Sensitivity evaluation of 2019 novel coronavirus (SARS-CoV-2) RT-PCR detection kits and strategy to reduce false negative

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Research

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Abstract

Background: In absence of effective vaccines, infection prevention and control of SARS-CoV-2 through diagnostic testing and quarantine is critical. Early detection and differential diagnosis of respiratory infections increases the chances for successful control of COVID-19 disease. The nucleic acid RT-PCR test is regarded as the current standard for molecular diagnosis with high sensitivity. However, the highest specificity confirmation target ORF1ab gene is considered to be less sensitive than other targets in clinical application. In addition, a large amount of recent evidence indicates that the initial missed diagnosis of asymptomatic patients with SARS-CoV-2 and discharged patients with “re-examination positive” may be due to low viral load, and the ability of rapid mutation of SARS-CoV-2 also increases the rate of false negative results. Moreover, the current used mixed sample nucleic acid detection is helpful to seek out the early community transmission of SARS-CoV-2, but the detection kit needs ultra-high detection sensitivity.

Methods: From January to March 2020, 10 confirmed specimens of 2019-nCoV were recruited from three 2019-nCOV nucleic acid testing center. Five different amplification kits with three different primers and probes sources were selected. RT-PCR and continuous amplification of nasopharyngeal and oropharyngeal swabs and environmental specimens were performed to validate the sensitivity of the commercially available Nucleic acid test kits.

Results: The results showed that ORF1ab gene can still be reported as positive at 1:10 dilution and the N gene even at 1:40 dilution with kit-1 through the verification of multiple positive samples, While other kits have less sensitive. The results in the suspicious range of weakly positive nasopharyngeal and oropharyngeal swabs and environmental specimens could be reported as positive after another re-amplification.

Conclusions: Through evaluate the sensitivity of different nucleic acid detection kits, this study provide direct evidence for the selection of kits for mixed sample detection or make recommendations for the selection of validation kit, which is of great significance for the prevention and control of the current epidemic and the discharge criteria of low viral load patients.

Background

The coronavirus that caused the outbreak was identified in the case of viral pneumonia in Wuhan in 2019[1-3], and was named 2019-nCoV/SARS-CoV-2 by the World Health Organization (WHO)[2, 4, 5]. SARS-CoV-2 belongs to the coronavirus genus β and its genome is single-stranded, non-segmented positive-sense RNA[6], which is the seventh known coronavirus that can infect humans[1, 7]. Similar to other pathogenic RNA viruses, the genetic material RNA is the first marker to be detected. Nucleic acid detection or sequencing is currently used in conjunction with pulmonary CT for clinical diagnosis of COVID-19[8, 9]. As the course of the disease progresses, antibodies IgM and IgG will be produce by the

human immune system. Although, antibody tests play a major role in monitoring the response to future immunization strategies and demonstrating previous exposure/immunity, the antibody positive rate often lags behind the nucleic acid detection[10-12], and cross-reactions existed in SARS-CoV antigen with autoantibodies[13].

Theoretically, fluorescence quantitative RT-PCR detection is widely used as the molecular diagnosis standard for SARS-CoV-2[14, 15]. Lately, the analysis showed that the pattern of viral load change in COVID-19 patients was similar to that in patients with influenza, but different from that in SARS and MERS (whose viral load peaked about 10 days after the onset of symptoms)[16-19].In COVID-19 patients, RT-PCR detection could be positive as early as one day before the onset of symptoms, while most COVID-19 patients cannot be detected before premorbid because of the low copy number of the virus[7, 17, 20]. In addition, some discharged patients appearing “re-examination positive” situation is also because of the persistence of a small number of viruses. Unfortunately, the positive rate of RT-PCR detection of SARS-CoV-2 is only 30%-50% at present[21, 22] due to improper sample collection, storage, and error detection[23]. Furthermore, once the target gene mutated or deleted, the test results will be invalid[24, 25].

RT-PCR nucleic acid detection not only has a high false negative rate[26], but also has a low sensitivity[27]. Moreover, the approved nucleic acid detection kits of the SARS-CoV-2 genome are based on the most conserved and specific open reading frame 1ab (ORF1ab), Envelope protein (E) and nucleocapsid protein (N)[6, 28, 29]. Athough ORF1ab is the highest specificity confirmation target gene, but is considered to be less sensitive than other targets in clinical application[30]. Currently, mixed sample testing has been widely used in large-scale population testing. However, some articles think that although this method can improve the detection efficiency, it may miss those individuals with low viral load[31]. In addition, none of the approved nucleic acid test kits mentioned whether they could be used for mixed sample detection. so does the pattern of ORF1ab positive reports cause missed tests? Is it feasible to report based on positive N or E genes? Clinically, it is recommended that samples with suspicious results or single channel positive results should be re-examined with another manufacturer's kit or method. However, what is the basis for choosing the validation kit? This is a problem that needs to be solved.

Methods

Patients

10 confirmed cases of COVID-2019 patients (2 female, 8 male, 5-50 years old) were collected from January to February 2020, in Jinan Central Hospital Affiliated to Shandong University and Jinan Infectious Disease Hospital, Shandong University, which were diagnosed by clinical symptoms, lung CT and nucleic acid test. And 100 suspected cases were collected in the first institution listed above, which had symptoms of fever, dry cough and pneumonia image. This research was approved by the Ethics Commission of Jinan Central Hospital and with informed consent of the patient.

Specimen collection

Nasopharyngeal and oropharyngeal swab specimens were collected with synthetic fiber swabs under the guideline of the Chinese Centre for Disease Control and Prevention (China's CDC) (http://www.chinacdc.cn/jkzt/crb/zl/szkb_11803/jszl_11815/202003/t20200309_214241.html). And the two swabs from nasopharyngeal and oropharyngeal were inserted into one sterile tube containing 3 ml of virus preservation solution. In addition, environmental specimens were collected from surface in direct contact with the patient, such as inner side of the mask, phone, doorknob, bedside, and etc. Each surface was wiped with one synthetic fiber swab, and then inserted the swab into a sterile tube listed above.

Virus RNA extractions

The virus RNA was extracted using magnetic bead method strictly according to the instructions of Nucleic acid extraction kit (Shanghai Zhijiang Biotechnology Co., Ltd, Shanghai, China). The RNA samples were diluted with RNA extract from nasopharyngeal and oropharyngeal swab of negative patients for detecting by RT-PCR.

Laboratory quality-control

Acceptable specimens are respiratory and serum specimens, the former including: nasopharyngeal and oropharyngeal swabs, bronchoalveolar lavage fluid, tracheal aspirates and sputum. Cotton swab heads are not allowed for swab specimens. Specimens should not be stored for more than 72 hours at 4℃. Positive control and negative control should be tested at the same time as all samples. The fluorescence amplification curve of negative control should not exceed the threshold. The CT value of all targets in the positive control should be within the expected range. The detection kit should contain the internal target gene, and the amplification curve should exceed the threshold line.

Real-time RT-PCR

Five different amplification kits were selected with three different primers and probes sources, among which one was from China's CDC, two was from the World Health Organization (WHO) [6], and the other two were self-designed by the kit manufacturer. Information for the five amplification kits was shown in table 1. Each kit contained 25 μl of reaction system including 5μl of RNA template. The amplification was operated separately according to the instructions of kits. The amplification result was detected by ABI7500 Real-time PCR system (Applied Biosystems, USA).

Continuous amplification

The RT-PCR products were re-amplified for another 40 cycles under the same amplification conditions. 53 nucleic acid samples of other respiratory pathogens with known concentrations were used for specificity test.

Statistical methods

SPSS18.0 software was used for statistical analysis. The Student *t* test was used to evaluate the differences between *Ct* values.

Results

Sensitivity evaluation of SARS-CoV-2 detection kits

To verify the sensitivity of the kits, we took nasopharyngeal and oropharyngeal swab samples from a confirmed positive patient. After RNA extraction, the RNA was diluted according to the following proportion gradient: 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320. Then, RT-PCR results showed that the dilution titer of ORF1ab was the highest in the kit-1 (Fig. 1A), indicating that the kit-1 was the most sensitive to SARS-CoV-2, followed by the kit-2. In addition, the CT value of the amplification curve was found to be positively correlated with the dilution titer (Fig. 1B). The Ct values of ORF1ab gene and N gene in kit-1 were still within the reportable interval at 1:20 and 1:160 dilution respectively, while reached the detection suspicious region in kit-2 at 1:5 and 1:40 dilution titer.

Then, if the N gene or E gene is positive when ORF1ab gene is negative, how to judge the result and how to select the validation kit? For example, three cases were presented in in Figure.1A. Our solution is as follows (Figure. 1C): 1. Both ORF1ab and N genes can be converted to positive after verification with kit 1; 2. When N gene is in a suspicious region with kit 2, it can be converted to positive after verification with kit 1; 3. When N gene was negative with kit 2, it can be converted to positive after verification with kit 1.

For the sake of further verify the sensitivity, another 9 positive samples were enrolled. The positive RNA extract was first quantified by digital PCR and then diluted to the same initial concentration. The results showed that ORF1ab gene can still be reported as positive at 1:10 dilution and the N gene even at 1:40 dilution (Figure. 1D) with kit-1, while they exceeded the detection line at 1:5 and 1:20 respectively with kit-2. Hence, we have reasons to believe that kit-1 has the highest sensitivity through the verification of multiple positive samples.

Clinical validation and Application

Besides choosing a more sensitive kit for validation, is there an easier method to increase the positive detection rate? First, the RT-PCR products of the above diluted samples in the suspicious range were amplified for another 40 cycles, and found that for the samples with dilution gradients of 1:10 and 1:20, the ORF1ab and N genes with large original amplified Ct values were expanded to the positive reportable

region, while other dilution gradients only with N or E genes were significantly amplified (figure. 2A). Moreover, 100 patients with clinical fever and dry cough who were suspected to be infected with the SARS-CoV-2 were enrolled for RT-PCR, and two positive cases and two suspicious cases were found (Table 2). Then, the suspicious cases were re-amplified to be positive by continuous amplification (Figure. 2B and Supplemental Fig. 1).

Meanwhile, the environmental samples from 3 COVID-19 patients were conducted nucleic acid testing and found that the sample inside the mask of one patient was weakly positive, which could be reported as positive after another re-amplification (Fig. 2C). Through analysis, we found that each target gene could reach the amplification plateau by adding another 30 cycles. In addition, we tried to add the initial RT-PCR amplification products of positive patients into a new amplification reaction system, and found that the results were not reliable (Data not shown).

Strategies to reduce false negatives of SARS-CoV-2

Above all, we suggest that the laboratory must evaluate the sensitivity of detection kits first and the sensitivity of the validation kit must be higher than that of the test kit. For those mixed samples, the ultra-high sensitivity kit should be preferred. Moreover, for these specimens with the suspicious interval region or single channel positive results, the continuous amplification can be used to increase the detection rate of low viral load specimens and greatly reduce the false negative rate of SARS-CoV-2.

Discussion

As of 3th July 2020, statistical data showed that the global number of confirmed cases of COVID-19 had surpassed 10900,000 with more than 520,000 deaths. With an increasing number of potential cases emerge, the SARS-CoV-2 poses a major threat to global public health[32]. A greater number of diagnostic tools have been developed such as virus isolation, PCR-based assays, IHC, and antibody assays, which are currently in place across different diagnostic laboratories around the world[33–37]. Although, RT-PCR is challenged by the "false negative" results[38], in view of the past major epidemic outbreaks[39], RT-PCR is still the preferred detection method. Although the detection rate of viral nucleic acid is closely related to the course of viral infection, which is not completely clear and the optimal sampling time is uncertain, so it is likely that the period of high viral load will be missed, resulting in false negatives[40]. Therefore, how to ensure the accuracy of nucleic acid test results is the currently facing problem. In this study, we aimed to evaluate the sensitivity of different RT-PCR kits for COVID-19 diagnosis.

An increasing number of articles showed that the SARS-CoV-2 is undergoing rapid mutation[41, 42], and multiple mutations were found over its entire genomes[43] (Table1). Fortunately, through gene comparison on BLAST, the primer or probe sequences published by CDC and WHO were not in these mutation regions. Moreover, study found that a deletion of 382 nucleotides in the ORF8 gene can enhances the transcription of the downstream N gene[25] which may increase the false negative

detection rate of SARS-CoV-2. Thus, attentions should be paid to the abnormally amplified N gene in clinical detection.

As with all viral nucleic acid testing projects, the RT-PCR results of SARS-CoV-2 are affected by various factors including before, during and after detection, thus sufficient laboratory quality-control measures should be taken. In addition, extending amplification cycles would naturally increase sensitivity, but it always comes with reduced specificity. Nucleic acid samples of other respiratory pathogens with known concentrations were used for continuous amplification. The cross reaction results showed that there was no cross-reaction with other pathogens, and its specificity did not decrease (Supplemental table). Moreover, the continuous amplification and other detection methods of SARS-CoV do exhibit false positive results[44, 45], we recommend to use it only when the amplification curve of target gene is in the specious region.

Conclusions

The emergence of mixed sample detection challenges the sensitivity of nucleic acid detection kits. As a perspective method to solve the dilemma of SARS-CoV-2 nucleic acid screening, mixed sample nucleic acid detection should be paid more attention.We believe that mixed samples detection is only applicable for low-risk population, and the use of high-sensitivity kits is necessary.More importantly, we also believe that antibody test and nucleic acid test should complement each other to improve the diagnosis effect, especially to screen asymptomatic patients better, so as to reduce the detection "false negative" phenomenon of "false recovered patients" or premorbid patients with low virus latency.

Declarations

Ethics approval and consent to participate

This study was undertaken with the approval of the Jinan Central Hospital Affiliated to Shandong University Ethics Service Committee. Informed consent was obtained from all the participants prior to sampling.

Availability of data and material

The data sets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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Authors' contributions

YW conceived and designed the study. YZ was the major contributor in drafting the manuscript. YZ and QZ performed the experiments. LW and HZ collected the samples. FP, HL, MJ, WY and QW extracted the RNA and made the clinical diagnoses. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, tables 1 and 2 are only available as a download in the supplemental files section.

Figures

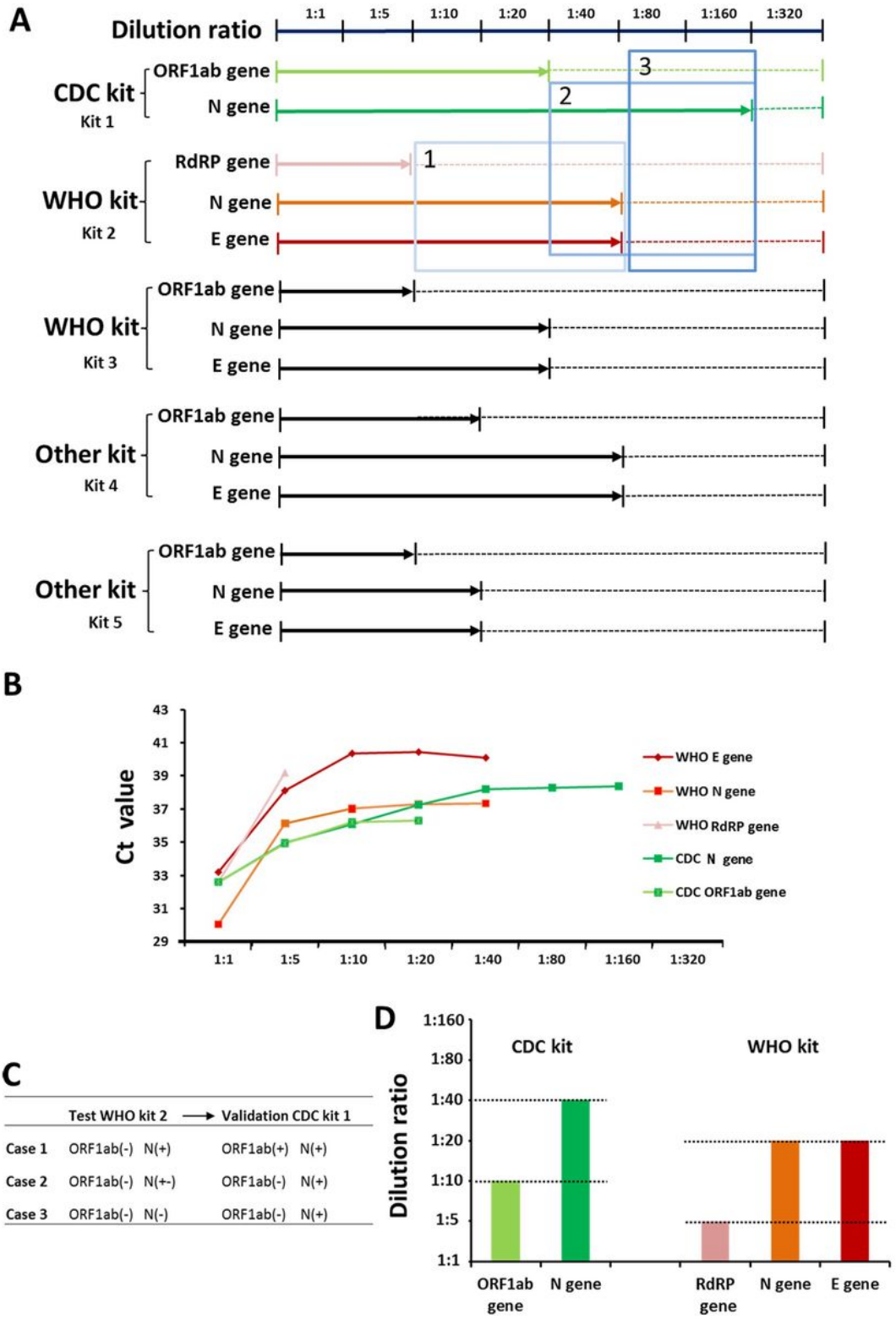

Figure 1

Sensitivity evaluation of SARS-CoV-2 detection kits. (A) Gradient dilution experiments showed that different kits have different sensitivity. (B) Ct value of different target genes were positively correlated

with the dilution concentration. (C) Selection of test kit and validation kit. (D) CDC kit has the highest sensitivity through the verification of multiple positive samples.

Figure 2

Strategies to reduce false negatives of SARS-CoV-2. (A) Continuous amplification of PCR products for gradient dilution samples. (B) Continuous amplification of PCR products for the nasopharyngeal and oropharyngeal swab specimens of clinical fever patients. (C) Continuous amplification of PCR products for the environmental samples of 3 positive patients. The specimen with a cycle threshold value of target genes above the baseline is interpreted as positive for

Supplementary Files

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