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

Fengting Yu[1,2#], Liting Yan[1,2#], Nan Wang[3,4#], Siyuan Yang[1,2], Linghang Wang[1,2], Yunxia Tang[1,2], Guiju Gao[1,2], Sa Wang[1,2], Chengjie Ma[1,2], Ruming Xie[1,2], Fang Wang[5], Chianru Tan[5], Lingxiang Zhu[3], Yong Guo[5*], Fujie Zhang[1,2*]

[1]Beijing Ditan Hospital, Capital Medical University, Beijing, China; [2]Clinical Center for HIV/AIDS, Capital Medical University, Beijing, China; [3]Human Genetic Resource Center, National Research Institute for Health and Family Planning, Beijing, China; [4]Chinese Academy of Medical Sciences, Graduate School of Peking Union Medical College, Beijing, China; [5]Department of Biomedical Engineering, School of Medicine, Tsinghua University, Beijing, China.

Contributions: [#]F.Y., L.Y., N.W. contributed equally to this study.

Correspondence: Professor Fujie Zhang, MD., PhD, Clinical and Research Center of Infectious Disease, Beijing Ditan Hospital, Capital Medical University, Beijing 100015, China. (E-mail: treatment@chinaaids.cn)

Summary: As a public health emergency, quantitative monitoring of Coronavirus disease 2019 (COVID-19) in lower respiratory tract samples in infected patients helps to evaluate disease progression, especially in cases of low viral load.

ABSTRACT

Background Coronavirus disease 2019 (COVID-19) has become a public health emergency. The widely used reverse transcription PCR (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 two 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 tested positive by both methods, the cycle threshold (Ct) of RT-PCR was highly correlated with the copy numbed of ddPCR (ORF1ab gene, $R^2 = 0.83$; N gene, $R^2 = 0.87$). 4 (4/161) negative and 41 (41/67) single-gene positive samples tested by RT-PCR were positive according to ddPCR with viral load ranging from 11.1 to 123.2 copies/test. Then the viral load of respiratory samples was compared and the average viral load in sputum (17429 ± 6920 copies/test) was found to be significantly higher than in throat swabs (2552 ± 1965 copies/test, $p < 0.001$) and nasal swabs (651 ± 501 copies/test, $p < 0.001$). Furthermore, the viral load in the early and progressive stages were significantly higher than that in the recovery stage (46800 ± 17272 vs 1252 ± 1027, $p < 0.001$) 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

INTRODUCTION

A novel coronavirus, 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 become a public health emergency of international concern [2-5]. There have been more than 80,000 infections and 2858 deaths reported as of February 28, 2020 since the first case was identified in December 2019 [6,7].

At present, viral nucleic acid detection by reverse transcription PCR (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 was 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 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 with SARS-CoV-2-infected patients.

METHODS

Participants

From February 5 to February 19, 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 did not meet the diagnostic criteria for suspected cases, which were as follows: a patient with one exposure history and two clinical conditions (a. Fever and/or respiratory symptoms; b. Imaging features of viral pneumonia; c. Normal or low white blood cell count and reduced lymphocyte in the earlier period of onset), or no clear exposure history but meet three clinical conditions [9]. The diagnostic criteria was that a suspected case with 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 SARS-Cov-2 tested positive in respiratory tract samples. As a result, 76 final 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, recovery phase, and clinical cure. The first three 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, prominent peripherally subpleural distribution and mainly in 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 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, the obvious improvement of respiratory symptoms, the absorption of pulmonary imaging lesions and two consecutive negative RT-PCR results of respiratory samples at least one day apart [9]. Those with normal chest imaging were defined as uncertain stage.

RT-PCR and ddPCR for SARS-CoV-2 detection

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

RT-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. The result was considered positive when the Ct values of both target genes were ≤ 38, negative when they were both > 38. If only one of the target genes had a Ct value ≤ 38 and the other > 38, it was interpreted as a single-gene positive.

ddPCR was performed via the COVID-19 digital PCR detection kit (TargetingOne, Beijing, China) and the TargetingOne Digital PCR System (TargetingOne, Beijing, China, Licensed by 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 were expressed as number (%) and continuous variables were described as mean ± SEM. Comparisons between two 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 0.05 (two-sided) was considered statistically significant. The above-mentioned analyses were performed using either Prism 7.0 (GraphPad, La Jolla, CA, USA) or SPSS 19.0 (College Station, TX, USA) software.

RESULTS

Patient characteristics

The 34 screened cases and 17 of the suspected cases were excluded from further analysis, as the 77 samples from them were tested negative by both methods. The 76 COVID-19 confirmed cases were included in the final analysis. The characteristics of confirmed participants are shown in Table 1. The median age was 40 years (IQR, 32-63; 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 were the mild type, while 22.4% were the severe type. 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 were 4 (range 2-6), 12 (range 7-19), and 20 (range 10-33) days after disease onset, respectively.

Comparison of RT-PCR and ddPCR

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 sample (14, 4.3%), and plasma sample (4, 1.2%) (Table2). 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 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 11.1-123.2 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) nasal swabs, 37.3% (50/134) throat swabs, and 66.4% (77/116) 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. Then, we further compared viral load among the three respiratory samples (Figure 3A). The average viral load in sputum (17429 ± 6920 copies/test)

was significantly higher than in throat swabs (2552 ± 1965 copies/test, $p < 0.001$) and nasal swabs (651 ± 501 copies/test, $p < 0.001$).

Analysis of viral load and time course of COVID-19

The above results showed 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 load in the early and progressive stages were significantly higher than that in the recovery stage (46800 ± 17272 vs 1252 ± 1027, $p < 0.001$). Owing to the limit samples, 6 patients were dynamically observed. Two patients in the progressive stage (patient A, patient B) were each observed 3 times, and the viral load increased over time (Figure 3C); 2 patients in the recovery stage (patient C, patient D) were each observed 4 times, and the viral load decreased over time (Figure 3D). In addition, the other two patients (patient E, patient F) were continuously observed from February 11 to 19 for 6 times and 7 times, respectively (Figure 3E). Patient E had 4 times of single-gene positive in RT-PCR, Patient F had 5 times, and ddPCR showed viral load fluctuations at 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 relative to RT-PCR 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 increased first and then decreased during the disease course of COVID-19.

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-vial-load samples.

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 patient E and patient 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 turning negative, suggesting that some patients may have a long asymptomatic virus-carrying state before clinical cure. Some patients who had at least twice negative nucleic acid tests and reached the discharge standard were found to be positive again during reexamination. There may be intermittent virus expulsion, leading to nucleic acid results positive again in patients

recovered from COVID-19 [20]. As time goes on, a large number of patients will enter the recovery stage and face discharge, and the monitoring of low viral load will be more frequent. Multiple tests of lower respiratory tract samples and by different methods maybe help to improve the sensitivity and 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 extremely urgent to develop drugs in the future. ddPCR 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 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 of 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.

Conclusion

RT-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.

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Potential conflicts: The authors have no conflicts of interest to declare.

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Table 1. Baseline characteristics of the final confirmed patients

Parameter	Value
Age, median (IQR), y	40 (32-63)
Male, No. (%)	38 (50)
Signs and symptoms at admission, No. (%)	
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, No. (%)	26 (34.2)
Clinical classification, No. (%)	
Mild type	59 (77.6)
Severe type	17 (22.4)
Clinical stage, No. (%)	
Early stage	7 (9.2)
Progressive stage	8 (10.5)
Recovery stage	49 (64.5)
Clinical cure	5 (6.6)
Uncertain	7 (9.2)

Table2. Performance of RT-PCR and ddPCR 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	96
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: Pos, positive; Neg, negative

Figure legends

Figure 1. Flow diagram of the study population. SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; coronavirus disease 2019 (COVID-19)

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.

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 patients. D. Dynamic changes of viral load in sputum samples in 2 convalescent patients. E. Low level fluctuation of viral load in two convalescent patients during 9 days detection.

Figure 1

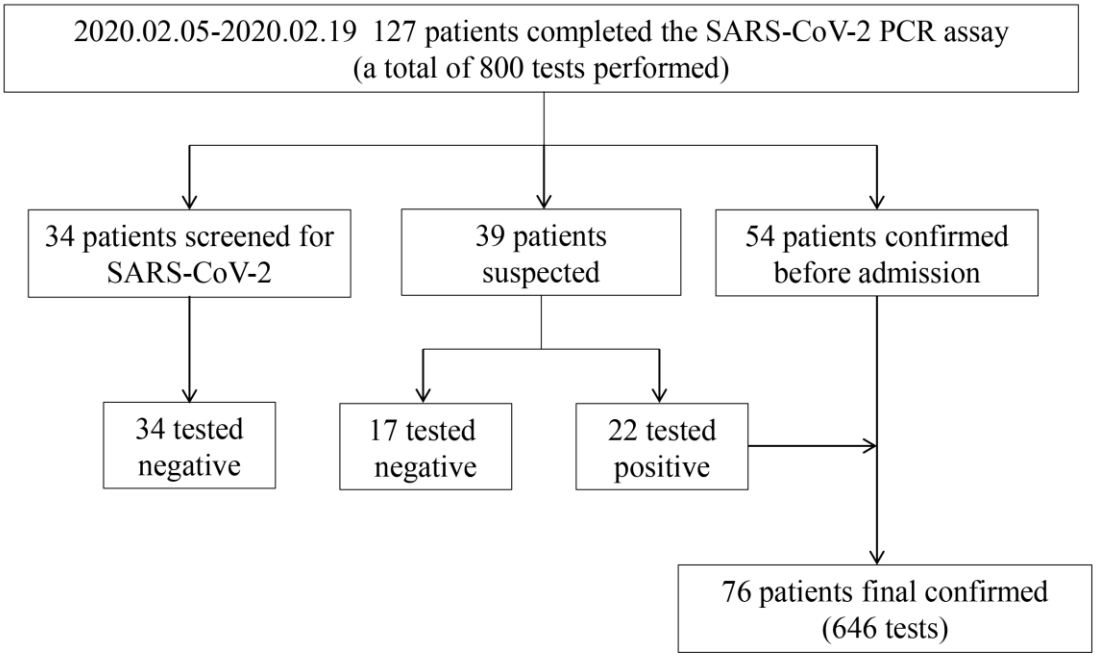

Figure 2

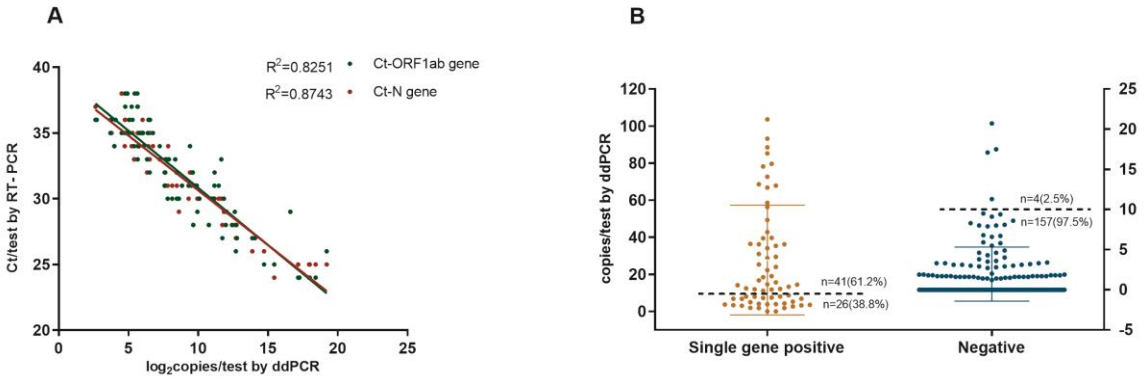

Figure 3