ORIGINAL ARTICLE

SARS-CoV-2 genome RNA detection based on multiplex Real-Time RT-PCR assay

Detecção de RNA do genoma de SARS-CoV-2 baseado em RT-PCR Multiplex em Tempo Real

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Abstract

Objectives: This work aimed to develop a multiplex Real-Time RT-PCR assay to detect the SARS-CoV-2 virus in a biological sample. **Methods:** Multiplex Real-Time one-step RT-PCR was performed compared with the singleplex CDC protocol. For the detection of the N1 and N2 viral regions and host human RNase P gene, the fluorescent dyes VIC-BHQ-1, FAM-BHQ-1, and TAMRA-BHQ-2, respectively, were tested in a one-tube triplex reaction. The swabs from 334 nasopharyngeal samples were tested in comparisons with the singleplex method, and a pUC18 plasmid was constructed with concatenating regions from nucleocapsid and RNAse P gene as a positive control. **Results**: Paired data between the two assays presented a positive correlation. Comparative data demonstrated that the qRT-PCR multiplex was an efficient method presenting 83% of concordance with the singleplex, and the RNA from SARS-CoV-2 was detected in 31 and 41%, respectively. In addition, in 3% of the samples, the viral RNA was only detected in the multiplex. **Conclusions**: A fast, accurate, and low-cost method for detecting SARS-CoV-2 was obtained, highlighting the choice of conventional fluorophores and probes assisting in epidemiological surveillance and the clinical management of this serious public health disease.

Keywords: COVID-19; Fluorophores; One-step; One-tube; Diagnosis.

Resumo

Objetivos: Este trabalho objetivou o desenvolvimento de um ensaio de RT-PCR multiplex em tempo real para detectar o vírus SARS-CoV-2 em amostra biológica. **Métodos**: A RT-PCR multiplex em tempo real foi realizada em comparação com o protocol do CDC singleplex. Para a detecção das regiões virais N1 e N2 e do gene da RNase P humana, as sondas fluorescentes VIC-BHQ-1, FAM-BHQ-1 e TAMRA-BHQ-2, respectivamente, foram testados em uma reação triplex em um único tubo. Os swabs de 334 amostras de nasofaringe foram testados em comparações com o método singleplex, e um plasmídeo pUC18 foi construído com regiões concatenadas do nucleocapsídeo viral e do gene RNAse P como controle positivo. **Resultados**: Os dados pareados entre os dois ensaios apresentaram correlação positiva. Dados comparativos demonstraram que a reação multiplex foi eficiente apresentando 83% de concordância com o singleplex, e o RNA de SARS-CoV-2 foi detectado em 31 e 41%, respectivamente. Além disso, em 3% das amostras, o RNA viral foi detectado apenas no multiplex. **Conclusões**: Obteve-se um método rápido, específico e de menor custo comparado às reações singleplex para a detecção de SARS-CoV-2 e em um único tubo, destacando-se a escolha de fluoróforos convencionais no desenho de sondas, auxiliando na vigilância epidemiológica e no manejo clínico desta grave doença de saúde pública.

Palavras-chave: COVID-19; Fluoróforos; Passo-único; Um tubo; Diagnóstico

INTRODUCTION

In March 2020, the World Health Organization (WHO) declared the infection caused by a novel strain of coronavirus, Severe Acute Respiratory Syndrome – Coronavirus 2 (SARS-CoV-2) as pandemic[1]. The first case was reported in Hubei Province, China, with non-specific symptoms which are common to other respiratory diseases such as fever, cough, fatigue, sputum production and shortness of breath[2]. Lymphopenia, prolonged prothrombin time, and elevated lactate dehydrogenase were also recently included as related symptoms to coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2[3].

COVID-19 dissemination is notorious, being transmitted mainly through person-to-person contact via respiratory drops released with a reproduction number (R0) ranging from 1.4 to 6.49[4-6]. More critically, the virus is efficiently spread by asymptomatic individuals, which characterizes an uncontrolled and silent spread of SARS-Cov-2[7, 8]. In Brazil, the confirmation of the first case of COVID-19 occurred on February 26, and in just over a month, cases of the disease have already been recorded in all states of the federation, including with community transmission. Globally, there are more than 24 million people diagnosed with the disease and the deaths have already surpassed the 820,000 mark in this country[9,10].

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The control of this massive infection relies on public policy and efficient detection and screening for infected patients with rapid and reliable testing. In fact, the identification of infected individuals is the cornerstone for contention strategies, patient management, and contact tracing. The gold standard method for diagnosis is based on indirect detection of viral RNA by qRT-PCR[11,12], mainly using Center for Disease Control (CDC - USA) guidelines and primers sets[13]. SARS-Cov-2 is an enveloped positive-sense single-stranded RNA virus whose sequences share 79.6% similarity to SARS-CoV and are 96% identical at the whole-genome level to a bat coronavirus[14,15]. The viral genome encodes non-structural (ORF1a and ORF1b) and structural (spike (S), envelope (E), membrane (M) and nucleocapsid (N)) proteins, which are involved in replication and the viral assembly, respectively[16,17]. CDC have approved primers and sequence-specific fluorescence probes against specific regions of nucleocapsid gene, named N1 and N2, which detect SARS-Cov-2 in nasopharyngeal and oropharyngeal swabs, sputum, bronchoalveolar lavage, bronchoscope brush samples, saliva, feces, blood, and urine[18-20]. The human RNase P gene is also included, in an amplification assay performed to each primer and probe separately[13]. However, large-scale testing is critical for mitigating outbreaks of SARS-Cov-2, which demands rapid and precise assays.

Some studies have been published describing the use of the multiplex RT-PCR assay to diagnose SARS-CoV-2 and this system offers increased throughput of viral detection with high efficiency. Also, companies in the field of molecular biology have developed kits based on assays with a multiplex proposal aiming to assist researchers, hospitals, clinics, and laboratories, optimizing the time between collection and release of the diagnosis. In this respect, we designed an in-house multiplex assay to simultaneously detect the two regions of the SARS-Cov-2 N gene and the human RNase P using CDC protocol, obtaining a fast, accurate and low-cost protocol to detect SARS-CoV-2.

MATERIALS AND METHODS

Clinical samples and RNA extraction

This work was developed in accordance with the ethical principles in Brazil under approval number CAAE 30848620.1.3037.5083 from the Ethics Committee in Research. The nasopharyngeal samples from 334 patients suspicious of COVID-19 infectious were collected according to CDC guidelines using rayon swab with plastic shaft and stored up to 24 hours at 4 °C before processing [13]. After this period, the samples were maintained at -80°C. The RNA was obtained in approximately 40 minutes in a room with controlled temperature (20-25°C) by using MagMaxTM Viral/Pathogen Nucleic Acid Isolation Kit (ThermoFisher) following the manufacturer's recommendations and KingFisher FlexTM for 96 deep well as an automated equipment system of extraction.

Singleplex Real-Time one-step RT-PCR

The singleplex was performed with primers (5'-3') and probes (P) labelled with 6-carboxyfluorescein (FAM at 5' and Black Hole Quencher (BHQ)-1 at 3'), all synthetized from IDT (Integrated DNA Technologies), as follow: (i) F: GACCCCAAAATCAGCGAAAT; R: TCTGGTTACTGCCAGTTGAATCTG; P: ACCCCGCATTACGTTTGGTGGACC, to amplify N1; (ii) F: TTACAAACATTGGCCGCAAA; R: GCGCGACATTCCGAAGAA; P: ACAATTTGCCCCCAGCGCTTCAG to amplify N2; and (iii) F: AGATTTGGACCTGCGAGCG; R: GAGCGGCTGTCTCCACAAGT; P: TTCTGACCTGAAGGCTCTGCGCG) to amplify RNAse P as human host control.

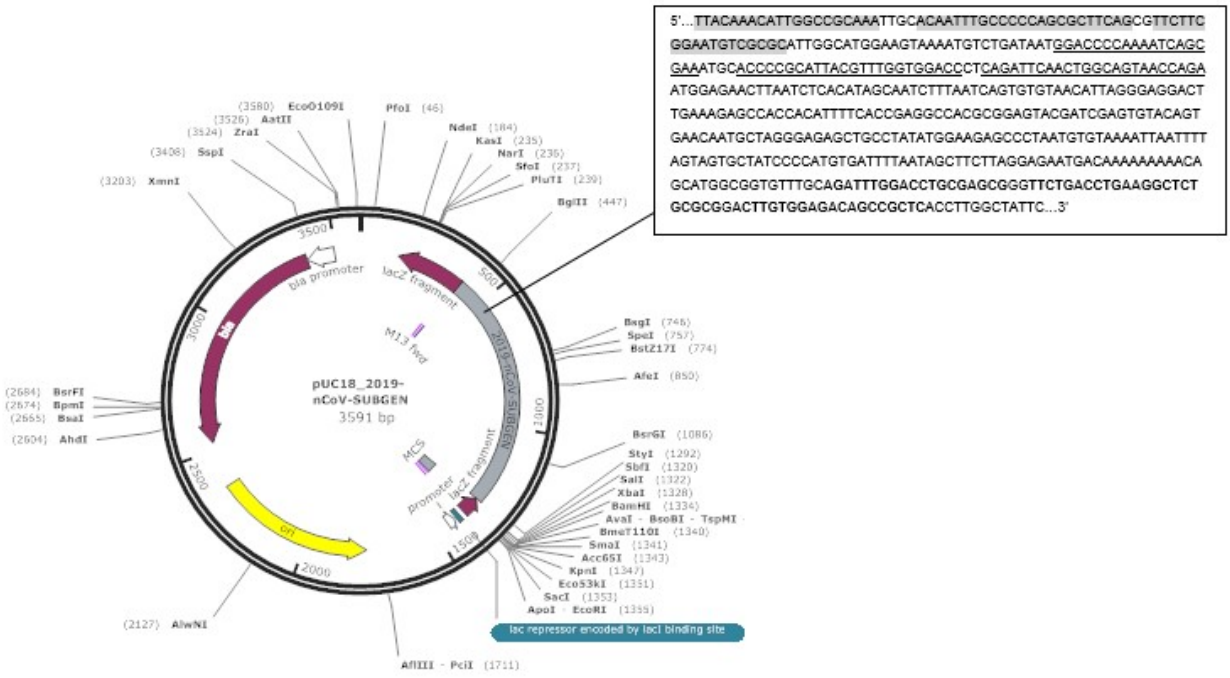
The RNA was submitted to qRT-PCR up to 48 hours after extraction as per CDC's instructions [13]. The workflow of the Taq PathTM 1-Step RT-qPCR Master Mix (Thermo Scientific) was used, including the positive control, in a 96 wells plate. Cycling conditions were reverse transcription for 15 minutes at 50°C, initial denaturation for 2 minutes at 95°C, followed by 45 cycles of 3 seconds at 95°C and 30 seconds at 55°C on ABI 7300 Real-Time PCR Systems (Applied Biosystems).

Multiplex Real-Time one-step RT-PCR

In-house multiplex assays were designed and performed to compare with the singleplex standard method. Reagents and conditions of multiplex were optimized using the above primers and N1 and N2 probes were labeled with VIC®-BHQ-1 (Applied Biosystems) and FAM-BHQ-1 fluorescent dyes, respectively and RNase P with TAMRA-BHQ-2, all purchased from GenOne Biotechnologies (Rio de Janeiro, RJ, Brazil). All CDC primers and probes were analyzed in the Primer Express software version 3.0 (Applied Biosystems/ Thermo Fisher Scientific), and the sequence of forwarding N1 primer was modified, 5'-gGACCCCAAAATCAGCGAA-3', to reach an annealing temperature of 59.4°C, 53% of GC in a length of 19 nucleotides. These modifications were important to promote similar kinetic amplification among primers and probes for multiplexing reactions. Reactions final volume of 15 µL were prepared to contain 750 nM of each primer and 187.5 nM of each probe. The Probe One-Step RT-PCR Low Rox Kit (catalog number 13-10507-05 LGC Biotechnologies, São Paulo, SP, Brazil) was used according to the manufacturer's recommendations.

As a positive control, a pUC18 plasmid containing, among others viral regions, N1, N2 and RNase P sequences (Figure 1), and named 2019 nCoV-Subgen (GenOne Biotechnologies, Rio de Janeiro, RJ, Brazil - WATSONbio Sciences), was constructed and serially diluted (10^4, 10^3 to 10^2 copies). The plasmid was cloned and stored in a chemically competent E. coli (Top 10). After transformation, cells containing the plasmid DNA were grown on LB agar plates containing ampicillin. After the formation of colonies, one of them was placed in LB media for replication of the transformed bacteria and subsequent extraction and purification of the plasmid DNA. Number of copies were calculated by using the equation: number of molecules = concentration of the plasmid (ng/µL) x 6.02 x 10^{23}/

Figure 1. pUC18 constructed with N1, N2 and RNAse P sequences, named (2019 nCoV-Subgen). This plasmid was used as positive control for SARS-CoV-2 detection through qRT-PCR. The sequences for the regions for N2, N1 and RNase P oligos are in grey, underlined, and bold, respectively. Plasmid figure was generated by SnapGene® Viewer software version 5.1.3.1.



length of the plasmid (3581) x 649 (g/mol = 1pb)[21]. All reactions were performed in a 7500 Real-Time PCR System (Applied Biosystems) and thermocycling conditions were 55 °C for 30 minutes, 95 °C for 15 minutes, followed by 45 cycles of 90 °C for 15 seconds and 60 °C for 60 seconds.

Data analysis

SARS-CoV-2 viral RNA was considered detectable when N1and N2 were amplified, even when RNase P sequence was not detected[22]. According to CDC guidelines, this situation can occur due to the particularity of each sample[13]. Additionally, the procedures adopted during collecting can promote the degradation of host target genes. Samples without curves for N1 and N2, and positive for RNAse P amplification, were considered as the virus was not detectable. Finally, when just one curve was observed for N1 or N2, the data were reported as inconclusive. Negative results for amplification of N1, N2 and RNAse P were discarded.

Data of multiplex were paired with singleplex detection, and curves higher than 40 cycles were considered as negative, due to the presence of some fluorescent noise signal in the no template control, avoiding false-positive results. The Fisher's Exact Test was used to determine the clinical parameters such as sensitivity, specificity, positive and negative predictive values of the multiplex considering singleplex as standard for SARS-CoV-2 diagnostic. The t-test was used to determine R square in a compared correlation analysis. All statistics were performed in GraphPrism 8.0.2 (263), and significance was considered when the P-value was lower than 0.05.

ETHICAL APPROVAL

CAAE 30848620.1.3037.5083 from the Ethics Committee in Research in Brazil.

RESULTS

The amplification curves for multiplex are shown in Figure 2. No competition was observed between N1 and N2 targets in the multiplex assay, with an average Ct of 28 and 23, respectively, and of Ct 26 for RNAse P gene expression. It was possible to observe that the viral targets were efficiently amplified and, if RNAse P was not detectable or its amplification curves presented low inclination, the viral load was higher, as observed by the Ct of the amplification curves. For data with paired samples between singleplex and multiplex, a positive correlation was obtained (r = 0.66 - CI 95% 0,05809 to 0,1457) with p <0.0001. For both, single and multiplex formats of RT-PCR, 2% of the samples were considered inconclusive in paired analyzes.

The SARS-CoV-2 RNA was detected by combining N1 and N2 regions in 41% (129/315) and 31% (97/315) of the samples in the singleplex and multiplex, respectively. A sensitivity of 93%, specificity of 99%, the positive predictive value of 97% and negative predictive value of 97% detection were observed in the multiplex assay, by using N1 and N2 detections. Additionally, the accuracy of the test was not affected by competition during the reaction and 3% (10/324) of the samples with positive

amplification curve were not previously detected in the singleplex. On the other hand, 13% (42/324) was detected only in the singleplex reaction. In general, the multiplex presented 83% of concordance with the singleplex. Some studies with multiplex qRT-PCR assays which also used primers designed for the regions recommended by WHO and CDC[13] are summarized in Table 1.

Figure 2. SARS-CoV-2 RNA detection based on one-step and multiplex N1, N2 and RNase P detection. Detection of the virus by presence of the amplification curves for N1 -Vic (A), N2 – Fam (B) and RNase P – Tamra (C), respectively. D) Virus was not detected, and RNase P were positive. E) Samples in which the virus was detected with reduction of the RNase P curve. Data analysis: Rn versus Cycle.

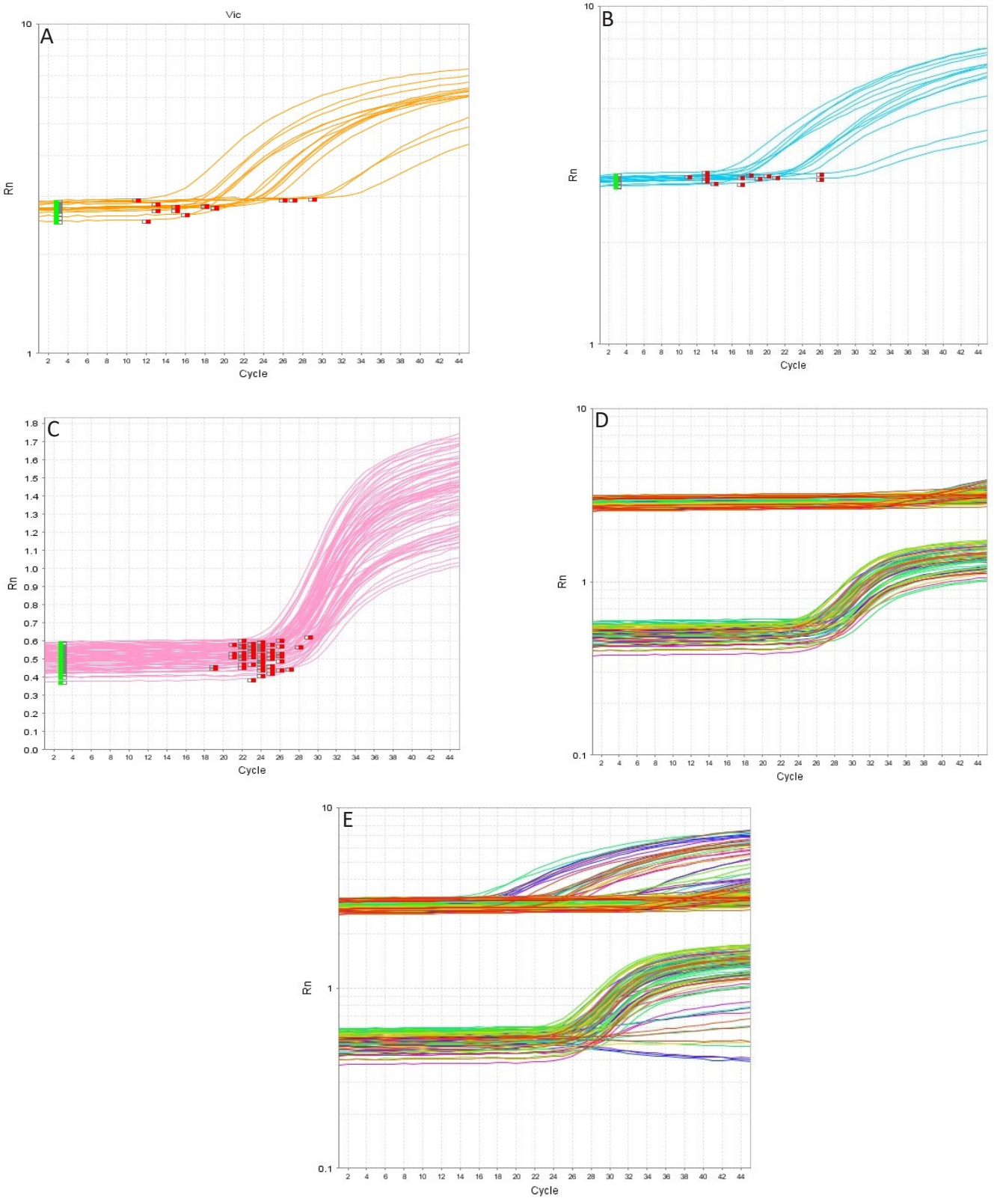


Table 1. One-tube assays for molecular diagnosis of SARS-CoV-2 based on Real Time PCR.

Probe targets and flurophores	RNA extraction/RT-PCR assays	Real time PCR equipment	References
FAM-N1-BHQ-1 FAM-N2-BHQ-1 HEX-RNAseP-BHQ-1 SYBR Green I	KiCqStart One-Step Probe RT-qPCR ReadyMix (Cat. KCQS07, Merck Life Science Private Limited)	Onestep TaqMan RT-PCR in QuantStudio 5 Real-Time PCR system (Applied BioSystems, Cat no. A34322)	[22]
FAM-SARS-CoV-2/N-TAMRA FAM-Influenza A/M1-BHQ-1 VIC-Influenza B/NP-BHQ-1	PrimeDirect (Takara), OmniTaq (DNA Polymerase Technology Inc), Alpha Taq(VitaNavi)/ TTX (TOYOBO), and a microfluidic disc-dirRT-qPCR reactions development	Microfluidic-discdirRT-qPCR equipment: microfluidic cassette, spinning, thermocycling and optical detection	[31]
FAM-N1-BHQ-1 HEX-N2- IBFQ Cy5-RNAseP-IBRQ	Luna Universal Probe One-Step RT-qPCR Kit (New England BioLabs, Ipswich, MA, US)	CFX96 Touch (Bio-Rad, Hercules, CA, US)	[25]
N1, N2 and EXO (RNA transcript derived from jellyfish DNA) FAM; Cy5-BHQ; VIC-MGB	Roche's MagNA Pure 96 instrument for RNA extraction using the pathogen universal kit/ AgPath-ID One-Step RT-PCR (Life Technologies, Carlsbad, CA)	ABI 7500 (Applied Biosystems, Foster City, CA)	[26]
FAM-N1-BHQ-1 FAM-N2- BHQ-1 HEX-RNAseP-BHQ-1	EZ1® Advanced XL instrument (Qiagen, Germantown, MD, USA) with EZ1® DSP Virus Kit (Qiagen, Germantown, MD, USA)/ qScript XLT One-Step RT-qPCR ToughMix (Quantabio, EUA)	7500 Fast Dx Real Time PCR (Applied Biosystems, Waltham, MA, EUA)	[30]
FAM-N1-IBFQ Cy5-RNAse P-IBRQ	MagMAX Viral/Pathogen Nucleic Acid Isolation Kit on the KingFisher Flex Magnetic Particle Processor/ Luna Universal Probe One-Step RT-qPCR Kit (New England Biolabs, Ipswich, MA, US) – salive; ThermoFisher Scientific TaqPath RT-qPCR - nasopharyngeal swabs	CFX96 qPCR machine (Bio-Rad, Hercules, CA, US) ABI 7500 Fast Dx	[28]
FAM-N1-IBFQ Cy5-RNAseP- BHQ-2	NucliSENS easyMag platform (BioMérieux, Durham, NC)/ TaqPath 1-step real-time quantitative PCR kit (catalog number A15299; Thermo Fisher Scientific)	7500 Fast Dx Real Time PCR (Thermo Fisher Scientific, Waltham, MA)	[27]

DISCUSSION

With the unprecedented spread of the SARS-CoV-2 virus causing the COVID-19 disease, worldwide efforts have been devoted to better managing this public health problem. "You cannot fight a fire blindfolded. And we cannot stop this pandemic if we don't know who is infected" was one of the various phrases from the World Health Organization director-general Tedros Adhanom Ghebreyesus emphasizing the importance of precise and rapid diagnostic tests for COVI-19[23].

Numerous efforts have been devoted to the optimization of rapid, sensitive, and effective diagnostic methods. In this scenario, multiplex assays have gained prominence since the amplification of viral RNA by qRT-PCR remains the gold standard methodology in the epidemiological control of this disease. When three genomic targets (two viral and one from the host) are analyzed in a single tube (or well) the benefits are clear, including fewer reactions, reagents, controls, thermocyclers, and skilled labor[22,24-26]. The most widely used protocol is based on one-step singleplex qRT-PCR reactions, performed separately for N1, N2 and RNase P amplifications and, therefore, requiring three distinct reactions for each collected sample, consuming resources, and time[25], often scarce in pandemic infections. As noted by Zhen et al. 2020[27], in a multiplex assay, about 90 samples can be analyzed in a single thermocycling, while only 29 are amplified on a single plate cycled with CDC protocol. In addition, regions codifying for spike (S), or other viral RNA sequences as the E (envelope), ORF1ab, and RdRP genes can be also considered as a SARS-CoV-2 RNA transcript to be analyzed by RT-PCR[27,28].

Kudo et al. 2020[25] performed the two methodologies and observed sensitivity of 100% in the multiplex compared to the singleplex, amplifying the three targets recommended by the CDC (N1, N2 and RNAse P) in the same tube. Arnaout et al. (2020)[29] performed 27,098 tests using primers sets for RdRp and N sequences of SARS-CoV-2, and 22% of the samples were

positive, with a detection limit of about 100 copies of RNA/mL of viral transport solution. In addition to the diagnosis, the amplification curves also provided quantitative data, which are interesting in assessing viral load. In this context, can be suggested that low viral loads and, therefore, undetectable in the trials, may reveal less infectious profiles of the disease, with a direct relationship between Ct, viral load, and infectivity.

In our study, although 3% of positive samples were not previously detected in the singleplex, the coincidence of detection was lost in 13% of the samples, probably due to the degradation of the sample during the process of handling, and our data is in consonance with as previously by Petrillo et al. (2020)[30]. The false-negative cases are plausible to occur by the qRT-PCR method and several factors must be optimized to improve the test sensitivity. Among the variables stand out sampling management affecting the sample integrity, the time between collection and extraction, the quality of the extracted RNA, qRT-PCR performed immediately after RNA extraction without defrosting after stock at -80 °C, and differences between handlers or reagents can interfere in the detection by qPCR method[31]. In this sense, clinical parameters and molecular tests combined can improve epidemiological surveillance, and cases with clinical indication of Covid-19 and qRT-PCR not detected must be repeated, and if necessary, another type of sample must be collected based on the time that the patient has been exposed to the virus[31-33].

In fact, false-negative and false-positive results have important implications. The false-negative report has been considered a priority since it directly impacts viral propagation. However, false-positive diagnostic may present substantial consequences in social life and the health system. The false-positive diagnostic can occur due to contamination (in samples, reagents or during q-RT-PCR amplification), cross-reactions with other viruses or misinterpretation of results. Therefore, whatever the diagnostic, it must be interpreted with caution within the context of the probability of disease[33].

Our in-house qRT-PCR multiplex maintained diagnostic accuracy consistent with other studies that developed rapid methodologies for the detection of SARS-CoV-2[22,25,26,28,30] enabling cost reduction, and speeding up the result releases. The choice of different fluorophores is in the technical aspects during the steps of optimization. So, the FAM, TAMRA and VIC are among the most useful fluorophores in molecular probes, which can reduce the assay cost. Moreover, a small reduction in reaction volume and the construction of our recombinant plasmid as a positive control for viral sequences and host control are details that must be considered in our work.

Finally, new kits for the detection of SARS-CoV-2 expand the diagnostic capacity, especially in places with budgetary constraints and in situations of the scarcity of inputs. In this way, optimization of multiplex tests directly assists in epidemiological and laboratory surveillance and clinical management of diseases, especially in this serious public health emergencies.

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