China, using a quantitative digital polymerase chain reaction and reverse-transcription PCR.

Methodology: A total of 103 clinical specimens from 31 patients were collected and tested by digital PCR and reverse-transcription PCR.

Results: The overall accuracy of digital PCR was 96.8%, which was higher than the overall accuracy of 87.1% for reverse-transcription PCR. 4 (3.88%) specimens for ORF1ab and 22 (21.36%) specimens for N gene were negative by reverse-transcription PCR but positive by digital PCR. 3 (2.91%, 3/103) specimens of ORF1ab were positive by reverse-transcription PCR but negative by digital PCR. The digital PCR assay exhibited higher sensitivity to measure the N gene than the ORF1ab gene ($p < 0.01$).

Conclusions: Our results showed that digital PCR assay provides more reliable detection of Coronavirus disease 2019 than reverse-transcription PCR, especially for low viral load specimens.

Key words: SARS-CoV-2; COVID-19; digital PCR; reverse-transcription PCR; pharyngeal; low viral load.

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Introduction

Coronavirus disease 2019 (COVID-19) has become a global public health emergency. One-step reverse-transcription polymerase chain reaction (RT-PCR) detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is recommended as the "gold standard" for the diagnosis of COVID-19 [1]. However, this commonly used method showed relatively low detection sensitivity (about 58%-96%) and had some false-negative results [2,3], which may be caused by the low viral load in the pharyngeal secretions of some patients, the inappropriate transport and storage of specimens, relatively low detection limit of RT-PCR and suboptimal biological sampling [4]. Patients with symptoms of COVID-19 but false-negative detection of SARS-CoV-2 by RT-PCR may be treated as common influenza or pneumonia, leading to - high risk of viral transmission and high mortality [5]. The false-negative result may also allow an asymptomatic or paucisymptomatic patient to be released from quarantine, further infect others and propagate the epidemic. Digital PCR (ddPCR) is a highly sensitive and direct quantification assay based on the principle of limiting dilution PCR and Poisson statistics. During the dPCR assay, the sample is dispersed into tens of thousands of reaction units. The fluorescent signals of each reaction unit are measured at the endpoint of the PCR e and then the absolute amount of target molecules present in the sample is calculated. dPCR technique does not rely on a standard curve for the quantification of nucleic acid molecules, which reduces error and improves accuracy. dPCR can detect a few copies of viral genomes and has been increasingly applied in the detection of infectious diseases. It can provide less false-negative results and provide critical support in COVID-19 diagnosis. dPCR has already been used in SARS-CoV-2 detection [6-8], however, it is still poorly investigated in clinical settings. In this retrospective study, we analyzed and compared the results of dPCR and RT-PCR assay for SARS-CoV-2 in clinical specimens from patients in Nantong city, China.

Methodology

Clinical specimen collection and RNA extraction

The Ethics Committee of the Nantong Third Hospital Affiliated to Nantong University approved this study. A total of 103 clinical specimens were collected

from 25 patients with confirmed COVID-19 and 6 non-confirmed patients in Nantong city, China from January 3 to March 6, 2020. Specimens included 67 oral pharyngeal swabs, 23 fecal swabs, 1 sputum specimen, and 12 serum specimens. Specimens, demographic and clinical data, laboratory test results, and CT images of the patients were obtained with informed consent from the patients.

RNA of all the specimens was extracted using automatic nucleic acid extractor, Model: EX3600/2400 (Liferiver Bio-tech, Shanghai, China) and RNA isolation kit, ME-0044 (Liferiver Bio-tech, Shanghai, China) following the manufacturer's instructions.

RT-PCR Assay

RT-PCR assay of the collected specimens was performed with Applied Biosystems™ Real-Time PCR System 7500 (Applied Biosystems, New York, USA) using Novel Coronavirus (2019-nCoV) real-time multiplex RT-PCR kit RR-0479-02 (Liferiver Bio-Tech, Shanghai, China).

A 25-μL of reaction solution 5 μL of RNA, 19 μL of reaction buffer provided by the RT-PCR kit, and 1μL of enzyme mix was used for the RT-PCR assay. Thermal cycling was performed at 45 °C for 10 minutes for reverse transcription, followed by 95°C for 3 minutes, then 45 cycles of 95 °C for 15 seconds, and 58 °C for 30 seconds. According to the instruction of the Liferiver RT-PCR kit, Ct values ≤43 for both ORF1ab and N gene were judged as positive, while Ct values > 43 for both genes was defined as negative.

dPCR Assay

The dPCR assay for SARS-CoV-2 virus in specimens was performed using the RainSure Novel Corona Virus (SARS-CoV-2) Nucleic Acid Detection Kit (Rainsure Scientific, Suzhou, China), which contains three fluorescent primers and probes targeting the ORF1ab and N genes of SARS-CoV-2 and the human RPP30 gene. The equipment was RainSure DropX-2000 Droplet Digital PCR System (Rainsure Scientific, Suzhou, China). The dPCR primers and probes are listed below.

Target 1 (ORF1ab), forward: 5'-CCCTGTGGGTTTTACACTTAA-3', reverse: 5'-ACGATTGTGCATCAGCTGA-3', probe: 5'-FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1-3'; Target 2 (N gene), forward: 5'-GGGGAACTTCTCCTGCTAGAAT-3', reverse: 5'-CAGACATTTTGCTCTCAAGCTG-3', probe: 5'-HEX-TTGCTGCTGCTTGACAGATT-TAMRA-3'; Internal references gene (RPP30), forward: 5'-AGTGCATGCTTATCTCTGACAG-3', reverse: 5'-GCAGGG CTATAGACAAGTTCA-3', probe: 5'-Cy5-TTTCCTGTGAAGGCGATTGACCGA-BHQ-3'.

A 25 μL of reaction solution composing of 14 μL RNA, 10 μL of SARS-CoV-2 one-step RT digital PCR master mixture, and 1 μL of enzyme mixture was used for the dPCR assay. 70 μL of droplet generation oil and 25 μL of the reaction mixture were loaded into the oil wells and the sample wells of the cartridge, respectively. The instrument automatically ran the droplet generation and performed PCR process according to the thermal cycling protocol: step 1, 49 °C for 20 minutes (reverse transcription); step 2, 97 °C for 12 minutes (DNA polymerase activation); step 3, 40 cycles of 95.3 °C for 20 seconds (denaturation) and 52 °C for 1 minute (annealing); step 4, 20 °C (cooling). The cartridge was then transferred and loaded into a DScanner-2000 (Rainsure Scientific, Suzhou, China) and the droplets were subjected to multi-channel fluorescence detection. The fluorescence channels of FAM, HEX, and Cy5 were scanned to detect the ORF1ab gene, N gene, and RPP30 gene, respectively. The data were analyzed with the GeneCount software, V1.62.0205 (RainSure Scientific, Suzhou, China). The criteria for positive result was positive droplets ≥ 2 and total droplets ≥ 10,000, while that for negative result was positive droplets < 2 and total droplets ≥ 10,000.

The limit of detection (LOD) of dPCR was determined using pseudovirus, cat# FNV2001, Lot: 20200302 (Fubio Biological Technology, Suzhou, China). 20 oropharyngeal swab specimens containing cells from a COVID-19 negative patient (SZCJF-20200331) were pooled to prepare a negative mock clinical matrix d using kit UTM 306 (Copan Collection, Murrita, USA). The pooled swab specimens were evenly divided into 6 tubes. 5 of these tubes were individually spiked with serially dilutions of quantitative SARS-CoV-2 pseudovirus to prepare a simulated, contrived clinical sample. Solutions of 25 copies/reaction, 8 copies/reaction, 4 copies/reaction, 2 copies/reaction, and 1 copy/ reaction were prepared by diluting the pseudoviral stock solution with a negative simulated clinical matrix. Four replicates of each concentration were extracted using the Rainsure SARS-CoV-2 Nucleic Acid Detection Kit and tested with a RainsureDropX-2000 digital PCR system (RainSure Scientific, Suzhou, China). The SARS-CoV-2 negative patient (SZCJF-20200331) was tested for SARS-CoV2 by FDA EUA approved Real-time Fluorescent RT-PCR Kit, cat#: MFG030010 (BGI Genomics,Shenzhen, China) and confirmed as negative.

Statistical Analysis

The dPCR data were analyzed using GeneCount Analysis System software, V1.62.0205 (Rainsure Scientific, Suzhou, China) to calculate the concentration of the targets.

Categorical variables were expressed as number (%) and continuous variables were described as mean ± SEM. Comparisons between the two groups were made using the T-test. A *p*-value less than 0.05 (two-sided) was considered statistically significant. The above analyses were performed using Excel (Microsoft, Redmond, USA) software.

Results

Baseline demographic and clinical information of the enrolled patients

Clinical data, blood test results, and CT scan information are shown in Table 1. T-test was used for comparison between the 2 groups. 8 patients with confirmed COVID-19 had chills, while 6 patients with non-confirmed COVID-19 did not have chills ($p < 0.01$). In this study, hypertension may be a relative risk factor for SARS-CoV-2 virus infection ($p < 0.01$). Blood tests showed that only the white cell count was significantly different between confirmed and non-confirmed patients ($p < 0.01$). 4 of the 25 confirmed patients (16%) had ground glass opacities (GGOs), while none of the non-confirmed patients had this symptom ($p < 0.05$).

LOD of the ddPCR

The lowest concentration of SARS-CoV-2 RNA with ≥ 95% detection was 0.32 copies/μL.

Comparison of the diagnostic accuracy of dPCR and RT-PCR

In this study, 31 specimens from 25 clinically confirmed patients (determined as positive based on a combination of diagnosis including RT-PCR results, CT images, and other features) and 6 non-confirmed patients were tested using dPCR and RT-PCR. 21 of the 25 COVID-19 patients were found to be positive by RT-PCR, with an accuracy rate of 87.1%; 24 of the 25 COVID-19 patients were found positive by ddPCR, with a positive accuracy of 96.8% (Table 2).

Consistency analysis of dPCR and RT-PCR for detection of ORF1ab and N gene

A total of 103 specimens were tested using dPCR and RT-PCR. The 100% positive results for the human RPP30 gene assured the success of each dPCR amplification. For ORF1ab gene, 4 (3.88%, 4/103) specimens were negative by RT-PCR but positive by ddPCR and 3 (2.91%, 3/103) specimens were positive

Table 1. Baseline demographic and clinical features of the enrolled patients. Comparisons between 2 groups were made using the T-test.

	Confirmed COVID-19	Non COVID-19	*p*-value
	Value	Value	
Patients, N (Total = 31)	25	6	
Age, Median (IQR), years	52 (26-73)	46 (26-66)	0.5232
Male, n (%)	14 (56)	2 (66.67)	0.3627
Signs and symptoms at admission, n (%)			
Fever	24 (96)	4 (66.67)	0.2261
Cough	17 (68)	4 (66.67)	0.9556
Chills	8 (32)	0 (0)	0.0026
Myalgia	6 (24)	2(33.33)	0.6950
Headache	5 (20)	1(16.67)	0.8622
Pharyngodynia	0 (0)	1 (16.67)	0.3632
Chest tightness	2 (8)	0 (0)	0.1615
Chest pain	1 (4)	0 (0)	0.3272
Diarrhea	1 (4)	0 (0)	0.3272
Nausea or Vomiting	1 (4)	1 (16.67)	0.4898
No signs or symptoms	1 (4)	0 (0)	0.3272
Underlying chronic diseases, n (%)			
Hypertension	8 (32)	0 (0)	0.0026
Hypothyroidism	1 (4)	0 (0)	0.3272
Chronic nephritis	1 (4)	0 (0)	0.3272
Chronic bronchitis	1 (4)	0 (0)	0.3272
Diabetes mellitus	0 (0)	1 (16.67)	0.3632
Rheumatoid arthritis	1 (4)	0 (0)	0.3272
Blood Test			
WBC (*10^9/L)	9 (57)	0 (0)	0.0012
PCT (ng/mL)	13 (52)	3 (50)	0.9373
ESR (mm/h)	22 (88)	5 (83)	0.8026
CT image diagnosis			
Infection/Inflammation	22 (88)	3 (50)	0.1551
Ground glass opacties	4 (16)	0 (0)	0.0429

by RT-PCR but negative by ddPCR. The results showed good consistency between dPCR and RT-PCR for the ORF1ab gene detection (93.20 %, 96/103) (Table 3). For N gene, dPCR showed 48 positive results, while RT-PCR showed only 26 positive results (Figure 1), resulting in 22 (21.36%, 22/103) false-negative results. The consistency between dPCR and RT-PCR results for N gene was 73.79% (76/103). The dPCR assay exhibited higher sensitivity to measure N gene than ORF1ab gene ($p < 0.01$). Those false-negative specimens had very low viral loads, as shown in Figure 1.

The dPCR assay provided a quantitative assay to measure the dynamic changes of the virus load in patients (Figure 2), and therefore may allow reliable monitoring of disease progression and treatment effect.

dPCR results of different specimens from the same patient

Pharyngeal, fecal, and serum specimens were collected on the same day in 6 patients confirmed Covid-19. dPCR results for ORF1ab were positive in all pharyngeal specimens and negative in all fecal and serum specimens. dPCR results for N gene were positive in both pharyngeal and fecal specimens and positive in 1 serum specimen. Virus load levels were pharynx > fecal > blood, implying that pharyngeal specimen may be superior to feces and serum specimens for sensitive diagnosis of the infection.

Discussion

Understanding the baseline demographic and clinical characteristics of patients with COVID-19 diseases is essential for performing effective clinical care. Hypertension is a common comorbidity in patients with COVID-19. It has been shown to be associated

Figure 1. Only positive dPCR results and both dPCR and RT-PCR positive results for SARS-Cov-2 test of the specimens with ORF1ab and N gene.

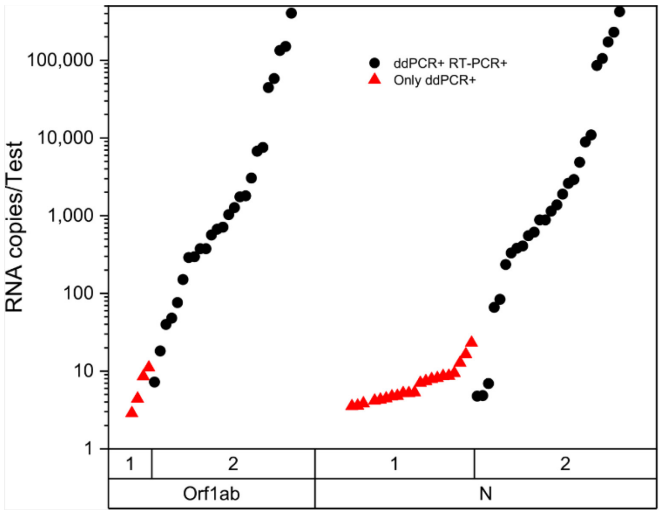

Figure 2. The dynamic changes of the virus load of 3 patients.

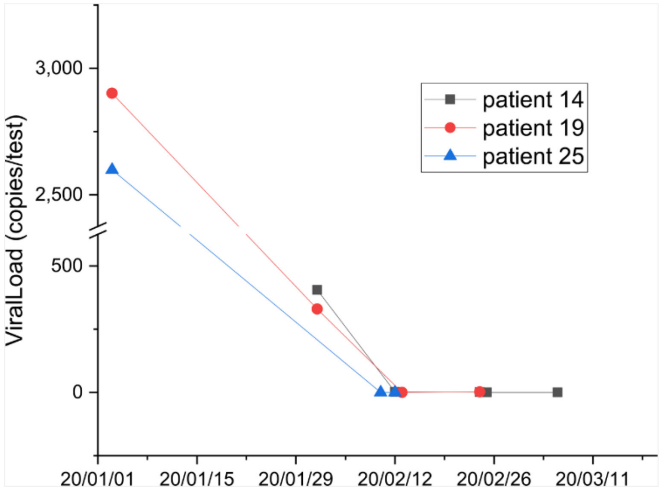

Table 2. Comparison of the accuracy between RT-PCR and dPCR with clinical diagnosis results.

Assay method	RT-PCR		dPCR	
	Positive	Negative	Positive	Negative
Clinical diagnosis results				
Positive	21[a]	5[b]	24[a]	1[b]
Negative	0[c]	6[d]	0[c]	6[d]
Positive detection rate	87.1%		96.8%	

Accuracy = [(a+b)/(a+b+c+d)]×100%.

Table 3. Comparison between RT-PCR and dPCR of all the specimens. Comparisons between ORF1ab and N gene of dPCR were made using the T test.

RT-PCR	ddPCR					
	ORF1ab gene			N gene		
	Positive	Negative	Total	Positive	Negative	Total
Positive	25	3	28	26	0	26
Negative	4	71	75	22	55	77
Total	29	74	103	48	55	103

with an increased risk of infection and adverse clinical outcomes in patients with COVID-19 [9]. Analysis of clinical data in this study also showed a higher proportion of confirmed patients had hypertension compared to non-confirmed patients ($p < 0.01$), and hypertension may be a risk factor for SARS-Cov-2 infection. Nevertheless, evidence for this risk factor is still limited, and further research is needed.

The LOD of the dPCR assay was determined to be 8 copies/test (0.32 copies/μL). The LOD of another dPCR assay was 10 copies/test using different dPCR instruments and assay kits [7]. The study using 7 commonly used RT-PCR assays showed that 1.267 copies/μL for ORF1ab gene and 1.392 copies/μL for N gene could be detected [10]. The high sensitivity and reliable quantification of low viral loads of dPCR assay can provide valuable diagnosis information to help clinicians choose appropriate treatment [11-13].

To explore the clinical application of ddPCR, we performed a head-to-head comparison of RT-PCR and dPCR assays using a cohort of 31 patients with a total of 103 pharyngeal, fecal, and serum specimens obtained at different treatment stages. The dPCR assay had higher positive accuracy compared to the RT-PCR (96.8% *versus* 87.1%). It was also more sensitive than RT-PCR for the N gene, making it a more reliable method for measuring the virus load in clinical specimen. In addition, dPCR assay could be used to track progress of the treatment by monitoring viral load in specimens obtained on different dates. dPCR results, combined with relevant radiological evidence and treatment history are believed to faithfully reflect the onset and healing of the COVID-19 disease.

The highly sensitive dPCR assay can provide quantitative information on viral load for different types of specimens collected from the same patient. In all the tested cases except one, viral load levels in specimens collected from patients on the same day were pharyngeal specimens > feces > serum. It was reported that viral loads in pharyngeal and anal swabs were correlated with different types of immune status, immune response phase, and resolution phase/ immunological tolerance, respectively [14]. Interestingly, a considerable amount of virus was found in the sputum of one patient with negative pharyngeal samples, and the amount of viral found in the sputum was significantly higher than in the pharyngeal and nasal swabs [7]. These observations by dPCR assay may provide valuable insight into the pathology of this emerging disease [15].

Conclusions

A retrospective study of clinically confirmed and non-confirmed patients with COVID-19 showed that dPCR assay has high sensitivity and accuracy for COVID-19 detection. Therefore, dPCR is a reliable assay for diagnosis, monitoring of COVID-19 progression and treatment, especially in clinical specimens with low viral load.

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