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Analytical sensitivity and clinical performance of a triplex RT-qPCR assay using CDC N1, N2, and RP targets for SARS-CoV-2 diagnosis

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ABSTRACT

Background: Several RT-qPCR kits are available for SARS-CoV-2 diagnosis and some have emergency use authorization from the US Food and Drug Administration. In particular, the nCoV19 CDC kit includes two targets for detecting SARS-CoV-2 (N1 and N2) and an RNaseP (RP) target for RNA extraction quality control, all of which are labeled with FAM, and thus three PCR reactions are required per sample.
Methods: We designed a triplex RT-qPCR assay based on nCoV19 primers and probes where N1, N2, and RP are labeled with FAM, HEX, and Cy5, respectively, so only a single PCR reaction is required for each sample for SARS-CoV-2 diagnosis.
Results: In total, 172 samples were analyzed in both singleplex and triplex assays, where 86 samples tested SARS-CoV-2 negative with both assays, so the triplex assay specificity was 100%. In addition, 86 samples tested SARS-Co-V 2 positive with the singleplex assay and 84 with the triplex assay, so the sensitivity was 97.7%. The limit of detection for the triplex assay was determined as 1000 copies/mL.
Conclusions: This new triplex RT-qPCR assay based on primers and probes from the CDC protocol is highly reliable for SARS-CoV-2 diagnosis, and it could speed up detection and save reagents during the current SARS-CoV-2 testing supplies shortage.

Introduction

The COVID-19 pandemic has challenged public health systems worldwide, mainly in terms of patient care or surveillance and control, as well as causing problems with the guaranteed supply and quality of SARS-CoV-2 related diagnostic tools. For instance, multiple in vitro RT-qPCR diagnosis kits are available on the market for the detection of SARS-CoV-2. Some of these kits have received emergency use authorization (EUA) from the US Food and Drug Administration (FDA), whereas only limited reported validations conducted by manufacturers are available for others. The CDC designed FDA EUA 2019-nCoV CDC kit (IDT, USA) based on N1 and N2 probes for detecting SARS-CoV-2 and RNaseP (RP) as an RNA extraction quality control has received positive evaluations in recent studies (Lu et al., 2020; Anon, 2020; Rhoads et al., 2020; Nallaa et al., 2020). However, the main limitation of the CDC protocol is the requirement to run three PCR reactions per sample because all of the probes are labeled with the dye FAM (Lu et al., 2020; Anon, 2020). This singleplex PCR protocol uses large amounts of reagents and reduces the laboratory testing capacity, especially in small-scale facilities, which are crucial during the ongoing coronavirus disease pandemic, particularly in developing countries. Moreover, several multiplex assays are commercially available but they usually depend on specific platforms or providers, and they are also substantially more expensive than the CDC protocol.

Thus, in the present study, we developed a triplex assay for detecting SARS-CoV-2 RNA from nasopharyngeal swabs based on the CDC designed probes and primers, N1 and N2, and RP, and we evaluated its clinical performance and analytical sensitivity using the singleplex 2019-nCoV CDC EUA kit as a gold standard.

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Materials and methods

Study design

In total, 172 clinical specimens (nasopharyngeal swabs collected in 0.5 mL of TE pH 8 buffer) included in this study were obtained from individuals selected for SARS-CoV-2 surveillance at "Universidad de Las Américas" SARS-CoV-2 diagnosis lab in Quito, Ecuador. Eight negative controls (TE pH 8 buffer) were included as controls for carryover contamination, with one for each set of RNA extractions.

RNA extraction and RT-qPCR for SARS-CoV-2 diagnosis using 2019-nCoV CDC kit. All samples included in the study were tested following an adapted version of the CDC protocol (Lu et al., 2020; Anon, 2020) using an AccuPrep Viral RNA extraction kit (Bioneer, South Korea), TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, USA), and a CFX96 thermal cycler (BioRad) (Freire-Paspuel et al., 2020a; Freire-Paspuel et al., 2020b; Freire-Paspuel et al., 2020c).

RT-qPCR for SARS-CoV-2 diagnosis using the N1, N2, and RP triplex assay. The same RNA extracts from all samples included in the study were tested using our triplex RT-qPCR assay. The N1 probe was labeled with FAM dye and BHQ1 quencher. The N2 probe was labeled with HEX dye and BHQ1 quencher. The RP probe was labeled with Cy5 dye, TAO, and Iowa Black RQ. All primers and probes used in the triplex assay were purchased from IDT (USA). The triplex reactions contained N1 and N2 primers and probes at final concentrations of 0.5 and 0.13 μM, respectively. For the RP primers and probe, the final concentrations were 0.3 and 0.075 μM, respectively. The triplex assay was also performed using a CFX96 Thermal cycler (BioRad). The RT-PCR conditions were: (a) retrotranscription: 5 min at 50 °C; (b) initial denaturalization: 1 min at 95 °C; and (c) cyclic amplification: 42 cycles for 15 s at 95 °C5 plus 1 min at 55 °C.

Analytical sensitivity

Limit of detection (LoD) analysis was performed using commercial positive control 2019-nCoV N positive control (IDT, USA), which was provided at 200,000 genome equivalents/mL.

Statistics

The Student's *t*-test was performed to compare Ct values.

Ethics statement

All samples have been submitted for routine patient care and diagnostics. Ethics approval was not sought because the study involves laboratory validation of test methods and the secondary use of anonymous pathological specimens that falls under the category 'exempted' by Comité de Etica para Investigación en Seres Humanos" from"Universidad de Las Américas".

Table 1
Performance of N1/N2/RP triplex assay compared to 2019-nCoV CDC EUA for RT-qPCR SARS-CoV-2 diagnosis.

	Triplex assay positive	Triplex assay negative
2019-nCoV CDC positive	**84** (97.7%)	**2**
2019-nCoV CDC negative	**0**	**86** (100%)

% values: sensitivity and specificity.

Results

Clinical performance of triplex assay compared with the CDC gold standard protocol

In total, 172 samples were tested for SARS-CoV-2 following both protocols described above. With the 2019-nCoV CDC EUA kit (singleplex assay), 86 samples tested positive and 86 samples tested negative (Table 1 and Supplementary Table 1). All 86 samples that tested negative with the 2019-nCoV CDC kit were also SARS-CoV-2 negative with the triplex assay, and thus the specificity determined in our study was 100%.

Among the 86 SARS-CoV-2 positive samples, 84 were also positive with the triplex assay, and thus the sensitivity of the triplex assay was 97.7% compared with the 2019-nCoV CDC EUA kit (Table 1 and Supplementary Table 1). Sample 65 was initially inconclusive because only the N1 probe was amplified, but it was positive for both N1 and N2 when the PCR was repeated (Supplementary Table 1).

The Ct values obtained for N1 and N2 in the singleplex assay were 30.70 ± 3.83 and 31.34 ± 4.09, respectively. The Ct values obtained for N1 and N2 in the triplex assay were 30.24 ± 3.82 and 32.57 ± 4.69, respectively. The Ct values did not differ significantly among the assays ($p = 0.79$ and $p = 0.18$ for N1 and N2, respectively).

Analytical sensitivity: LoD for N1, N2, and RP triplex assay

The viral loads shown in Supplementary Table 1 were calculated based on a calibration curve obtained with the 2019-nCoV N positive control (IDT, USA). The LoD is defined as the lowest viral load in which all replicates are detected (100% sensitivity), and thus our data indicate that the LoD for the N1 and N2 probes was 1000 viral RNA copies/mL of sample (under our conditions, RNA was extracted from 0.2 mL of viral transport media and eluted in 40 μL at the end of the extraction process to yield 5 RNA copies/μL of RNA extraction solution), as shown in Table 2.

The viral loads for the only two samples determined as singleplex positive but triplex assay negative were 1.56 copies/μL (sample 85, Table 2) and 2.41 copies/μL (sample 84, Supplementary Table 1).

Discussion

In the present study, we developed a triplex assay for SARS-CoV-2 RT-qPCR based on the same targets used in the EUA FDA approved CDC singleplex assay. The primers and probes for N1, N2, and RP used in our triplex assay were purchased from IDT (USA), which is among the few companies endorsed by the CDC for purchasing its CDC designed SARS-CoV-2 singleplex kit. The main limitation of our study was the sample size (172 samples) but our results indicate that the proposed triplex assay for N1, N2, and RP performed well in terms of its sensitivity and specificity compared with 2019-nCoV CDC EUA, with values of 97.7% and 100%, respectively. Moreover, our the results showed that the LoD for the triplex assay was 1000 viral RNA copies/mL of sample, which is equivalent to 5 viral RNA copies/μL of RNA extraction solution according to our experimental procedure. The only 2 SARS-CoV-2 positive samples that failed in the triplex assay actually had viral loads below the calculated LoD. It is important to note that this triplex assay for SARS-CoV-2 RT-qPCR was only validated for the instruments (CFX96 from BioRad) and chemistries described in the present study, and further validation may be required for other systems.

Other triplex assay protocols have been published recently that employ CDC designed primers and probes (Waggoner et al., 2020;

Table 2
Analytical sensitivity for the N1/N2/RP triplex RT-qPCR assay.

Viral load (copies/mL)	N1 replicates	N1 sensitivity	N2 replicates	N2 sensitivity
2000	5/5	100%	5/5	100%
1500	5/5	100%	5/5	100%
1000[a]	5/5	100%	5/5	100%
500	4/5	80%	4/5	80%

[a] Limit of detection or lower viral load where sensitivity keeps 100%.

Perchetti et al., 2020), but our triplex assay protocol is the first to use exactly the same set of primers and probes for the N1, N2, and RP gene targets as the CDC FDA EUA singleplex protocol.

Our N1, N2, and RP triplex RT-qPCR method represents an affordable alternative to other commercial triplex assays. For instance, in Ecuador, the cost per reaction is below 8 USD for the triplex assay, whereas the triplex assays provided by other commercial brands usually cost above 15 USD per reaction. For any laboratory throughout the world that currently uses the CDC protocol, the proposed triplex assay would provide improvements and speed up diagnosis as well as reducing the usage of reagents, which are necessary to enhance the testing capacity for SARS-CoV-2.

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Authorship contribution statement

All authors contributed to study conceptualization, experimental procedures, and revising and approving the final version of the manuscript.

Byron Freire-Paspuel and Miguel Angel Garcia-Bereguiain analyzed the data and wrote the manuscript.

Declaration of competing interest

All authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2020.10.047.

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