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MAJOR ARTICLE

Quantitative Detection and Viral Load Analysis of SARS-CoV-2 in Infected Patients

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Background. Coronavirus disease 2019 (COVID-19) has become a public health emergency. The widely used reverse transcription–polymerase chain reaction (RT-PCR) method has limitations for clinical diagnosis and treatment.

Methods. A total of 323 samples from 76 COVID-19–confirmed patients were analyzed by droplet digital PCR (ddPCR) and RT-PCR based 2 target genes (*ORF1ab* and *N*). Nasal swabs, throat swabs, sputum, blood, and urine were collected. Clinical and imaging data were obtained for clinical staging.

Results. In 95 samples that tested positive by both methods, the cycle threshold (Ct) of RT-PCR was highly correlated with the copy number of ddPCR (*ORF1ab* gene, $R^2 = 0.83$; *N* gene, $R^2 = 0.87$). Four (4/161) negative and 41 (41/67) single-gene positive samples tested by RT-PCR were positive according to ddPCR with viral loads ranging from 11.1 to 123.2 copies/test. The viral load of respiratory samples was then compared and the average viral load in sputum (17 429 ± 6920 copies/test) was found to be significantly higher than in throat swabs (2552 ± 1965 copies/test, $P < .001$) and nasal swabs (651 ± 501 copies/test, $P < .001$). Furthermore, the viral loads in the early and progressive stages were significantly higher than that in the recovery stage (46 800 ± 17 272 vs 1252 ± 1027, $P < .001$) analyzed by sputum samples.

Conclusions. Quantitative monitoring of viral load in lower respiratory tract samples helps to evaluate disease progression, especially in cases of low viral load.

Keywords. COVID-19; SARS-CoV-2; RT-PCR; ddPCR; viral load.

A novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been identified as the pathogen of the coronavirus disease 2019 (COVID-19) [1]. The outbreak of COVID-19 has spread around the world and has become a public health emergency of international concern [2–5]. There have been more than 80 000 infections and 2858 deaths reported as of 28 February 2020 since the first case was identified in December 2019 [6, 7].

At present, viral nucleic acid detection by reverse transcription–polymerase chain reaction (RT-PCR) is regarded as the gold standard for the etiological diagnosis of COVID-19 [8, 9]. However, the sensitivity and reliability of RT-PCR were questioned due to the presence of negative results in some patients who were highly suspected of having the disease based on clinical presentation and exposure history, as well as positive results in some confirmed cases after recovery [10, 11]. In addition, the RT-PCR method has limitations on viral load analysis for evaluating disease progression and prognosis, and is unable to evaluate the efficacy of antiviral drugs.

Several studies have shown that droplet digital PCR (ddPCR) has the advantages of absolute quantification and is more sensitive for virus detection than RT-PCR [12, 13]. In this study, we compared RT-PCR and ddPCR for COVID-19 diagnosis, and explored the changes in viral load in different tissue samples and during disease progression in patients with SARS-CoV-2.

METHODS

Participants

From 5 February to 19 February 2020, 400 samples from 127 patients were tested simultaneously by RT-PCR and ddPCR in Beijing Ditan Hospital, Capital Medical University. As shown in Figure 1, the enrolled 127 subjects included 54 confirmed cases, 39 suspected cases, and 34 patients who were screened due to fever or respiratory symptoms but who did not meet the diagnostic criteria for suspected cases, which were as follows: a patient with 1 exposure history and 2 clinical conditions (fever and/or respiratory symptoms, imaging features of viral pneumonia, normal or low white blood cell count and reduced

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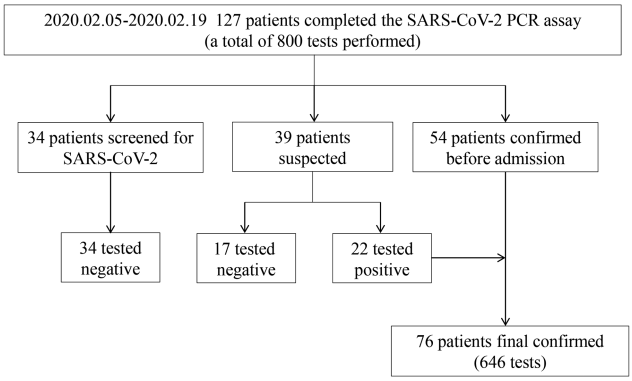

Figure 1. Flow diagram of the study population. Abbreviations: PCR, polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

lymphocyte count in the earlier period of onset), or no clear exposure history but who met 3 clinical conditions [9]. The diagnostic criteria were that a patient met the suspected conditions firstly and had positive RT-PCR assay or viral gene sequencing that was highly homologous with SARS-CoV-2 [9]. Among the suspected cases, 17 were found not to be COVID-19 and 22 became confirmed cases with a positive SARS-CoV-2 result in respiratory tract samples. As a result, 76 confirmed patients were included in the present study.

The clinical data of the 76 confirmed patients were collected, including sex, age, symptoms and signs, other chronic diseases, laboratory examination, imaging data, and clinical typing information. The clinical stages were divided into early, progressive, and recovery phase and clinical cure. The first 3 stages were determined according to chest computed tomography (CT). In the early phase, the typical CT manifestations were multifocal bilateral or isolated round ground-glass opacity with or without patchy consolidations and prominent peripherally subpleural distribution, mainly in the posterior part or lower lobe. In the progressive phase, the number, range, and density of the lesions increased significantly, and the distribution moved from peripheral to central. In the recovery phase, the lesions were gradually absorbed, leaving a few cord-like high-density shades [14]. Clinical cure was considered to be the recovery of temperature for more than 3 days, an obvious improvement in respiratory symptoms, the absorption of pulmonary imaging lesions, and 2 consecutive negative RT-PCR results of respiratory samples at least 1 day apart [9]. Those with normal chest imaging were defined as uncertain stage.

Reverse Transcription–Polymerase Chain Reaction and Droplet Digital Polymerase Chain Reaction for SARS-CoV-2 Detection

Nasal swabs, throat swabs, sputum, blood, and urine samples were collected. Viral RNA was extracted within 2 hours using the QIAamp QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT-PCR and ddPCR were performed subsequently.

Reverse transcription–PCR was conducted with primers and probes targeting the *ORF1ab* and *N* genes and a positive reference gene. Reaction system and amplification conditions were performed according to the manufacturer's specifications (Shanghai BioGerm Medical Technology Co Ltd, China). The result was considered valid only when the cycle threshold (Ct) value of the reference gene was 38 or less. The result was considered positive when the Ct values of both target genes were 38 or less and negative when they were both greater than 38. If only one of the target genes had a Ct value of 38 or less and the other was more than 38, it was interpreted as a single-gene positive.

Droplet digital PCR was performed via the COVID-19 digital PCR detection kit (TargetingOne, Beijing, China) and the TargetingOne Digital PCR System (TargetingOne; licensed by China Food and Drug Administration (CFDA), registration number: 20170025; 20190097; 20192220517). The kit allows the detection of the *ORF1ab* gene, *N* gene, and a positive reference gene. The limit of detection was 10 copies/test.

Statistical Analysis

Categorical variables are expressed as number (%) and continuous variables are described as means ± SEMs. Comparisons between 2 groups were made using the Mann-Whitney *U* test. The correlation between the Ct values of RT-PCR and viral load determined by ddPCR was analyzed with the Spearman correlation test. A *P* value less than .05 (2-sided) was considered statistically significant. The above-mentioned analyses were performed using either Prism 7.0 (GraphPad Software, La Jolla, CA) or SPSS 19.0 (IBM Corporation, Armonk, NY) software.

RESULTS

Patient Characteristics

The 34 screened cases and 17 of the suspected cases were excluded from further analysis, as the 77 samples from these patients tested negative by both methods. The 76 COVID-19–confirmed cases were included in the final analysis. The characteristics of confirmed participants were shown in Table 1. The median age was 40 years (interquartile range [IQR], 32–63 years; range, 6 months to 92 years) and the proportion of men was 50%. The most prevalent signs and symptoms at admission were fever (88.2%) and cough (69.7%). Two patients had no symptoms or sign. Furthermore, 77.6% of patients had mild type disease, while 22.4% had severe type disease. In terms of clinical stage, 49 patients were in the recovery phase, which accounted for the largest proportion (64.5%), followed by the progressive phase (10.5%), early phase (9.2%), and clinical cure phase (6.6%). Seven patients were of uncertain stage because their chest imaging was normal. The average days from symptom onset to the early, progressive, and recovery phases

Table 1. Baseline Characteristics of the Final Confirmed Patients

Parameter	Value
Age, median (interquartile range), years	40 (32–63)
Male, n (%)	38 (50)
Signs and symptoms at admission, n (%)	
Fever	67 (88.2)
Cough	53 (69.7)
Fatigue	27 (35.5)
Myalgia	20 (26.3)
Chills	12 (15.8)
Anorexia	9 (11.8)
Dyspnea	8 (10.5)
Pharyngodynia	7 (9.2)
Headache	4 (5.3)
Nausea and vomiting	4 (5.3)
Diarrhea	3 (4.0)
No sign or symptom	2 (2.6)
Combined with chronic diseases, n (%)	26 (34.2)
Clinical classification, n (%)	
Mild type	59 (77.6)
Severe type	17 (22.4)
Clinical stage, n (%)	
Early stage	7 (9.2)
Progressive stage	8 (10.5)
Recovery stage	49 (64.5)
Clinical cure	5 (6.6)
Uncertain	7 (9.2)

were 4 (range, 2–6), 12 (range, 7–19), and 20 (range, 10–33) days after disease onset, respectively.

Comparison of Reverse Transcription–Polymerase Chain Reaction and Droplet Digital Polymerase Chain Reaction

A total of 323 samples from the 76 confirmed patients were tested by both RT-PCR and ddPCR, including sputum (116, 35.9%), throat swabs (134, 41.5%), nasal swabs (55, 17.0%), urine samples (14, 4.3%), and plasma samples (4, 1.2%) (Table 2). According to the RT-PCR results, 95 samples were positive, 67 were single-gene positive, and 161 were negative. The ddPCR results of the 95 positive samples were also positive, and the Ct value of RT-PCR was highly correlated with the copy number determined by ddPCR (*ORF1ab*, $R^2 = 0.83$; *N*, $R^2 = 0.87$). However, when the Ct value was between 34 and 38, there was no correlation or only a poor correlation (*ORF1ab*, $R^2 = 0.08$; *N*, $R^2 = 0.16$) (data not shown). Among the 67 single-gene positive samples, 26 (38.8%) were negative in ddPCR and 41 (61.2%) were positive, with copy numbers ranging from 11.1 to 123.2 copies/test (Figure 2B). Among the 161 negative samples identified by RT-PCR, 157 (97.5%) samples were negative by ddPCR, and 4 samples was positive, with the copy number ranging between 11.3 copies/test and 20.7 copies/test.

The results showed that both RT-PCR and ddPCR were accurate and reliable in high-viral-load samples and negative samples, but ddPCR was better at detecting samples with low viral load.

Viral Load of Different Tissue Samples

According to the results of ddPCR, 16.4% (9/55) of nasal swabs, 38.1% (51/134) of throat swabs, and 69.0%% (80/116) of sputum samples were positive. No positive results were found in blood or urine (Table 2). The positive rate of sputum samples was significantly higher than that of throat swabs and nasal swabs. We then further compared viral load among the 3 respiratory samples (Figure 3A). The average viral load in sputum (17 429 ± 6920 copies/test) was significantly higher than that in throat swabs (2552 ± 1965 copies/test, $P < .001$) and nasal swabs (651 ± 501 copies/test, $P < .001$).

Analysis of Viral Load and Time Course of COVID-19

The above results show that sputum samples may better reflect the level of virus replication in vivo. Therefore, we further analyzed the dynamic changes in viral load in the disease stages with 116 sputum samples from 44 confirmed patients. The results showed that the viral loads in the early and progressive stages were significantly higher than that in the recovery stage (46 800 ± 17 272 vs 1252 ± 1027, $P < .001$). Due to the limited samples, 6 patients were dynamically observed. Two patients in the progressive stage (patients A and B) were each observed 3 times, and the viral load increased over time (Figure 3C); 2 patients in the recovery stage (patients C and D) were each observed 4 times, and the viral load decreased over time (Figure 3D). In addition, the other 2 patients (patients E and F) were observed from 11 to 19 February for 6 and 7 times, respectively (Figure 3E). Patient E had 4 instances

Table 2. Performance of Reverse Transcription–Polymerase Chain Reaction and Droplet Digital Polymerase Chain Reaction for COVID-19 Clinical Specimens

Specimens	Pos (RT-PCR)		Single-gene Pos (RT-PCR)		Neg (RT-PCR)		Total
	Neg (ddPCR)	Pos (ddPCR)	Neg (ddPCR)	Pos (ddPCR)	Neg (ddPCR)	Pos (ddPCR)	
Nasal swabs	0	8	5	1	41	0	55
Throat swabs	0	43	12	7	71	1	134
Sputum	0	44	9	33	27	3	116
Urine	0	0	0	0	14	0	14
Blood	0	0	0	0	4	0	4
Total	0	95	26	41	157	4	323

Abbreviations: COVID-19, coronavirus disease 2019; ddPCR, droplet digital polymerase chain reaction; Neg, negative; Pos, positive; RT-PCR, reverse transcription–polymerase chain reaction.

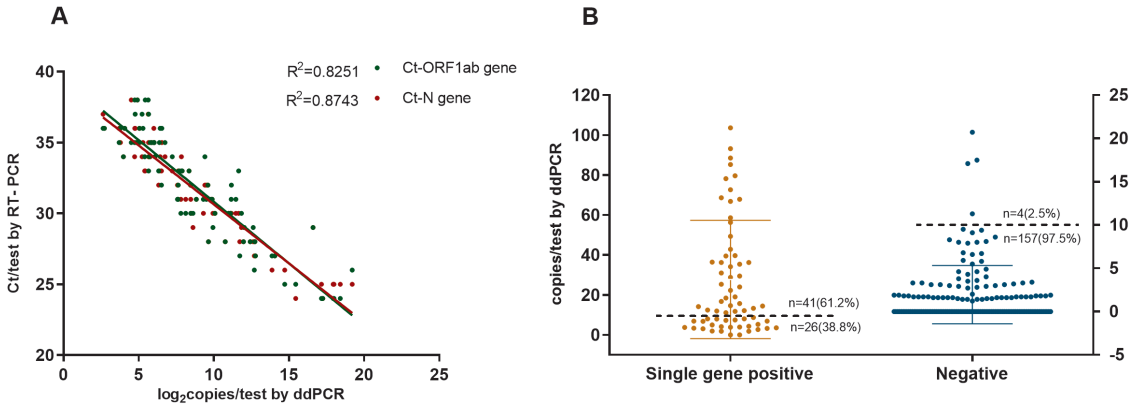

Figure 2. *A*, Correlation analysis between the Ct value of RT-PCR and the viral load of ddPCR. *B*, Viral load distribution tested by ddPCR in single-gene positive and negative samples of RT-PCR. Abbreviations: Ct, cycle threshold; ddPCR, droplet digital polymerase chain reaction; ORF, opening-reading frame; RT-PCR, reverse transcription–polymerase chain reaction.

of single-gene positive results with RT-PCR, patient F had 5 instances, and ddPCR showed viral load fluctuations below 150 copies/test.

DISCUSSION

To determine the viral load levels of SARS-CoV-2 in different tissue samples, the dynamic changes during disease progression, and performance of ddPCR in detecting the virus, a total of 323 samples from 76 confirmed patients were analyzed. We found that RT-PCR and ddPCR gave consistent results for high-viral-load samples; however, ddPCR was better in detecting low-viral-load samples. The viral load of different tissues revealed that sputum samples contained more virus than throat and nasal swabs. The quantitative results of sputum samples showed that the viral load first increased and then decreased during the disease course of COVID-19.

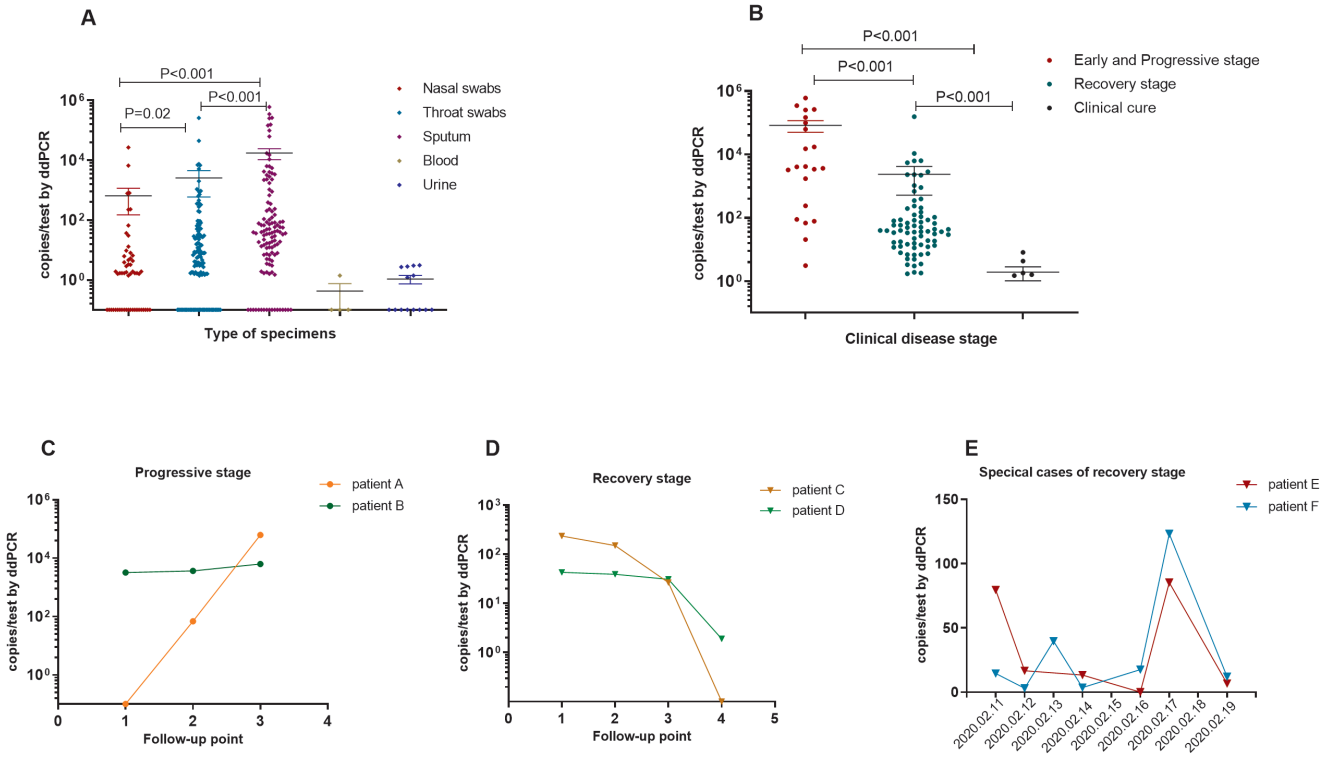

Figure 3. *A*, Viral load of different tissue samples. *B*, Analysis of viral load in different clinical stages of COVID-19. *C*, Dynamic changes of viral load in sputum samples in 2 progressive-stage patients. *D*, Dynamic changes of viral load in sputum samples in 2 convalescent patients. *E*, Low-level fluctuation of viral load in 2 convalescent patients during 9 days of detection. Abbreviations: COVID-19, coronavirus disease 2019; ddPCR, droplet digital polymerase chain reaction.

The results of RT-PCR and ddPCR were consistent in the 95 positive samples, and the Ct value of RT-PCR was highly correlated with the copy number value of ddPCR. However, when Ct values were between 34 and 38, the viral load of samples with the same Ct value was significantly different, indicating that the Ct value of RT-PCR may not sensitively reflect the level of viral load when the viral load is low. This result is consistent with previous reports [15, 16]. In 67 single-gene–positive samples and 4 RT-PCR–negative samples, ddPCR gave positive results with low viral load, suggesting that RT-PCR was unstable in the detection of low-viral-load samples.

A previous study showed that SARS-CoV-2 existed in both the upper and lower respiratory tract [17]. We analyzed the viral load of samples from different tissues with ddPCR and found that the positive rate and viral load of sputum were higher than those of throat swabs and nasal swabs. These results demonstrated that, although SARS-CoV-2 can colonize the upper respiratory tract, lower respiratory tract samples could better reflect the viral replication level in infected patients. In addition, lower respiratory tract samples may also be more suitable than throat and nasal swabs for the etiological diagnosis of COVID-19, which may increase the low detection rate among positive patients.

Furthermore, the dynamic changes in the viral load during the course of COVID-19 were analyzed using the sputum samples. Consistent with recently reported studies [18, 19], we found that the viral load increased in the early and progressive stages and decreased in the recovery stage. In patients E and F who entered the recovery stage as determined by chest CT, the viral load was found to fluctuate at a low level for more than 9 days before becoming negative, suggesting that some patients may have a long asymptomatic virus-carrying state before clinical cure. Some patients who had at least 2 negative nucleic acid tests and who reached the discharge standard were found to be positive again during re-examination. There may be intermittent virus shedding, leading to nucleic acid results being positive again in patients who recovered from COVID-19 [20]. As time goes on, a large number of patients will enter the recovery stage and be discharged from the hospital, and the monitoring of low viral load will be more frequent. Multiple tests of lower respiratory tract samples and by different methods may help to improve the sensitivity and to assess whether discharge criteria are met.

According to the changes in viral load and chest CT in the course of COVID-19, high-level replication of the virus may indicate the progress of the disease. Effective antiviral therapy can shorten the course of disease and reduce the severity [21]. However, there is no effective antiviral therapy for COVID-19, and it is therefore extremely urgent to develop drugs in the future. Droplet digital PCR may play an important role in evaluating the efficacy of antiviral drugs by dynamically detecting viral load.

This study has several limitations. First, although the result showed that the viral load in the samples of the lower respiratory tract was high, the sputum, throat swabs, and nasal swabs of the subjects were not matched during comparison. Matched analysis will be performed in another prospective cohort study by our team. Second, due to the limited sample size, we did not analyze the relationship between the viral load and the severity of COVID-19 at different stages, and further research is needed.

Conclusions

Reverse transcription–PCR is sensitive and reliable, but ddPCR performed better in detecting low-viral-load samples. Sputum is a better indicator of viral replication in the body than throat and nasal swabs, and the viral load of sputum samples tends to increase and then decrease during the course of the disease.

Notes

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Potential conflicts of interest. The authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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