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An external quality assessment for the molecular testing of the SARS-CoV-2 virus genome in Zhejiang Province, China

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ABSTRACT

The COVID-19 pandemic has necessitated the rapid expansion of laboratories that conduct SARS-CoV-2 tests. A provincial external quality assessment (EQA) scheme on SARS-CoV-2 tests was organized by Zhejiang Provincial CDC to assess the accuracy of the tests in individual CDC municipal and county laboratories in Zhejiang Province, China. Three positive samples in high, medium, and low concentrations, respectively, were prepared using the serial dilutions from the culture with the viral titer concentration of $1\times10^{6.3}$ TCID50/mL, and one negative sample were included. A total of 93 laboratories participated, contributing results from 36 distinct combinations of nucleic acid extraction methods and PCR reagents. There was 100% concordance among all laboratories for all EQA samples, and no false-positive or false-negative results were observed. The EQA survey provides confidence in the identification of infected individuals or asymptomatic populations and assurance for clinical and public health decision-making based on test results.

1. Introduction

Since the first outbreak of Coronavirus Disease 19 (COVID-19) in Wuhan, China (Zhu et al., 2020), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused about 120 million cases and over 2,656,822 deaths worldwide (n.d.).

According to the guidelines for COVID-19 control and prevention in China, early detection, immediate isolation, case investigation, contact tracing, and early treatment of the infected patients are the key measures for preventing the spread of COVID-19. To build a network of testing laboratories for SARS-CoV-2 throughout the country, the National Health Commission of the People's Republic of China issued the technical guidelines which required the laboratories to meet the conditions (including biosafety level [BSL], instruments, qualified personnel, etc.) for large-scale SARS-CoV-2 nucleic acid testing, including secondary hospitals, Centers for Disease Control and Prevention (CDCs), and medical testing laboratories (third-party testing agencies) in each county. However, the CDC's laboratories are one of the most significant units for SARS-COV-2 nucleic acid testing, and they're also the final result reviewers for positive samples related to the COVID-19 outbreaks all over the country.

A major bottleneck in managing the COVID-19 pandemic in China and the rest of the world is diagnostic testing, which is performed primarily on symptomatic patients because of limited laboratory capabilities and limited access to nucleic acid extraction and real-time reverse transcription polymerase chain reaction (RT-PCR) reagents. Recent studies have showed that many asymptomatic infections are also sources of infection. Therefore, there is an urgent need to increase capabilities to screen asymptomatic and presymptomatic populations. The large-scale and rapid screening of nucleic acid of SARS-CoV-2 has become an important means to find infected patients and asymptomatic people. Recently, this method has been performed in Suihua (Heilongjiang Province), Dalian (Liaoning Province), and Beijing to control the COVID-19 outbreak (Wang et al., 2020).

In practice, a reliable laboratory result is the most important element for quick and accurate decision-making in patients and asymptomatic carrier control. However, the quality and diagnostic performance of these tests have not been adequately validated, and WHO has encouraged laboratories to participate in external quality assessment (EQA) schemes for this novel virus (Laboratory testing for 2019 novel coronavirus (2019-nCoV) in suspected human cases, 2020). EQA is a fundamental element, especially when using emergency use authorization diagnostic kits for newly emerging pathogens. Recently, some countries have carried out the EQA for SARS-CoV-2 molecular testing and identified the potential weakness of nucleic acid extraction and PCR reagent kits [(Fischer et al., 2021; Görzer et al., 2020; Matheeussen et al., 2020; Sung et al., 2020; Wang et al., 2021)]. Therefore, the Zhejiang Provincial Center for Disease Control and Prevention organized the first province-wide EQA scheme for qualitative molecular detection of SARS-CoV-2 in July 2020.

In this report, we present the results of the first study of EQA for SARS-CoV-2 in CDC laboratories in Zhejiang Province, China, and the

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data from this study provide a snapshot of current laboratory practices and accuracy.

2. Materials and methods

2.1. Viruses and specimen preparation

To prepare the SARS-CoV-2 stock for the EQA, the SARS-CoV-2 strain (SARS-CoV-2/human/CHN/WZ122/2020), was isolated from the throat swab specimen from a patient with COVID-19 in Zhejiang Province, on January 26, 2020, was used to inoculate Vero-E6 cells (ATCC C1008) in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies; Bleiswijk, The Netherlands) supplemented with 2% fetal bovine serum (Life Technologies; Thornton, Australia) and cultured at 37℃ in 5% CO_2 for 5 days in a BSL-3 laboratory Zhou et al., 2020; Drosten et al., 2003. When complete cytopathic effects were observed, the culture supernatant was collected, and real-time RT-PCR using the Novel Coronavirus (2019-nCoV) Nucleic Acid Detection Kit (BioGerm, Shanghai, China) was performed to confirm the positive culture. Using the Reed-Muench method (LaBarre and Lowy, 2001), The viral titer was $1\times10^{6.3}$ TCID50/mL, and the virus culture was inactivated with β-propiolactone (Wako, Japan), and no infectious virus was confirmed for residual infectivity by inoculation in cell culture after 3 generations. A series of ten-fold dilutions of the culture were made with DMEM, and dilutions in 3 different concentrations were selected to prepare EQA samples according to the cycle threshold (C_t) values in RT-PCR, and the diluent (DMEM) was added as a negative control. Samples were quantified with the SARS-CoV-2 molecular detection assay (Yu et al., 2020). The nucleic acid was extracted from a 200 μL aliquot of the original sample using the RNeasy Mini Kit (Qiagen, Germany), and the elution volume was 50 μL. For real-time RT-PCR, each 25 μL reaction mixture contained 20 μL of reaction buffer (including enzymes, primers, and probes) (BioGerm, Shanghai, China) and 5 μL of eluted RNA as a template. Amplification was performed according to the manual, and the C_t values of positive samples in 3 concentrations were: 22.0, 22.4 (Sample 1), 24.4, 25.0 (Sample 2), and 31.0, 31.6 (Sample 3) for the *ORF1ab* gene and *N gene* of the SARS-CoV-2 genome, respectively. The control sample was negative as expected.

2.2. Dispatch of panels

Located in East China, Zhejiang Province is 105,500 square km with a population of 58.5 million in 2019 (n.d.). In this study, 11 municipal CDC laboratories and 82 county CDC laboratories participated in this EQA scheme in Zhejiang Province, China. All municipal CDC laboratories were required to receive the EQA panels between June 30 and July 3, 2020, which were in turn shipped on the day of receipt to the county CDC laboratories. Panels were shipped on dry ice and transported by sample transfer vehicle. Laboratories were instructed to either test the material immediately or store it at -80 ℃ until testing. The results were required to return within 7 days after receiving the test samples.

There were 4 samples in each EQA panel for county CDC laboratories, 3 samples (300 μL each) containing serial 10-fold dilutions of non-infectious SARS-CoV-2 positive culture and one negative control (300 μL each). In these panels for municipal CDC laboratories, each has 4 positive samples (one duplicated of low concentration) and one negative control. All CDC laboratories were asked to treat and test the panels as clinical samples using their routine molecular assay and associated workflows in BSL-2 laboratories.

2.3. EQA data collection

Since the dispatch for EQA panels of SARS-CoV-2, all municipal and county CDC laboratories have been required to submit the raw data and interpreted results (positive or negative) with the information including nucleic acid extraction platforms, reagents for real-time RT-PCR, and the PCR instruments via email and paper within 7 days. A questionnaire for participation was sent out to collect information about the molecular test systems in each laboratory.

This EQA approved the most commonly used nucleic acid extraction methods, real-time PCR reagents, and detection systems for SARS-CoV-2, and the participants were able to report C_t values of the target region(s) based on respective real-time RT-PCR assays. To compare data from semiquantitative, the median and interquartile ranges of the C_t values were converted to box-and-whisker plots. The outlier was determined by the ROUT method using GraphPad Prism 8 (GraphPad Software Ltd). A negative result for a positive sample or any C_t values in negative samples was also defined as outliers. Statistical analyses were conducted in R version 4.0.2.

3. Results

3.1. SARS-CoV-2 molecular test performance

In this EQA, 11 municipal CDC laboratories and 82 county CDC laboratories were included. All participants responded on time and submitted their results with the raw data to the EQA provider before the deadline of July 8, 2020. The protocols used for SARS-CoV-2 real-time RT-PCR varied among the 93 CDC laboratories, and the flow diagram was showed in Fig. 1. A total of 14 different nucleic acid extraction platforms or methods were used, including 11 semi-automatic nucleic acid extraction or pipetting platforms, 2 column-based nucleic acid isolation kits, and 1 magnetic bead-based manual nucleic acid isolation kit. The 4 most frequently used extraction platforms were TIANLONG, Thermo, Bioperfectus, and TIANGEN, respectively.

For SARS-CoV-2 detection, 8 distinct real-time RT-PCR reagents were used. Three frequently used reagent kits were Novel Coronavirus (2019-nCoV) Nucleic Acid Detection Kit (BioGerm, Shanghai, China), Detection Kit for 2019 Novel Coronavirus (2019-nCoV) RNA (PCR-Fluorescence Probing) (DaAn Gene, Guangzhou, China) and COVID-19 Coronavirus Real-Time PCR Kit (Bioperfectus, Taizhou, China), respectively. The characteristics of commercial RT-PCRkits for SARS-CoV-2 virus RNA are showed in Table S1. In terms of real-time PCR platforms, 10 amplification platforms were used in this EQA, and the main real-time PCR instrumentation platform was Applied Biosystems.

In this study, detecting all SARS-CoV-2-positive samples and the negative sample correctly was defined as an acceptable level of proficiency. The reported target regions were the *ORF1ab* gene, *N* gene, and *E* gene. All 93 laboratories performed well, and there was 100% concordance for the qualitative detection of SARS-CoV-2 among all laboratories for all EQA samples, and no false-positive or false-negative results were obtained by any of the laboratories. The boxplot of C_t values for each sample is showed in Fig. 2 and no outliers exist.

Among the C_t values of the *ORF1ab* gene for the samples in 3 different concentrations (S1, S2, and S3), there were 2, 2, and 3 results that deviated from the mean value by >2 SD in S1, S2, and S3, respectively, and no result that deviated from the mean value by >2 SD was found in the *N* gene. The summary of C_t values for 2 frequently reported gene targets is showed in Table S2. Regardless of the molecular testing reagents and PCR amplification platforms, there was no significant difference ($P > 0.05$) between the results obtained by manual and automated nucleic acid extraction methods. However, there was also no significant difference ($P > 0.05$) between the results obtained by municipal and county CDC laboratories.

4. Discussion

The most important means for reducing the transmission of SARS-CoV-2 is the early detection of SARS-CoV-2 in patients and

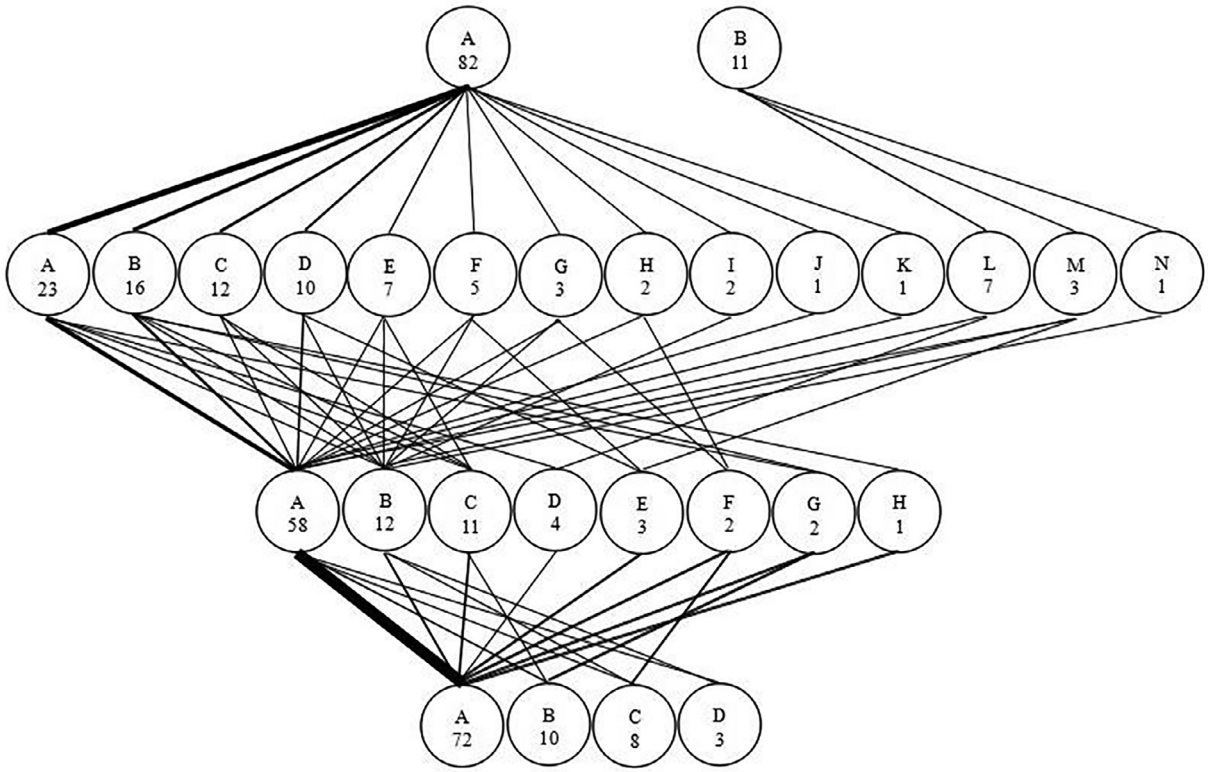

Fig. 1. Protocols used for real-time RT-PCR in 93 laboratories participating in an external quality assessment of SARS-CoV-2 testing, Zhejiang Province, China, June 30–July 8, 2020. The workflow diagram shows the variations in RNA extraction platforms, PCR reagents, and amplification platforms. The weight of the lines reflects the number of laboratories using a particular step. Numbers in the circles indicate the number of laboratories. RT-PCR, reverse transcription PCR; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.Extraction Methods:

A: Automated
B: Manual
Nucleic acid extraction:
A: NP968-C/NP968-S/GeneRotex 96 (TIANLONG)
B: KingFisher 24/KingFisher Duo Prime (Thermo)
C: SSNP-2000A/SSNP-3000A/SSNP-9600A (Bioperfectus)
D: TGuide S32 (TIANGEN)
E: MagMax Express (Thermo)
F: Maxwell 16/Maxwell RSC 48/Maxwell RSC AS4500 (Promega)
G: MagNA Pure 96/MagNA Pure LC 2.0 (Roche)
H: GenAct NE-48/GenAct NL-48 (GeneoDx)
I: NX-48 (Genolution)
J: QIAcube HT (Qiagen)
K: Emagpure-32A (Emerther)
L: Qiagen RNA Mini KIT (Qiagen)
M: Nucleic acid extraction kit (magnetic bead method) (Liferiver)
N: High Pure Viral Nucleic Acid Kit (Roche)
Real-time RT-PCR reagents:
A: Novel Coronavirus (2019-nCoV) Nucleic Acid Detection Kit (BioGerm)
B: Detection Kit for 2019 Novel Coronavirus (2019-nCoV) RNA (PCR-Fluorescence Probing) (DaAn Gene)
C: COVID-19 Coronavirus Real Time PCR Kit (Bioperfectus)
D: Multiple Real-Time PCR Kit for Detection of 2019-nCoV (X-ABT)
E: Novel Coronavirus (2019-nCoV) Real-Time Multiplex RT-PCR Kit (Liferiver)
F: COVID-19 (SARS-CoV-2) Nucleic Acid Test Kit (EasyDiagnosis)
G: Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing) (Sansure)
H: Novel Coronavirus (2019-nCoV) RT-PCR Detection Kit (Fosun)
PCR platforms:
A: ABI 7500/7500 fast/ABI QuantStudio 5/ABI QuantStudio 7 Flex/ABI QuantStudio Dx/ABI ViiA 7 (Applied Biosystems, Thermo Fisher Scientific)
B: BIO-RAD CFX96 Touch (BIO-RAD)
C: Roche 480/Roche 480 II (Roche)
D: Agilent StrataGene Mx3000P (Agilent)

asymptomatic carriers (Gao et al., 2020). Real-time RT-PCR is the gold standard method for the diagnosis of COVID-19 infection. However, the SARS-CoV-2 RNA detection capabilities are limited in hospitals and CDCs in the early stages of the outbreak of COVID-19 in China, and most county-level CDC laboratories lack the capability to test the viral RNA. In China's 4-level disease containment system, the important roles and responsibilities of CDCs at the national, provincial, municipal, and county levels are preventing and tackling acute infectious diseases. During the fight against COVID-19, the CDC laboratories are the most significant units to detect SARS-COV-2. They're also the final result reviewers for positive samples related to the COVID-19 outbreaks all over the country.

After June 2020, the large-scale high-throughput SARS-CoV-2 testing capability has been established in Zhejiang Province, China,

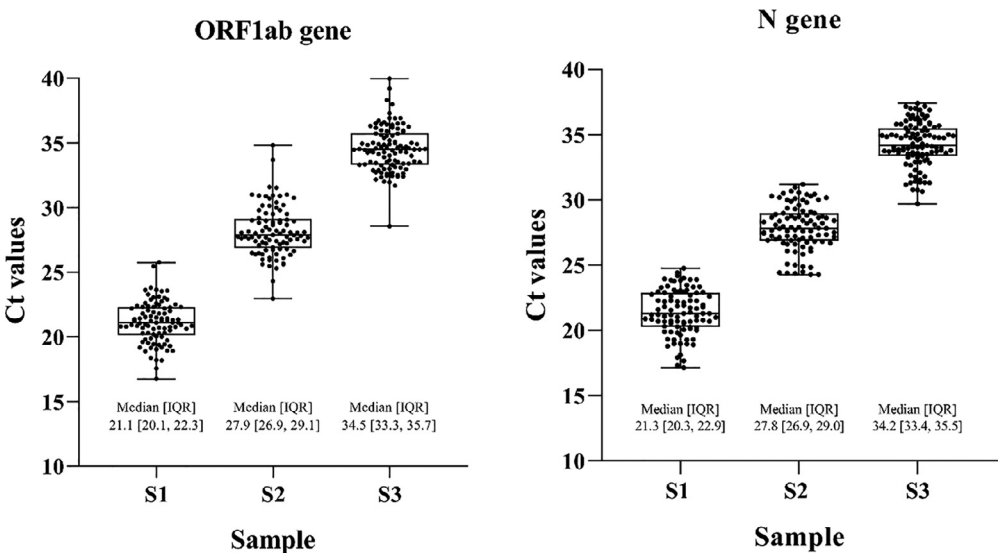

Fig. 2. Box-whisker-plot of cycle threshold values for EQA panel samples tested by CDC laboratories in Zhejiang Province, China, June 30 - July 8, 2020. A horizontal line within each box denotes the median value; the top and bottom of each box indicate the third and first quartiles, respectively; and error bars indicate the minimum and maximum values. S1, sample 1; S2, sample 2; S3, sample 3.

and the tests of the SARS-CoV-2 virus genome were also widely carried out in municipal and county-level CDC laboratories. Monitoring and analyzing EQA results from a large group of participating laboratories help to assess the accuracy of methods applied in a variety of individual laboratory performances.

Zhejiang Province has a 58.5 million population and 11 municipal cities (one municipal city includes several districts and counties), and there are 90 districts and counties in this province. The population of each county and district ranges from tens of thousands to 2 million, and it is the first time detects nucleic acid of viruses using real-time RT-PCR in several counties CDC laboratories in Zhejiang Province. EQA scheme is a good opportunity to assess the performance of their assays against municipal laboratories in line with agreed clinical practice, based on well-characterized virus samples, to identify any weaknesses in their procedures or methods. Therefore, we organized the CDC laboratories at the municipal and county levels to carry out the EQA of SARS-COV-2 nucleic acid testing. Table S3 shows the locations of 11 municipal and 82 county CDC laboratories that participated in this EQA for molecular testing of the SARS-CoV-2 genome. All participating laboratories achieved the correct qualitative results, and all laboratories are capable of generating the SARS-CoV-2 RT-PCR results within 24 hours after receiving the samples.

Twenty-five kinds of real-time RT-PCR SARS-CoV-2 RNA assays kits have been approved by the China National Medical Products Administration, and 8 kinds of kits were used in this EQA (Fig. 1). Notably, these commercial kits are different in their target genes and interpretation criteria (see Table S1). The kits from Liferiver and X-ABT have 3 target regions: *ORF1ab* gene, *N* gene, and *E* gene. However, there were only *ORF1ab* gene and *N* gene in other kits. All laboratories reported the C_t values of the *ORF1ab* gene and *N* gene for each sample, but only 7 laboratories reported the Ct values of the *E* gene. Though no outlier was detected in 3 different concentrations for the *ORF1ab* gene and the *N* gene, deviations were found in these reported results. There was one C_t value of *ORF1ab* in a low concentration sample with a C_t value of 39.98, it nearly exceeded the interpretation criteria C_t value of 40 for the kit (DaAn Gene). As highly sensitive methods are required for early COVID-19 diagnostic screening, one low-concentration sample was included in this EQA panel close to the limits of detection in the published or commercial assays (Wang et al., 2021). Laboratories that were unable to detect low concentration samples, or whose methods showed Ct values greatly different from the provided medians, should strive to improve the sensitivity of their molecular assays to prevent false-negative results in respiratory samples with low viral concentrations from SARS-CoV-2 infected patients, e.g., during the early phase of infection or asymptomatic populations.

It is worth noting that there are several limitations to this study. First, according to the information collected in this EQA, most laboratories may have deployed multiple testing methods, with different nucleic acid extraction platforms, RT-PCR reagents, and PCR machines, but results are reported for only one method per laboratory. However, some participants did not report the equipment calibration status, so the deviation of the PCR instrument cannot be excluded. This study may not accurately represent the true scope of method deployment. Second is the small number of samples designed in this EQA. There are only 3 or 4 positive samples and one negative sample, and the data is relatively limited. The combination of lower concentration and negative samples should be considered. Third, the minimal essential medium was used to mimic patient specimens, and the real matrix effect on the detection was not evaluated properly. The lower respiratory tract samples, such as sputum samples, have not been assessed. Fourth, the interpretation criteria of critical values for determining the qualitative results in different kits are not consistent. The threshold value of C_t is 36 in certain kits, and some are less than 40 or 43, so the same samples would show completely distinct results using different reagents according to their instructions, which also reflect the need for a more reasonable detection limit for each kit.

In conclusion, this manuscript summarized the first-time province-wide EQA of SARS-CoV-2 molecular testing carried out by CDC laboratories in Zhejiang Province, China. Overall, laboratories achieved reasonable test sensitivity, providing confidence in the results of these new molecular tests and assurance of the clinical and public health decisions based on these test results. The methodology used in this study provides practical experience for those planning to conduct EQA for testing of SARS-CoV-2 and other emerging pathogens in the future. Recently, new variants of SARS-CoV-2 (such as B.1.1.7, B.1.351, and B.1.617.2 variants) have been detected in numerous countries around the world (n.d.; Dougherty et al., 2021)), including China. A more extensive EQA that includes new variants and replicate samples for consistency evaluation is needed in the follow-up national or international EQA.

Ethical approval and consent to participate

This study complied with the Declaration of Helsinki Principles and was approved by the Institutional Ethical Committee of Zhejiang Provincial Center for Disease Control and Prevention.

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Declarations of competing interest

All authors have no conflicts of interest.

Authors' contributions

Junhang Pan: Data curation, Writing- Original draft preparation. Hao Yan: Conceptualization, Writing - review & editing. Zhen Li: Data curation. Xiuyu Lou: Data curation. Haiyan Mao: Conceptualization, Methodology. Wen Shi: Visualization, Writing - review & editing. Wenwu Yao: Supervision, Writing - review & editing. Yanjun Zhang: Resources, Conceptualization, Supervision, Writing – review & editing.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.diagmicrobio.2022.115766.

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