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Comparison of analytical sensitivity of SARS-CoV-2 molecular detection kits

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A B S T R A C T
Objectives: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has had a significant impact on global public health systems, making nucleic acid detection an important tool in epidemic prevention and control. Detection kits based on real-time reverse transcriptase PCR (rRT-PCR) have been used widely in clinics, but their analytical sensitivity (limit of detection, LOD) remains controversial. Moreover, there is limited research evaluating the analytical sensitivity of other molecular detection kits.

Methods: In this study, armored ribonucleic acid reference materials developed in-house were used to evaluate the analytical sensitivity of SARS-CoV-2 detection kits approved by the National Medical Products Administration. These were based on rRT-PCR and other molecular detection assays.

Results: The percentage retesting required with rRT-PCR kits was as follows: 0%, 7.69%, 15.38%, and 23.08% for samples with concentrations ranging from 50 000 to 781 copies/ml. In total, 93% of rRT-PCR kits had a LOD < 1000 copies/ml. Only one kit had an LOD > 1000 copies/ml. The LOD of other molecular detection kits ranged from 68 to 2264 copies/ml.

Conclusions: The study findings can help pharmaceutical companies optimize and improve detection kits, guide laboratories in selecting kits, and assist medical workers in their daily work. © 2021 The Authors. Published by Elsevier Ltd on behalf of International Society for Infectious Diseases.

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Introduction

The outbreak of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was first reported in December 2019. To date, more than 207 million people have been infected with SARS-CoV-2 and over 4 million people have died from COVID-19 globally (WHO, 2021). Asymptomatic infection in individuals has accelerated the transmission of the virus, making containment and mitigation difficult (Han et al., 2020). To prevent the situation from worsening, governments need to implement stronger prevention and control strategies to test and track patients, suspected cases, and asymptomatic cases.

Currently, the diagnostic methods for COVID-19 include not only traditional molecular tests, serology tests, and computed tomography, but also field-effect transistor-based sensing and plasmonic sensing, as well as the use of high-throughput sensors (Taleghani N et al., 2021; Seo G et al., 2020; Ahmadivand A et al., 2021). Among these, molecular detection is recommended as the gold standard for SARS-CoV-2 detection (Green et al., 2020). Real-time reverse transcriptase PCR (rRT-PCR) is a robust technology with a high specificity and sensitivity (Yuce et al., 2021). However, false-negative results from rRT-PCR are associated with substantial risks and public health implications. Many factors can lead to false-negative results, especially low viral loads (Yu et al., 2020; Kucirka et al., 2020; Wikramaratna et al., 2020). Accurate detection can help effectively identify infected individuals and limit the spread of the virus. Therefore, it is necessary to improve the analytical sensitivity to ensure the accuracy and reliability of the test results. The limit of detection (LOD) is the lowest concentration of SARS-CoV-2 RNA at which the positivity rate of the detection kit is ≥95%, known as the analytical sensitivity. It is an important performance parameter for evaluating a detection kit. At the beginning of the pandemic, only a few kits were evaluated for detection performance (Alcoa-Florez et al., 2020; Wang B et al., 2020; Wang X et al., 2020; Yan et al., 2021). Currently, the number of kits ap-

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proved by the National Medical Products Administration (NMPA) has increased significantly, and previously approved kits have been optimized. Notably, some of these kits are CE-certified and US Food and Drug Administration-approved for use in various countries. In addition, the evaluation of detection kits has mainly focused on the rRT-PCR method, with little focus on other molecular detection assays.

Therefore, the aim of this study was to evaluate the analytical sensitivity of kits currently approved by the NMPA, including those using rRT-PCR and those using other molecular detection techniques, to assess whether each SARS-CoV-2 detection kit meets the LOD claimed by their manufacturer and to provide a theoretical basis for laboratories in selecting kits.

**Methods**

**Preparation of the evaluation sample**

As shown in Figure 1a, SARS-CoV-2 ORF1a, RdRP, ORF1b/S, and N/E virus-like particles (VLPs) were prepared in the laboratory and used as evaluation samples for this study. Four gene fragment sequences were synthesized (Geneary, Shanghai, China). These recombinant plasmids were transformed into BL21 (DE3) cells, and protein expression was induced using isopropyl-

β-d-thiogalactopyranoside. The VLPs were purified and digested with DNase and RNase to remove free nucleic acids from the surface. Specific primers and probes were used to identify the four target sequences contained in the VLPs by quantitative PCR (qPCR) and were quantified by droplet digital PCR (ddPCR) (Supplementary Material Table S1). According to the results, four VLPs were mixed in equal proportions at a concentration of 1 × 10^8 copies/ml. The four-fold serially diluted mixture was used as the evaluation sample at concentrations of 50 000, 12 500, 3125, 781, 195, 49, and 12.25 copies/ml. Each concentration group included 21 samples. For each detection kit, there were two negative and 147 positive samples. The procedure was performed according to the manufacturer’s instructions. RNA was obtained by extraction and purification of nucleic acid, or lysis release. The viral RNA extraction kit (Qiagen, Shanghai, China) was used for RNA extraction and purification in the rRT-PCR-based kits, except for Da an 2, for which the lysis method for RNA preparation was used. Results were interpreted according to the manufacturer’s instructions (Supplementary Material Table S2). In cases in which false positivity was suspected, the sample was retested. The results of the retest were interpreted according to the interpretation criteria of the retest result.

**Preparation of the detection kits**

The LODs of the SARS-CoV-2 detection kits approved by the NMPA were evaluated in this study. The 13 commercial detection kits for rRT-PCR were as follows: Sansure, Da an (Da an 1 and Da an 2), BioGerm, Liferiver, Maccura, EasyDiagnosis, Bioperfect, Applied Biological, Fosun Long March, Kinghawk, GeneoDx, and BGI. The following five kits based on other molecular detection assays were also evaluated in this study: CapitalBio, Rendu, Zhongzhi 1, Zhongzhi 2, and Anbio. Evaluation of the 13 kits using the rRT-PCR method was performed in our laboratory. Biopharmaceutical companies were entrusted to complete the evaluation of the other five detection kits.

Da an has two rRT-PCR kits approved by the NMPA, namely Da an 1 and Da an 2. Although the detection principles of these two kits are the same, the RNA preparation method, thermal cycling conditions, and cycling number differ. The method used by Anbio is a hybrid capture immunofluorescence assay. Zhongzhi 1 uses a dual amplification assay method, whereas Zhongzhi 2 uses a ribonucleic acid (RNA) isothermal amplification gold probe chromatography assay. The method used by Rendu is an RNA capture probe assay. CapitalBio uses an integrated isothermal amplification chip assay.

**Evaluation of the LOD of the rRT-PCR-based commercial kits**

As shown in Figure 1b, the rRT-PCR evaluation samples were grouped according to seven concentrations: 50 000, 12 500, 3125, 781, 195, 49, and 12.25 copies/ml. Each concentration group included 21 samples. For each detection kit, there were two negative and 147 positive samples. The procedure was performed according to the manufacturer’s instructions. RNA was obtained by extraction and purification of nucleic acid, or lysis release. The viral RNA extraction kit (Qiagen, Shanghai, China) was used for RNA extraction and purification in the rRT-PCR-based kits, except for Da an 2, for which the lysis method for RNA preparation was used. Results were interpreted according to the manufacturer’s instructions (Supplementary Material Table S2). In cases in which false positivity was suspected, the sample was retested. The results of the retest were interpreted according to the interpretation criteria of the retest result.

**Evaluation of the LOD of other commercial kits**

As shown in Figure 1b, five detection kits were tested by biopharmaceutical companies. All companies were provided with evaluation samples with concentrations of 50 000, 12 500, 3125, 781, 195, and 49 copies/ml. Each concentration group included 21 samples. There were three negative and 126 positive samples. The order of all samples was disrupted before handing them to the biopharmaceutical company, and the samples were transported through cold-chain transportation to maintain a low temperature environment. The evaluation samples were tested according to the manufacturer’s instructions. All companies were required to report results within a week.

**Statistical analysis**

As shown in Figure 1c, probit regression analysis was used to evaluate the LOD of the test results in MedCalc Statistical Software version 19.6.1 (MedCalc Software Ltd, Ostend, Belgium). The Pearson Chi-square test was used to evaluate the positivity rate of target genes in IBM SPSS Statistics version 19.0 (IBM Corp., Armonk, NY, USA).

**Results**

**Characteristics of SARS-CoV-2 VLPs**

Four VLPs were prepared, and specific probe primers for ORF1a, RdRP, ORF1b/S, and N/E were used to detect VLPs containing the target sequence (Supplementary Material Table S1). The four VLPs were quantified by droplet digital PCR using specific probe primers. The results showed ORF1a, RdRP, ORF1b/S, and N/E VLPs at concentrations of 5.44 × 10^11, 1.273 × 10^12, 4.48 × 10^11, and 1.9 × 10^12 copies/ml, respectively (Supplementary Material Table S3). The four VLPs were diluted and mixed to form a high-concentration sample with a concentration of 1 × 10^8 copies/ml. Three commercial kits could detect ORF1a, RdRP, ORF1b/S, and N/E fragments were used to confirm that all target sequences were present in the mixture (Supplementary Material Tables S4–S6).

**Rate of retest calculation**

Serially diluted samples were detected using commercial detection kits, and the results were presented as positive or negative according to the manufacturer’s instructions (Table 1). As described in some of the instructions, samples that were suspected to be positive in the first test were retested. The percentage of rRT-PCR kits that required retesting was 0% (0/13), 7.69% (1/13), 15.38% (2/13), and 23.08% (3/13) for samples with concentrations ranging from 50 000 to 781 copies/ml, respectively. With the decrease in sample concentration, the number of kits that required retesting increased. Therefore, the percentage of rRT-PCR kits retested was
Figure 1. Flow chart of the analytical sensitivity evaluation of the SARS-CoV-2 detection kits. (a) Preparation of the evaluation sample. (b) Evaluation of the LOD of the detection kits. (c) Statistical analysis.

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; LOD, limit of detection; PEG, polyethylene glycol; VLP, virus-like particles; ddPCR, droplet digital PCR; qPCR, quantitative PCR.

69.23% (9/13), 92.31% (12/13), and 61.54% (8/13) for samples with concentrations from 195 to 12.25 copies/ml, respectively (Table 2, Figure 2a).

The retest rate was calculated as follows: number of retest/number of replicates (retest rate, %) (Table 2). The retest rate of the Da an 2 detection kit was the highest. The retest rates were 14.29% (3/21), 57.14% (12/21), 71.43% (15/21), 57.14% (12/21), 23.81% (5/21), and 9.52% (2/21) for the samples of concentrations 12, 500, 3125, 781, 195, 49, and 12.25 copies/ml, respectively. The Maccura kit had the second highest retest rate, which were as follows: 23.81% (5/21), 14.29% (3/21), and 71.43% (15/21) for concentrations of 3125, 781, and 195 copies/ml, respectively. For samples
with low concentrations, the retest rates were zero because all test results were considered negative. For Sansure and kits based on other molecular detection assays, retesting of samples of each concentration was not required; therefore, the retest rates of these kits were not calculated (Figure 2a). Evaluation of the LOD of target genes

All kits were designed to simultaneously detect different target genes. Hence, the positivity rate for each gene was calculated. The positivity rate was calculated as follows: number of
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Figure 3. Estimated limit of detection (LOD) of the molecular detection assay kits using probit regression analysis. (a)–(m) Analysis graphs of probit regression for each of the 13 commercial detection kits investigated.

The positivity rate of the ORF gene in the Da an 2 kit was much higher than that of the N gene (10/21 vs 19/21, Pearson Chi-square test, \( P = 0.003 \)), which affected the positivity rate of the 781 copies/ml sample. In contrast, the difference in the positivity rates of the 195 copies/ml samples showed that the N and E genes were more sensitive than the ORF gene (17/21 vs 21/21 vs 8/21, \( P < 0.001 \)) in the Maccura kit. This phenomenon was more obvious in the low-concentration samples. However, Da an 1 showed similar positivity rates for the N and ORF genes (20/21 vs 20/21).

Evaluation of the LOD of rRT-PCR kits

Probit regression analysis of the positivity rate of 147 samples was performed to obtain the LOD of the rRT-PCR kit (Table 3). The LODs of Sansure, Liferiver, Fosun Long March, and Da an 1 were 48 copies/ml (95% confidence interval (CI) 32–131 copies/ml), 113 copies/ml (95% CI 69–321 copies/ml), 176 copies/ml (95% CI 115–432 copies/ml), and 185 copies/ml (95% CI 115–469 copies/ml), respectively. The 95% hit rate of the Applied Biological, BioGerm, Kinghawk, BGI, Bioperfect, and Easy-Diagnosis kits were 311 copies/ml (95% CI 195–775 copies/ml), 331 copies/ml (95% CI 229–725 copies/ml), 392 copies/ml (95% CI 239–999 copies/ml), 439 copies/ml (95% CI 235–1385 copies/ml), 480 copies/ml (95% CI 275–1320 copies/ml), and 711 copies/ml (95% CI 420–1853 copies/ml), respectively. The LODs of Maccura, GeneoDx, and Da an 2 were 724 copies/ml (95% CI 478–1776 copies/ml), 827 copies/ml (95% CI 432–28 953 copies/ml), and 1217 copies/ml (95% CI 756–3039 copies/ml), respectively. The results of the probit regression analysis are shown in Figure 2c and Figure 3.
Evaluation of the LOD of kits using other detection methods

The LODs of kits using other detection methods were obtained to analyze the feedback from the biopharmaceutical companies. Similarly, probit regression analysis was performed on the positivity rate of 126 samples to obtain the LODs of the other detection assay kits (Table 3). As shown in Figure 4, the LOD of Zhongzi 2 was 761 copies/ml (95% CI 457–2056 copies/ml); that of Anbio was 803 copies/ml (95% CI 365–5930 copies/ml); those of Zhongzi 1 and CapitalBio were 820 copies/ml (95% CI 477–2365 copies/ml) and 2264 copies/ml (95% CI 1204–7041 copies/ml), respectively; and that of Rendu was 68 copies/ml. Notably, the Rendu kit reported 12 samples as positive, with a concentration of 49 copies/ml. Other positive samples with other concentrations were also completely detected. Therefore, the CI was not obtained after the probit regression analysis (Figure 4).

Discussion

During the process of RNA preparation, the volumes of the sample and elution buffer were different (Table 1). They are summarized as follows: (1) 140 μl of sample for extraction and 60 μl of buffer for elution; (2) the volume of the sample is 200 μl; (3) the elution volume is 80 μl. This difference could affect the RNA concentration. For instance, RNA obtained from (2) was two-fold more concentrated than that from (3). Similarly, the volumes of the reaction system and RNA template were also different. The discrepancy in the amount of template may affect the detection performance of a kit (Wang B et al., 2020). The RNA templates of the kits tested were 2/5, 1/5, 1/2, and 1/3 of the total reaction system. The largest difference between them was up to 2.5-fold. Remarkably, the LOD of Da an 2 was much worse than that of Da an 1, and the method of RNA preparation may be the reason for this huge
Table 1
Characteristics of National Medical Products Administration approved SARS-CoV-2 rRT-PCR detection kits.

| Study kits | Specimens | Fluorescence channel | Target gene(s) | LOD (copies/ml) | Input Vol. (μl) | Elution Vol. (μl) | RNA Vol. (μl) | Total reaction Vol. (μl) | Cycling number |
|------------|-----------|----------------------|----------------|----------------|----------------|----------------|----------------|-------------------------|----------------|
| Sansure    | OP, BALF  | FAM, ROX             | ORF1ab, N      | 200            | 140            | 60             | 20             | 50                      | 45             |
| Da an 1    | OP, sputum| FAM, VIC             | ORF1ab, N      | 500            | 200            | 60             | 5              | 25                      | 45             |
| BioGerm    | NP, OP,  | FAM, HEX/VIC         | ORF1ab, N      | 1000           | 140            | 60             | 5              | 25                      | 40             |
| Lifesaver  | NP, sputum| FAM, HEX/VIC, TEXAS  | RdRP, N, E     | 200            | 140            | 60             | 5              | 25                      | 45             |
| Maccura    | EasyDiagnosis | OP, sputum | FAM, ROX, Cy5| ORF1ab, N, E  | 1000           | 140            | 80             | 20                       | 40             |
| Bioperfect | NP, OP, | FAM, VIC             | ORF1ab, N      | 350            | 140            | 60             | 5              | 25                      | 45             |
| Applied Biological | OP, sputum | FAM, VIC | ORF1ab, N | 200            | 200            | 60             | 5              | 25                      | 45             |
| Fosun Long March | OP, sputum | FAM, ROX | ORF1ab, N, E | 1000           | 140            | 80             | 20             | 40                      | 40             |
| Kinghawk   | NP, sputum| FAM, VIC             | ORF1ab, N      | 350            | 140            | 60             | 5              | 25                      | 45             |
| GeneoDX    | NJ, OP,  | FAM, VIC             | ORF1ab, N      | 350            | 140            | 60             | 5              | 25                      | 45             |
| BGI        | BALF      | FAM, VIC             | ORF1ab, N      | 100            | 140            | 60             | 10             | 30                      | 40             |

BALF, bronchoalveolar lavage fluid; LOD, limit of detection; OP, nasal pharyngeal; NP, oral pharyngeal; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; Vol., volume.

Table 2
Summary of the retest rate of SARS-CoV-2 rRT-PCR detection kits.

| Detection kits | Number of retest/number of replicates (retest rate, % at diluted concentrations (copies/ml) |
|----------------|-----------------------------------------------------------------------------------------|
| Da an 1        | 0/21 (0)                                                                               |
| BioGerm        | 0/21 (0)                                                                               |
| Lifesaver      | 0/21 (0)                                                                               |
| Maccura        | 0/21 (0)                                                                               |
| EasyDiagnosis  | 0/21 (0)                                                                               |
| Bioperfect     | 0/21 (0)                                                                               |
| Applied Biological | 0/21 (0)                              |
| Fosun Long March | 0/21 (0)                              |
| Kinghawk       | 0/21 (0)                                                                               |
| GeneoDX        | 0/21 (0)                                                                               |
| Da an 2        | 0/21 (0)                                                                               |
| BGI            | 0/21 (0)                                                                               |

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Table 3
Summary of results for LOD evaluation.

| Detection kits | Number of positive/number of replicates (positivity rate, %) at diluted concentrations (copies/ml) |
|----------------|--------------------------------------------------------------------------------------------------|
| Sansure        | 21/21 (100)                                                                                |
| Da an 1        | 21/21 (100)                                                                                |
| BioGerm        | 21/21 (100)                                                                                |
| Lifesaver      | 21/21 (100)                                                                                |
| Maccura        | 21/21 (100)                                                                                |
| EasyDiagnosis  | 21/21 (100)                                                                                |
| Bioperfect     | 21/21 (100)                                                                                |
| Applied Biological | 21/21 (100)                              |
| Fosun Long March | 21/21 (100)                              |
| Kinghawk       | 21/21 (100)                                                                                |
| GeneoDX        | 21/21 (100)                                                                                |
| Da an 2        | 21/21 (100)                                                                                |
| BGI            | 21/21 (100)                                                                                |

LOD, limit of detection.
to reduce the discrepancy in the sensitivity of different target genes and reduce the retest rate. The detection region of the target gene should be carefully selected to avoid competition between primers during the test. The kit instructions should clearly indicate the RNA extraction method and the corresponding kit. If the lysis method is used for RNA preparation, it should be indicated by the manufacturer of the sample preservation solution. All recommended kits, reaction reagents, and procedures in the instruction manual should be strictly verified. The kit should be fully verified with accurate quantitative international standard reference materials to determine the LOD of the kit. In the application process, weak positive quality control materials should be used to regularly evaluate the detection performance of the kit.

Although we tried to perfect our research, there are limitations to this study. Compared with VLPs, real samples may contain more interference or inhibitory substances, and clinical samples should be used for sensitivity and specificity evaluations.

In conclusion, although the measured LODs of several kits were inferior to those claimed, the analytical sensitivities of the kits approved by the NMPA are able to meet the needs of COVID-19 diagnosis in clinics. Next, biopharmaceutical companies should focus on the pitfalls of their detection kits and improve the detection performance of the kits. Laboratories need to emphasize the importance of quality control in daily work. All of these measures are essential for ensuring reliable test results.

Author contributions

JML and RZ conceived and designed the study. JY and RLZ completed the experiment. JY prepared the first version of the manuscript. JML, RZ, and YXH provided valuable comments on the manuscript. JY revised and finalized the manuscript. All authors read and approved the final version of the manuscript.

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Ethical approval and consent to participate

Not applicable.

Conflict of interest

None declared.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijijd.2021.08.043.

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