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Novel One-Step Single-Tube Nested Quantitative Real-Time PCR Assay for Highly Sensitive Detection of SARS-CoV-2

Ji Wang,[#] Kun Cai,[#] Ruiqing Zhang,[#] Xiaozhou He, Xinxin Shen, Jun Liu, Junqiang Xu, Feng Qiu, Wenwen Lei, Jinrong Wang, Xinna Li, Yuan Gao, Yongzhong Jiang,[*] Wenbo Xu,[*] and Xuejun Ma[*]

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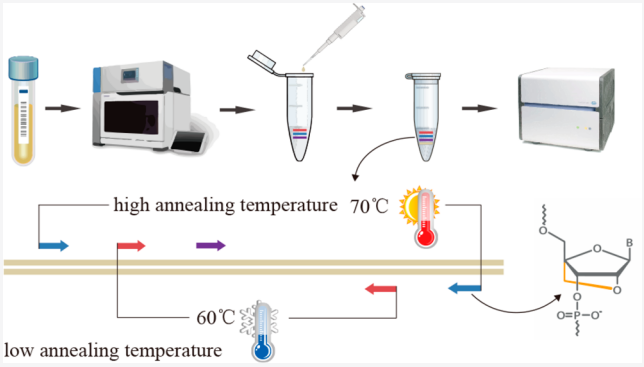
ABSTRACT: Coronavirus disease 2019 (COVID-19) has become a public health emergency. The reverse transcriptase real-time quantitative PCR (qRT-PCR) test is currently considered as the gold standard in the laboratory for the etiological detection of COVID-19. However, qRT-PCR results could be false-negative due to the inadequate sensitivity of qRT-PCR. In this study, we have developed and evaluated a novel one-step single-tube nested quantitative real-time PCR (OSN-qRT-PCR) assay for the highly sensitive detection of SARS-CoV-2 targeting the *ORF1ab* and *N* genes. The sensitivity of the OSN-qRT-PCR assay was 1 copy/reaction and 10-fold higher than that of the commercial qRT-PCR kit (10 copies/reaction). The clinical performance of the OSN-qRT-PCR assay was evaluated using 181 clinical samples. Among them, 14 qRT-PCR-negative samples (7 had no repetitive results and 7 had no cycle threshold (CT) values) were detected by OSN-qRT-PCR. Moreover, the 7 qRT-PCR-positives in the qRT-PCR gray zone (CT values of *ORF1ab* ranged from 37.48 to 39.07, and CT values of *N* ranged from 37.34 to 38.75) were out of the gray zone and thus were deemed to be positive by OSN-qRT-PCR, indicating that the positivity of these samples is confirmative. Compared to the qRT-PCR kit, the OSN-qRT-PCR assay revealed higher sensitivity and specificity, showing better suitability to clinical applications for the detection of SARS-CoV-2 in patients with low viral load.



Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been identified as the causative pathogen of the coronavirus disease 2019 (COVID-19).[1] Globally, reported to the World Health Organization (WHO), as of April 29, 2020, there have been 3,018,952 confirmed cases of COVID-19, including 207,973 deaths.[2] The epidemic has been reported as a "global pandemic" by WHO, involving 212 countries, areas, or territories with cases.[3]

At present, there is no specific antiviral drug or therapeutic vaccine for COVID-19. The key to prevention and control lies in early etiological diagnosis. The reverse transcriptase real-time quantitative PCR (qRT-PCR) test is considered as a gold standard in the laboratory for the etiological detection of COVID-19.[4] But, the reliability and sensitivity of the qRT-PCR method have been questioned in the clinical settings because of the uncertainty of testing results. For instance, the clinical diagnosis and qRT-PCR detection is not always consistent. Moreover, the qRT-PCR results of some patients change from negative to positive after multiple tests and some confirmed cases have qRT-PCR positive results after recovery, suggesting that the initial qRT-PCR results could be false-negatives.[5,6] Apart from the inaccurate collection of samples or the insufficient viral quantity in the patients, the false-negatives might be also due to the inadequate sensitivity of qRT-PCR. To address this challenge, it is therefore very important to develop a highly sensitive diagnostic method of SARS-CoV-2.

Nested PCR (N-PCR) is a derivative method based on the principle of PCR. N-PCR has higher sensitivity and specificity by two rounds of amplification, and it is more suitable for the detection of samples with low viral load. However, the operation of the N-PCR is more complex, and the lid opening after the first round of PCR increases the risk of cross-contamination. Our team previously developed a novel locked nucleic acid (LNA)-based one-step single-tube nested real-time qRT-PCR strategy (OSN-qRT-PCR) to detect viral and bacterial pathogens with higher sensitivity and specificity than qRT-PCR without the need of lid opening.[7−10]

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In this study, we developed and evaluated an OSN-qRT-PCR assay for the highly sensitive detection of SARS-CoV-2, targeting the *ORF1ab* and *N* genes on the basis of a commercial qRT-PCR kit for SARS-CoV-2 (Sansure, Hunan, China). The mentioned qRT-PCR kit showed detection sensitivity of 200 copies/mL or 10 copies/reaction and was officially approved by the National Medical Products Administration (NMPA) and used in the detection of COVID-19 nationwide. Compared to this qRT-PCR kit, the OSN-qRT-PCR assay revealed higher sensitivity and specificity, showing better suitability to clinical applications for the detection of SARS-CoV-2 in patients with low viral load.

EXPERIMENTAL SECTION

Specimens. The specificity evaluation panel preserved in the Sansure company consisted of an inactivated culture of SARS-CoV-2, human coronavirus (NL63, HKU1, 229E, OC43), middle east respiratory syndrome coronavirus (MERS-CoV), severe acute respiratory syndrome coronavirus (SARS-CoV), influenza virus types B (Flu B), influenza virus types A (Flu A), FluA-H1N1, FluA-H3N2, FluA-H5N1, and FluA-H7N9, human rhinovirus (HRV) type A, type B, and type C, Boca virus (HBoV), parainfluenza virus (PIV), human adenovirus (HAdV), human metapneumovirus (HMPV), human metapneumovirus (HMPV), respiratory syncytial virus (RSV) type A and B, *Cryptococcus neoformans* (CN), *Streptococcus pyogenes* (SPY), *Acinetobacter baumannii* (Ab), *Pneumocystis carinii* (PC), *Klebsiella pneumoniae* (KPN), *Streptococcus pneumoniae* (SP), *Haemophilus influenzae* (HIN), *Pseudomonas aeruginosa* (PAE), *Legionella pneumophilia* (LP), *Bordetella pertussis* (BP), *Staphylococcus aureus* (SAU), *Mycoplasma pneumoniae* (MP), *Chlamydia pneumoniae* (CP), *Aspergillus fumigatus* (AF), *Candida albicans* (CAL), *Candida glabrata* (CGL), *Mycobacterium tuberculosis* (MTB), *Nontuberculous mycobacterium* (NTM), Epstein−Barr virus (EB), measles virus (MeV), human cytomegalo virus (HCV), norovirus (NV), rotavirus (RV), varicella zoster virus (VZV), mumps virus (MuV), and human genome DNA. The qRT-PCR kit (Sansure, Hunan, China) showed 100% specificity for SARS-CoV-2, and no cross-reaction was observed with the other viral and bacterial pathogens described above.

From January 18 to January 22, 2020, a total of 181 throat swabs with clinically suspected COVID-19 were collected and transported to the Hubei Provincial Center for Disease Control and Prevention, China. All aspects of the study were performed per national ethics regulations and approved by the Institutional Review Boards of China CDC. Written consent was obtained from patients or children's parents.

Nucleic Acid Extraction. The collection, transportation, storage, and detection of specimens were strictly carried out according to SARS-CoV-2 Laboratory Biosafety Guidelines (Second Edition)[11] and the Technical Guide for Laboratory Testing of COVID-19 (Fourth Edition)[12] issued by the General Office of the State Health Commission. According to the instructions recommended by the manufacturer, the total RNA was extracted from 200 μL of sample preservation solution using the Tian Long automatic extraction kit (Tian Long, Xi'an, China). The RNA was eluted in 80 μL of elution buffer and stored at −80 °C until further use.

Outer Primers Design for OSN-qRT-PCR Assay. OSN-qRT-PCR outer primers were designed according to the principle described in our previous publications.[7,10] The schematic diagram of OSN-qRT-PCR is shown in Figure 1.

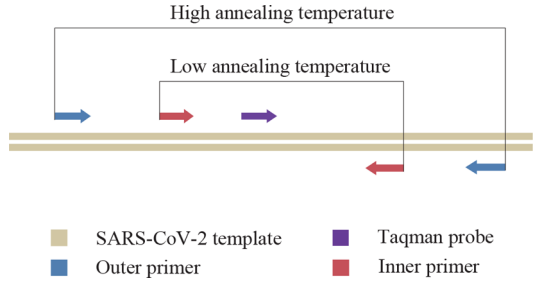

Figure 1. Schematic diagram of the OSN-qRT-PCR assay for the detection of SARS-CoV-2.

All of the outer primers for the *ORF1ab* gene and *N* gene were designed by Oligo 7 software, and several locked nucleic acids (LNAs) were incorporated into outer primer nucleotides. For each incorporated LNA monomer, the temperature of the duplex was increased by about 2−8 °C.[13,14] Temperature gradient PCR (annealing temperatures from 55 to 72 °C) was performed to determine the optimum annealing temperature and the maximum range at which both inner and LNA-outer primers could work. After primer annealing temperatures were determined, inner and LNA-outer primer concentrations were optimized. As shown in Figure 1, at a high annealing temperature, only the LNA-outer primers worked and the outer primers were consumed as much as possible after 10 cycles, and then at low annealing temperature, the inner primers and probes worked and went through 40 cycles to detect fluorescence signal.[10]

OSN-qRT-PCR Assay. The reaction components included 20 μL of template, 26 μL of reaction buffer, and 4 μL of the mixture enzyme. After vortexing and centrifugation, the reaction tube was then transferred to the detector Life Technologies480. The OSN-qRT-PCR amplification contained the following steps: 50 °C for 30 min, 95 °C for 1 min, 10 cycles at 95 °C for 30 s, 70 °C for 40 s, and 72 °C for 40 s, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and then 25 °C for 10 s of instrument cooling. The FAM (*ORF1ab* gene) channel and ROX (*N* gene) channel were selected to detect SARS-CoV-2, and the HEX channel was chosen to detect the reference gene. Each run contained a positive control and a negative control. All FAM, HEX, and ROX channels showed a typical S-shaped amplification curve, and a cycle threshold (CT) ≤ 35 was considered to be positive.

Analytical Specificity and Sensitivity of the OSN-qRT-PCR Assay. We used the specificity evaluation panel described above to explore the specificity of the OSN-qRT-PCR method for SARS-CoV-2. The fragments of the *ORF1ab* gene and *N* gene (NCBI Reference Sequence: NC_045512.2) of SARS-CoV-2 were cloned into the pUC57 vector. We then used the recombinant plasmids as standards for the sensitivity analysis of OSN-qRT-PCR assay. The recombinant plasmids were diluted 10-fold from 10^8 to 10° copies/test, and nonribozyme water was used as a negative control.

qRT-PCR Kit for SARS-CoV-2 Detection. The qRT-PCR kit (Sansure, Hunan, China) targeting the *ORF1ab* and *N* genes was officially approved by the National Medical Products Administration (NMPA). The operational procedures were performed according to the manufacturer's specifications (Sansure Biotech, Hunan, China). The result was considered positive when the CT values of both target genes (*ORF1ab* and *N*) were ≤40 and was considered negative when they were >40. If only one of the target genes had a CT value ≤40 and

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the other was >40, which is the uncertainty case when CT values (37−40) happen to fall in the gray zone, it needed to be retested. If the repeated result remained positive for only one of the two targets, it was interpreted as a single-gene positive, otherwise known as negative.

Statistical Analysis. The OSN-qRT-PCR and qRT-PCR (Sansure kit) methods were applied to detect 181 clinical samples with clinically suspected COVID-19 in parallel. IBM SPSS Statistics, version 21 (IBM Corporation) was used to perform all of the statistical analysis. The results of qRT-PCR assay and OSN-qRT-PCR assay were analyzed using Kappa and McNemar's tests, and a value of $P < 0.05$ was considered statistically significant. The CT values of four groups were analyzed by scatter plots, including the *ORF1ab* group of qRT-PCR, *ORF1ab* group of OSN-qRT-PCR, *N* group of qRT-PCR, and *N* group of OSN-qRT-PCR. We compared the differences of the CT values of *ORF1ab* between qRT-PCR and OSN-qRT-PCR by linear regression and correlation analysis. In addition, the differences of CT values of *N* between OSN-qRT-PCR and qRT-PCR were also analyzed by linear regression and correlation analysis.

RESULTS AND DISCUSSION

Analytical Specificity and Sensitivity of the OSN-qRT-PCR Assay. Using the specificity evaluation panel, the OSN-qRT-PCR method showed 100% specificity to SARS-CoV-2 and was negative for other human coronaviruses (NL63, HKU1, 229E, OC43), middle east respiratory syndrome coronavirus (MERS-CoV), severe acute respiratory syndrome coronavirus (SARS-CoV), 38 other pathogenic viruses or bacteria, and human genome DNA. A panel of diluted recombinant plasmids (from 10^8 to 10^0 copies/test) were tested to ascertain the end point dilution. The sensitivity of the OSN-qRT-PCR assay was 1 copy/reaction, which is 10-fold higher than that of the commercial qRT-PCR kit (10 copies/reaction).

The uniqueness of OSN-qRT-PCR is that two steps of PCR at an optimum annealing temperature are relatively independent and do not interfere with each other in a closed single tube. OSN-qRT-PCR for SARS-CoV-2 uses five oligonucleotides for each gene site in a closed reaction and thus has a total of 10 oligonucleotides matching the template sequence (two genes), which therefore indicates its high analytical specificity. The initiation of the second PCR depends on the success of the first PCR, and the sensitive detection of fluorescence signals depends on the successful match between the Taqman probe and template.[10]

Clinical Evaluation of OSN-qRT-PCR. We evaluated the diagnostic performance of the OSN-qRT-PCR assay for SARS-CoV-2 using 181 clinical samples. Of the 181 clinical samples, OSN-qRT-PCR detected 39 positive samples, of which 25 were detected by qRT-PCR. Fourteen qRT-PCR-negative samples (7 had no repetitive results and 7 had no CT values) were detected by OSN-qRT-PCR. The results of the two methods in these 14 clinical samples are shown in Table 1. Because only one of the two target genes was positive, samples (number: 1−7) were retested by qRT-PCR. At last, these samples were considered as negative with a CT value >40 (both targets genes). Additionally, the results of the OSN-qRT-PCR and the qRT-PCR assay were analyzed by Kappa and McNemar's tests. Kappa values were 0.737 and $P < 0.001$, as shown in Table 2. The results showed that there was a significant difference between the two methods, indicating that

Table 1. CT Values of 14 Clinical Specimens between OSN-qRT-PCR and qRT-PCR Methods

	qRT-PCR		OSN-qRT-PCR	
number	*ORF1ab*	*N*	*ORF1ab*	*N*
1[a]	38.99	40.50	24.54	30.43
2[a]	38.22	40.89	22.73	28.87
3[a]	40.64	39.79	22.65	29.19
4[a]	40.32	40.96	23.99	30.61
5[a]	40.75	39.40	27.89	34.98
6[a]	39.07		24.21	34.49
7[a]		36.64	20.30	27.27
8			25.68	34.25
9			21.72	29.06
10			21.55	26.90
11			13.71	19.74
12			26.14	33.91
13			24.94	31.40
14			23.54	32.86

[a]Only one of the two target genes was positive so samples (number: 1−7) were retested by qRT-PCR. At last, these samples were considered as negative with CT values >40 (both targets genes) by qRT-PCR.

Table 2. Comparison of the Results of Clinical Samples Detected by Two Methods

OSN-qRT-PCR	qRT-PCR		Total
	positive	negative	
positive	25	14	39
negative	0	142	142
total	25	156	181

the OSN-qRT-PCR method is more sensitive than the qRT-PCR method. We conclude that the OSN-qRT-PCR method is highly sensitive with an improved positive rate of SARS-CoV-2 detection, as justified by the fact that the sensitivity of OSN-qRT-PCR assay is 1 copy/reaction while the qRT-PCR is reported to have a sensitivity of 10 copies/reaction. Though, the positivity of 14 samples was not validated by a third method but tended to be true-positives given the strict specificity evaluation of this assay and our previous working experiences.[7,8,10]

Currently, more than 10 qRT-PCR-based detection kits for SARS-CoV-2 with varied detection sensitivity (PCR-Fluorescence Probing) have been officially approved by NMPA in China.[15] The qRT-PCR is a gold standard and a widely accessible method in many diagnostic laboratories in China. However, under some circumstances, the level of viral load might be lower than the detection limit of the qRT-PCR due to the poor quality of sample collection, improper RNA extraction, or the different sample types and course of the disease, leading to false-negative results. Digital PCR is suitable for detecting samples with low viral load and has access to the viral load of samples.[16] However, digital PCR often needs unique supporting reagents, instruments, and professional operators, making the cost very high with moderate throughput. Our results demonstrated that samples with low viral load could also be detected and quantified by the OSN-qRT-PCR method. Compared to digital PCR, the operation of the OSN-qRT-PCR method is the same as qRT-PCR and there is no need for extra professional training. Besides, the OSN-qRT-PCR assay is feasible in any qRT-PCR-instrument-

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equipped laboratory. The cost of OSN-qRT-PCR is lower than digital PCR and the turn-around time of OSN-qRT-PCR is shorter (2 h) than that of digital PCR (3−4 h) though a bit longer than that of qRT-PCR (1.5 h). We therefore conclude that OSN-qRT-PCR is more fit and advantageous for the detection of SARS-CoV-2 in suspected patients with low viral load.

CT Value Analysis. The CT values of the *ORF1ab* and *N* genes using OSN-qRT-PCR were significantly lower than those of qRT-PCR (Figure 2). A significant difference was

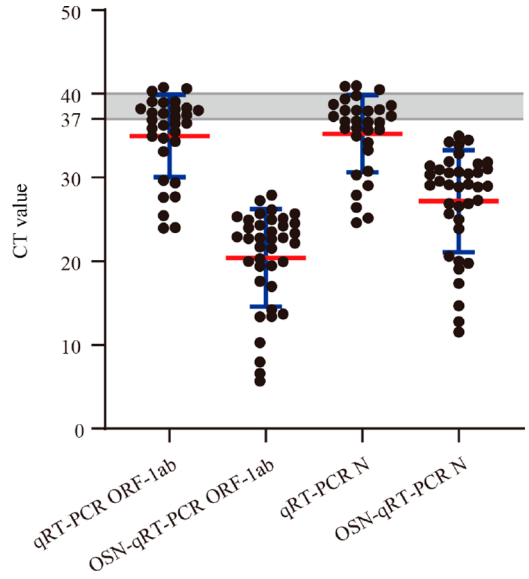

Figure 2. CT values of four groups were analyzed by scatter plots, including the *ORF1ab* group of qRT-PCR, *ORF1ab* group of OSN-qRT-PCR, *N* group of qRT-PCR, and *N* group of OSN-qRT-PCR.

observed in the CT values of the *ORF1ab* gene using OSN-qRT-PCR (mean, 20.4; range, 5.69−27.89; standard deviation, 5.836) vs the CT values of the *ORF1ab* gene using qRT-PCR (mean, 34.97; range, 23.97−40.75; standard deviation, 4.913). A similar result was observed for the CT values of the *N* gene using OSN-qRT-PCR (mean, 27.2; range, 11.55−34.98; standard deviation, 6.093) vs the CT values of the *N* gene using qRT-PCR (mean, 35.24; range, 24.62−40.96; standard deviation, 4.608). According to the guidelines for COVID-19 laboratory detection techniques by China CDC, the samples with no CT value or CT ≥ 40 of qRT-PCR are considered as negative and the samples with CT values ranging from 37 to 40 are identified as undetermined (the gray zone) and repeated tests are recommended. Among 25 qRT-PCR-positive cases, 7 cases in the qRT-PCR gray zone (CT value of *ORF1ab* ranged from 37.48 to 39.07 and CT value of *N* ranged from 37.34 to 38.75) were out of the gray zone and thus were deemed to be positive by OSN-qRT-PCR, indicating that the positivity of these samples is confirmative. As shown in Table 1, the CT values of 14 cases using qRT-PCR were above 40; however, after amplification using OSN-qRT-PCR, these 14 samples showed clear positive results.

The CT values of *ORF1ab* between qRT-PCR and OSN-qRT-PCR had a strong correlation (r = 0.9135, 95% confidence interval between 0.8246 and 0.9584). $P < 0.0001$ (α = 0.05) indicated a significant linear correlation between the CT values of the two methods. The regression curve of the two groups had good goodness of fit with a regression equation $y = 1.147x - 20.44$ (R^2 = 0.7676). If x is 37 or 40, then y is 22.00 or 25.44. This means that the CT value of the samples with the gray zone will be increased to the range 22.00−25.44 after OSN-qRT-PCR testing, making the obscure results clear and easy to read. If x is 45, then y is 31.18. The CT value of 45 is the upper limit of qRT-PCR, and 31.18 is the highest CT value of OSN-qRT-PCR in theory. The CT values of *N* between qRT-PCR and OSN-qRT-PCR also had a significant linear correlation (r = 0.8761, 95% confidence interval between 0.7689 and 0.9354, $P < 0.0001$). Its regression equation was $y = 1.338x - 20.21$ (R^2 = 0.8345). The results are shown in Figure 3.

The CT values of both *ORF1ab* and *N* genes between qRT-PCR and OSN-qRT-PCR have a strong correlation, indicating that the presence of the external amplification step of the OSN-qRT-PCR assay does not affect the stability of the inner PCR and that the CT value still shows a similar regularity of qRT-PCR. Additionally, the regression curve between qRT-PCR and OSN-qRT-PCR has good goodness of fit, indicating that OSN-qRT-PCR can be used to quantify the RNA load of SARS-CoV-2.

Characterization of the Samples. A total of 181 samples (patients) were tested. Of these, 39 (21.5%) samples were positive by the OSN-qRT-PCR assay. Of the 39 patients with OSN-qRT-PCR positive results, the median age was 55 years

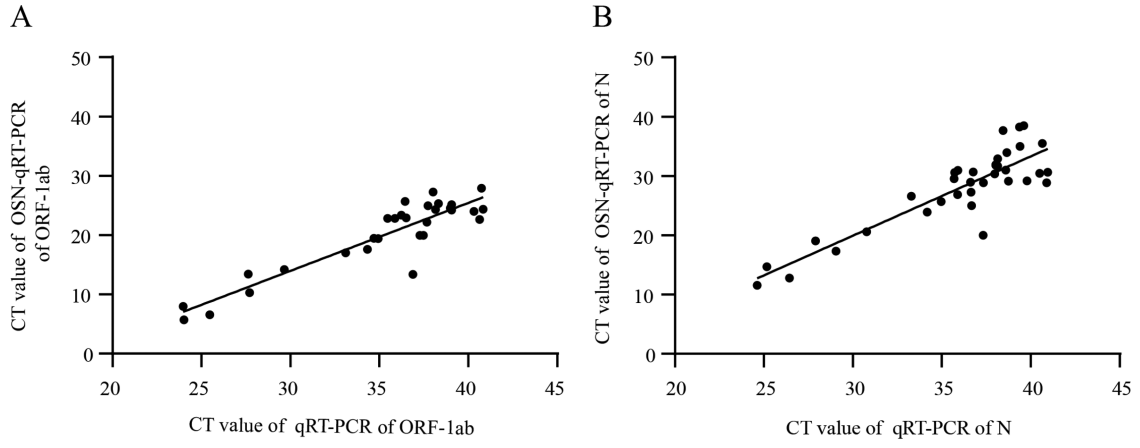

Figure 3. (A) CT values of *ORF1ab* between qRT-PCR and OSN-qRT-PCR analyzed by linear regression and correlation analysis. (B) CT values of *N* between qRT-PCR and OSN-qRT-PCR analyzed by linear regression and correlation analysis.

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(range, 27−83 years) and the proportion of men was 46.2%. In addition, the median time from illness onset to PCR testing was 9.85 days (range, 1−19 days). Among them, 1 case (2.6%) had exposure history to the spouse (confirmed patient), 1 case (2.6%) had exposure history to a COVID-19 suspected human, and 2 cases (5.1%) were medical workers. Many patients presented with signs and symptoms at the time of admission, including 28 (71.8%) with a fever, 17 (43.6%) with a cough, 5 (12.8%) with muscle ache, 5 (12.8%) with chest distress, 3 (7.7%) with shortness of breath, 3 (7.7%) with a poor spirit, 2 (5.1%) with sputum production, and 1 (2.6%) with a headache (Table 3). Fever and cough were the most prevalent clinical symptoms.[17,18]

Table 3. Baseline Characteristics of the Confirmed Patients by OSN-qRT-PCR Assay

parameter	confirmed patients (n = 39)
age (years)	
median	55
range	27−83
sex	
female	21 (53.8%)
male	18 (46.2%)
days from illness onset to PCR testing	
mean (SD)	9.85
range	1−19
exposure history	
spouse	1 (2.6%)
suspected human	1 (2.6%)
medical workers	2 (5.1%)
signs and symptoms	
fever	28 (71.8%)
cough	17 (43.6%)
muscle ache	5 (12.8%)
chest distress	5 (12.8%)
shortness of breath	3 (7.7%)
poor spirit	3 (7.7%)
sputum production	2 (5.1%)
headache	1 (2.6%)

Nevertheless, as the clinical information on the samples is limited, the correlation between disease progression and viral load has not been analyzed in this study. Moreover, since the sample type is limited and size of clinical samples is small, a more comprehensive evaluation of the OSN-qRT-PCR assay using bigger and more diverse samples is therefore needed later.

Applicable Scene. The distinctive features of high sensitivity, simple operation, and easy applicability make OSN-qRT-PCR assay an ideal tool to capture patients with low viral load and a fit to the current strategy in controlling the spread of COVID-19 in China. This assay might be suitable for the following scenarios. First, it is suitable for the detection of asymptomatic infected persons. Due to the existence of the detection window or inadequate sensitivity, the qRT-PCR method makes it difficult to identify all asymptomatic infections. Right now, all parts of the country have lifted the blockade and various industries have fully resumed work. The asymptomatic infected persons with a low viral load of SARS-CoV-2 are a major hidden danger, as the healthy people infected by the asymptomatic person will probably cause a new round of outbreak.[19] The monitoring data of close contact released by the National Health Commission clearly showed that some second-generation cases had exposure history to asymptomatic infected persons and that cluster outbreaks caused by asymptomatic infections were found in epidemiological investigations.[20] Second, it can accurately identify positive results and avoid repeated testing, which saves a lot of time, money, and material resources, particularly alleviating the difficulty and pressure of front-line inspectors in reporting the results. A study reported that the results of qRT-PCR for many patients were negative several times and then finally turned positive, which was considered to be related to the insensitivity of the detection method.[21] Third, it might be promisingly used for the testing of blood samples or urine samples, of which the positive rate and viral load are normally lower than those of sputum, throat swabs, and nasal swabs. Another advantage of collecting blood or urine samples is to ensure the sampling quality by reducing the sampling deviation and infection risk.

CONCLUSION

Collectively, the OSN-qRT-PCR assay has advantages of high sensitivity and easy applicability for the detection of SARS-CoV-2 in clinical samples. Moreover, the method is a promising tool to be potentially used for the detection of clinical samples with low viral load.

AUTHOR INFORMATION

Corresponding Authors

Yongzhong Jiang − *Hubei Center for Disease Control and Prevention, Wuhan 430000, China*; Email: 575652423@qq.com

Wenbo Xu − *National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China*; Email: wenbo_xu1@aliyun.com

Xuejun Ma − *National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China; Center for Biosafety Mega-Science, Chinese Academy of Sciences, Wuhan 430071, China*; orcid.org/0000-0001-5891-5260; Phone: +86-010-58900810; Email: maxj@ivdc.chinacdc.cn

Authors

Ji Wang − *National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China*

Kun Cai − *Hubei Center for Disease Control and Prevention, Wuhan 430000, China*

Ruiqing Zhang − *National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China; Hebei General Hospital, Shijiazhuang 050051, China*

Xiaozhou He − *National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China*

Xinxin Shen − *National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China*

Jun Liu − *National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China*

Junqiang Xu − *Hubei Center for Disease Control and Prevention, Wuhan 430000, China*

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Feng Qiu − *National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China*
Wenwen Lei − *National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China*
Jinrong Wang − *National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China; Hebei Medical University, Shijiazhuang 050031, China*
Xinna Li − *National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China*
Yuan Gao − *National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China; Hebei Medical University, Shijiazhuang 050031, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.analchem.0c01884

Author Contributions

[#]J.W., K.C., and R.Z. contributed equally to this article. X.M., W.X., and Y.J. designed the study; J.W., K.C., R.Z., X.H., J.L., J.X., F.Q., W.L., and X.M. performed the experiments; J.W., X.S., J.W., X.L., and Y.G. analyzed and interpreted the data; X.M., J.W., and R.Z. wrote the paper. All authors provided a critical review and approved the final manuscript.

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Notes

The authors declare no competing financial interest.

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