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COMPARISON OF SINGLEPLEX AND MULTIPLEX REAL-TIME REVERSE TRANSCRIPTION - POLYMERASE CHAIN REACTION FOR THE DETECTION OF SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS

COMPARAÇÃO DA REAÇÃO EM CADEIA DA POLIMERASE EM TEMPO REAL VIA TRANSCRIÇÃO REVERSA SINGLEPLEX E MULTIPLEX PARA DETECÇÃO DO CORONAVÍRUS DA SÍNDROME RESPIRATÓRIA AGUDA GRAVE 2

Francielle Liz Monteiro[1], Fabiana Caroline Zempulski Volpato[2], Priscila Lamb Wink[3], Julia Biz Willig[4], Rodrigo Minuto Paiva[5], Fernanda De-Paris[6], Rafaela Bianchin Mozzaquatro[7] e Afonso Luis Barth[8]

ABSTRACT

The main laboratorial test for the diagnosis of COVID-19 (Coronavirus disease 2019) is the detection of SARS-CoV-2 (Severe acute respiratory syndrome coronavirus 2) RNA by the RT-qPCR (real-time reverse transcription - polymerase chain reaction) technique. To optimize the diagnosis of COVID-19, we have developed a multiplex real-time reverse transcription polymerase chain reaction (RT-qPCR) technique, which targets the nucleocapsid protein of SARS-CoV-2. The multiplex RT-qPCR assay was compared with the US CDC singleplex protocol. Protocol 1 included 113 RNA samples previously tested for SARS-CoV-2 and Protocol 2, included 107 fresh RNA samples that were tested simultaneously by the singleplex and multiplex. Protocols 1 and 2 presented agreement between singleplex and multiplex RT-qPCR of 88.5% and 98,1%, respectively. After the validation of the multiplex RT-qPCR assay, this methodology was applied in the routine of the diagnostic laboratory and 2,015 samples were analyzed in the first month of the multiplex use. In this period, we found that the multiplex assay proved to be a practical approach which provided reliable results. In conclusion, the multiplex RT-qPCR using primers for targets N1 and N2 is comparable to singleplex.

Keywords: Coronavirus disease, COVID-19, molecular diagnosis, SARS-CoV-2.

1 Docente do Curso de Biomedicina e Mestrado em Ciências da Saúde e da Vida da Universidade Franciscana, Santa Maria, Rio Grande do Sul, Brasil. ORCID: 0000-0002-4698-9657. E-mail: francielle.monteiro@ufn.edu.br

2 Docente colaboradora da Universidade Estadual do Oeste do Paraná, Brasil. ORCID: 0000-0002-5225-2256. E-mail: fabiana_volpato@yahoo.com.br

3 Laboratório de Biologia Molecular, Santa Casa de Misericórdia de Porto Alegre, Rio Grande do Sul Brasil. ORCID: 0000-0002-8126- 2365. E-mail: pris_farma@yahoo.com.br

4 Laboratório Central da Santa Casa de Misericórdia de Porto Alegre, Rio Grande do Sul, Brasil. Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul. ORCID: 0000-0003-4518-6809. E-mail: juhbiz@hotmail.com

5 Hospital de Clínicas de Porto Alegre, Unidade de Microbiologia, Setor de Biologia Molecular e Esterilização, Porto Alegre, Rio Grande do Sul, Brasil. ORCID: 0000-0002-4112-0880. E-mail: rpaiva@hcpa.edu.br

6 Hospital de Clínicas de Porto Alegre, Programa de Medicina Personalizada, Porto Alegre, Rio Grande do Sul, Brasil. ORCID: 0000-0003-2563-9487. E-mail: fparis@hcpa.edu.br

7 Discente do Curso de Biomedicina da Universidade Franciscana, Santa Maria, Rio Grande do Sul, Brasil. ORCID: 0000-0003-0652-1387. E-mail: rafaela.bmozzaquatro@ufn.edu.br

8 Hospital de Clínicas de Porto Alegre, Centro de Pesquisa Experimental, Laboratório de Pesquisa em Resistência Bacteriana, Porto Alegre, Rio Grande do Sul, Brasil. ORCID: 0000-0002-7969-3908. E-mail: albarth@hcpa.edu.br

RESUMO

O principal exame laboratorial para diagnóstico da COVID-19 (Doença do Coronavírus de 2019) é a detecção do RNA do SARS-CoV-2 (Severe acute respiratory syndrome coronavirus 2) pela técnica de RT-qPCR (reação em cadeia da polimerase em tempo real via transcrição reversa em tempo real). Para otimizar o diagnóstico da COVID-19, desenvolvemos uma técnica RT-qPCR multiplex, que tem como alvo a proteína do nucleocapsídeo do SARS-CoV-2. O ensaio de RT-qPCR multiplex foi comparado com o protocolo singleplex do CDC (EUA). O Protocolo 1 incluiu 113 amostras de RNA previamente testadas para SARS-CoV-2 e o Protocolo 2 incluiu 107 amostras frescas de RNA que foram testadas simultaneamente pelo singleplex e multiplex. A comparação do RT-qPCR singleplex e multiplex demonstrou concordância de 88,5% e 98,1% nos protocolos 1 e 2. Após a validação do ensaio de RT-qPCR multiplex, esta metodologia foi aplicada na rotina do laboratório de diagnóstico e 2.015 amostras foram analisadas no primeiro mês de uso do multiplex. Neste período, descobrimos que o ensaio multiplex provou ser uma abordagem prática que forneceu resultados confiáveis. Em conclusão, a RT-qPCR multiplex utilizando primers para os alvos N1 e N2 é comparável ao singleplex.

Palavras-chave: *Doença do Coronavírus, COVID-19, diagnóstico molecular, SARS-CoV-2.*

INTRODUCTION

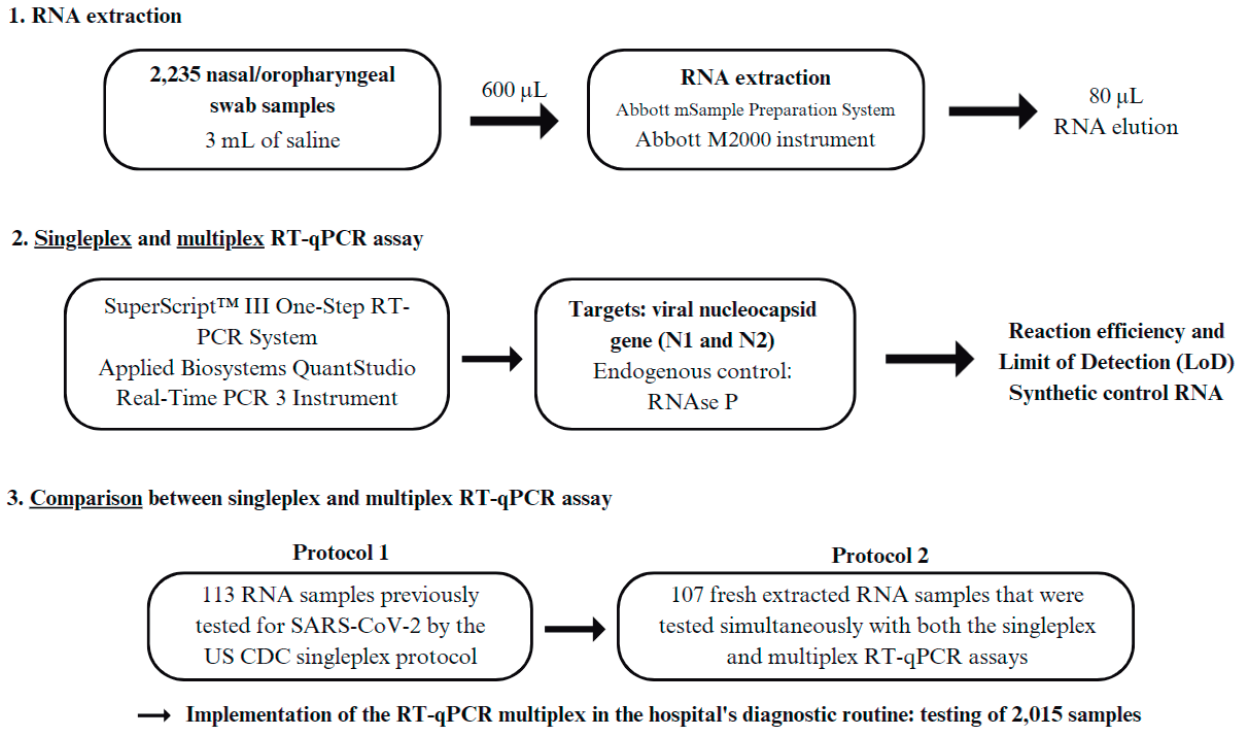
The main laboratorial test for the diagnosis of COVID-19 (Coronavirus disease 2019) is the detection of *SARS-CoV-2* (*Severe acute respiratory syndrome coronavirus 2*) RNA by the RT-qPCR (real-time reverse transcription - polymerase chain reaction) technique, due to its high sensitivity and specificity. This test makes it possible to detect the genome virus during the acute phase of the infection, even with low viral load in the analyzed sample (CORMAN *et al*., 2020; LU *et al*., 2020; SHIRATO *et al*., 2020). In order to increase reaction specificity and to avoid false-negative results using only one target on amplification, the World Health Organization (WHO) recommends the detection of at least two different targets in the *SARS-CoV-2* genome. The targets used for the diagnosis of COVID-19 vary according to the protocols, and the most used regions are the RdRp, the E gene and the N gene (WHO, 2020).

The method proposed by the Centers for Disease Control and Prevention (CDC) has targeted two conserved segments of the viral nucleocapsid gene (N1 and N2) and a human ribonuclease P gene, RNAse P (RP), as an internal control (LU *et al.,* 2020; SHIRATO *et al.,* 2020; WHO, 2020). As the same fluorescent reporter is used for the amplification of N1, N2 and RP probes, the three PCR reactions must be performed separately, limiting the number of clinical specimens to be tested per 96-well plate. An alternative is to perform a multiplex RT-qPCR assay, where two *SARS-CoV-2* target regions are amplified simultaneously. With this, it is possible to test a greater number of patients in a shorter time and reduce the risk of contamination due to sample manipulation. In order to increase the laboratory testing capacity and to decrease the turnaround time, as well as to reduce the consumption of PCR reagents and plastic inputs, the objective of this study was to validate a multiplex RT-qPCR assay to detect SARS-CoV-2 and comparison with singleplex RT-qPCR.

MATERIAL AND METHODS

Figure 1 shows the experimental design of the present study. The project was approved by the Research Ethics Committee (CAAE 30767420.2.0000.5327).

Figure 1 - Experimental design.



Elaborated by the authors (2023).

A total of 2,235 nasal/oropharyngeal swab samples from health care workers and patients attending in a hospital in the city of Porto Alegre (Rio Grande do Sul, Brazil) were evaluated in this study. Swabs were immersed and mixed in 3 mL of saline and 600 µL of this mixture was used for RNA extraction using the Abbott mSample Preparation System (Promega, Madison, WI, USA) with an Abbott M2000 instrument (Abbott, Chicago, EUA) following the manufacturer's instructions. The total RNA extracted was eluted in 80 µL of Abbott mElution buffer.

The multiplex RT-qPCR assay was carried out in comparison to the US CDC singleplex protocol. Two different approaches were carried out: "Protocol 1", included 113 RNA samples previously tested for *SARS-CoV-2* by the US CDC singleplex protocol (92 *SARS-CoV-2*-positive, 10 *SARS-CoV-2*-negative, and 11 inconclusive) that were thawed prior to the singlexplex and multiplex RT-qPCR assays after being kept at -80˚C for a maximum period of two weeks and "Protocol 2", included 107 fresh extracted RNA samples that were tested simultaneously with both the singleplex and multiplex RT-qPCR assays. After the validation of the multiplex RT-qPCR assay, this methodology was applied in the routine of the diagnostic laboratory and 2,015 samples were analyzed.

Both the singleplex RT-qPCR (standard) and the multiplex RT-qPCR were performed using the SuperScript™ III One-Step RT-PCR System (Thermo Fisher Scientific, Massachusetts, EUA). The singleplex RT-qPCR for *SARS-CoV-2* was based on the US CDC protocol, where two targets of the viral nucleocapsid gene (N1 and N2) and RP were amplified (Table 1) (LU *et al.*, 2020; SHIRATO *et al.*, 2020, US CDC, 2020; WHO, 2020). The reaction contained 7.5 µL of 2X Reaction Mix (0.4 mM of each dNTP and 6 mM $MgSO_4$), 0.125 µM of probe, 0.5 µM of forward and reverse primers; 0.2 µL of enzyme, 0.03 µM of ROX™ as reference dye, 2.17 mM of magnesium sulfate, 4 µL of RNA (100-200 ng) and water to complete the 15 µL final volume. For the multiplex RT-qPCR, the N1 and N2 probes were marked with the HEX and FAM fluorescent reporters, respectively. The same concentration of the reagent used in the singleplex were used in the multiplex protocol, without adding water to the mixture. We conducted both assays (singleplex and multiplex reactions) in 96-well plates using an Applied Biosystems QuantStudio Real-Time PCR 3 Instrument (Thermo Fisher Scientific, Massachusetts, EUA). Cycling conditions consisted of 15 min at 50°C for reverse transcription, 2 min at 95°C for activation of the Taq enzyme, and 40 cycles of 15 s at 95°C and 30 s at 60°C. We used the threshold automatically established by the equipment. A cycle threshold (Ct) value lower than 40 for N1 and N2 targets was reported as RT-qPCR positive. The result was considered negative whether the Ct was undetectable or greater than 40; the result was considered inconclusive whether only a single target (N1 or N2) was amplified. For results validation, the Ct obtained for the RP should be lower than 32 - clinical samples with Ct greater than 32 were excluded from the study.

Table 1 - Primers and probes used in singleplex and multiplex RT-qPCR.

Primer/probe name	Sequence (5' - 3')
N1-F2019-nCoVN1	GAC CCC AAA ATC AGC GAA AT
N1-R2019-nCoVN1	TCT GGT ACT GCC AGT TGA ATC TG
N1-P2019-nCoVN1	ACC CCG CAT TAC GTT TGG TGG ACC
N2-F2019-nCoVN2	TTA CAA ACA TTG GCC GCA AA
N2-R2019-nCoVN2	GCG CGA CAT TCC GAA GAA
N2-P2019-nCoVN2	ACA ATT TGC CCC CAG CGC TTC AG
RP-F	AGA TTT GGA CCT GCG AGC G
RP-R	GAG CGG CTGTCT CCA CAA GT
RP-Probe	TTC TGA CCT GAA GGC TCT GCG CG

Elaborated by the authors (2023).

The reaction efficiency carrying out the multiplex RT-qPCR was evaluated for both N1 and N2 regions in comparison to the singleplex RT-qPCR reaction by using a standard curve with five points established from results of a serial dilution (from 10 to 1 x 10^5 copies/µL) of a synthetic control RNA (Integrated DNA Technologies, Coralville, IA, USA) in triplicate. The data of the Ct values of the serial dilutions were plotted against the target concentration (number of the virus copies). We determined the slope of the curve by linear regression and defined the required levels for PCR efficiency ([100 x 10(-1/slope)-1]) and linearity (R2) of each RT-qPCR target to be 90-110% and >0.95,

respectively (BROEDERS *et al*., 2014). Data were automatically calculated by the Thermo Fisher Cloud Dashboard. Limit of detection (LoD) was determined for the singleplex and multiplex approaches using a commercial *SARS-CoV-2* RNA molecule which was diluted at the following concentrations: 10, 20, 30, 40, 50 and 100 copies/µL. The LoD was defined as the lowest RNA concentration, which was detected in 20 replicates with 95% probability of obtaining a correct result.

RESULTS

"Protocol 1": Of the 92 *SARS-CoV-2*-positive, only 6 (6.52%) presented negative or inconclusive results by the multiplex RT-qPCR technique (Table 2). Of the 11 inconclusive samples, four were detected in the multiplex RT-qPCR assay and the other seven presented negative results. All the 10 *SARS-CoV-2*-negative samples presented negative results for the multiplex protocol. The variation the Ct in the singleplex in comparison with the multiplex was 1.72 ± 0.82 (N1) and 1.13 ± 1.87 (N2). The agreement between singleplex and multiplex was 88.5%.

Table 2 - Protocol 1 results: Comparison of Ct values of singleplex RT-qPCR and the multiplex RT-qPCR.

	Singleplex			Multiplex				
Sample	**N1**	**N2**	**Variation N1-N2**	**N1**	**N2**	**Variation N1-N2**	**Variation N1**	**Variation N2**
1A	32.76	36.14	3.38	35.54	36.28	0.74	2.78	0.14
2A	15.78	16.78	1	17.17	18.79	1.62	1.39	2.01
3A	26.13	27.41	1.28	28.7	31.93	3.23	2.57	4.52
4A	31.95	40.74	8.79	33.94	34.22	0.28	1.99	-6.52
5A	20.34	21.24	0.9	21.64	23.05	1.41	1.3	1.81
6A	24.25	26.13	1.88	26.3	27.65	1.35	2.05	1.52
7A	22.62	22.6	0.02	24.57	24.34	0.23	1.95	1.74
8A	33.44	33.45	0.01	35.13	35.12	0.01	1.69	1.67
9A	23.55	23.23	0.32	25.81	25.89	0.08	2.26	2.66
10A	22.11	21.23	0.88	23.71	23.62	0.09	1.6	2.39
11A	16.96	16.86	0.1	17.7	17.53	0.17	0.74	0.67
12A	31.32	35.95	4.63	33.99	34.84	0.85	2.67	-1.11
13A	19.79	22.17	2.38	20.91	20.49	0.42	1.12	-1.68
14A	34.37	35.79	1.42	37.93	37.47	0.46	3.56	1.68
15A	35.09	34.33	0.76	36.53	35.86	0.67	1.44	1.53
16A	25.6	29.41	3.81	27.09	27.51	0.42	1.49	-1.9
17A	34.67	38.3	3.63	39.2	37.58	1.62	4.53	-0.72
18A	36.69	38.02	1.33	*ndt*	38.68	-	-	0.66
19A	24.65	25.09	0.44	26.96	27.16	0.2	2.31	2.07
20A	11.28	10.25	1.03	11.63	11.65	0.02	0.35	1.4
21A	18.74	19.11	0.37	20.4	20.37	0.03	1.66	1.26
22A	31.82	32.35	0.53	33.48	33.77	0.29	1.66	1.42
23A	19.34	19.15	0.19	19.91	20.17	0.26	0.57	1.02
24A	26.96	26.08	0.88	27.97	27.05	0.92	1.01	0.97
25A	33.29	33.68	0.39	34.33	35.83	1.5	1.04	2.15
26A	23.3	22.95	0.35	24.18	23.78	0.4	0.88	0.83

27A	26.1	27.5	1.4	28.05	27.35	0.7	1.95	-0.15
28A	15	15.6	0.6	16.61	16.59	0.02	1.61	0.99
29A	27.96	29.01	1.05	29.57	30.31	0.74	1.61	1.3
30A	25.98	27.12	1.14	27.88	28.37	0.49	1.9	1.25
31A	23.83	25.05	1.22	25.19	25.42	0.23	1.36	0.37
32A	17.9	17.65	0.25	19.51	19.91	0.4	1.61	2.26
33A	29.22	30.33	1.11	31.61	32.17	0.56	2.39	1.84
34A	20.49	20.54	0.05	21.97	22.46	0.49	1.48	1.92
35A	23.3	23.97	0.67	25.19	25.59	0.4	1.89	1.62
36A	21.7	21.6	0.1	23.56	23.21	0.35	1.86	1.61
37A	21.37	21.34	0.03	22.83	22.94	0.11	1.46	1.6
38A	20.6	19.63	0.97	21.31	20.68	0.63	0.71	1.05
39A	33.68	36.17	2.48	37.04	39.53	2.49	3.36	3.36
40A	32.64	35.2	2.56	35.56	35.41	0.15	2.92	0.21
41A	23.94	25.44	1.5	25.28	25.59	0.31	1.34	0.15
42A	24.97	28.61	3.64	26.47	26.77	0.3	1.5	-1.84
43A	19.29	19.99	0.7	20.29	20.4	0.11	1	0.41
44A	16.29	16.83	0.54	17.16	16.94	0.22	0.87	0.11
45A	14.95	15.32	0.37	16.02	16.3	0.28	1.07	0.98
46A	14.94	16	1.06	16.44	16.65	0.21	1.5	0.65
47A	32.59	34.91	2.32	36.04	36.34	0.3	3.45	1.43
48A	25.53	26.58	1.05	26.98	27.55	0.57	1.45	0.97
49A	16.12	17.77	1.65	17.51	20.89	3.38	1.39	3.12
50A	28.48	28.13	0.35	30.01	29.79	0.22	1.53	11.66
51A	36.27	37.59	1.32	*ndt*	*ndt*	-	-	-
52A	31.77	31.69	0.08	34.45	33.69	0.76	2.68	2.03
53A	25.11	27.65	2.54	27.65	27.44	0.21	2.54	-0.21
54A	29.67	32.47	2.8	32.07	32.53	0.46	2.4	0.06
55A	36.88	38.16	1.28	38.2	*ndt*	-	1.32	-
56A	35.04	36.71	1.67	38.02	39.54	1.52	2.98	2.83
57A	22.51	23.54	1.03	24.62	24.81	0.19	2.11	1.27
58A	16.04	16.14	0.1	17.19	17.32	0.13	1.15	1.18
59A	24.8	24.28	0.52	26.24	25.6	0.64	1.44	1.32
60A	33.54	36.05	2.51	35.07	37.86	2.79	1.53	1.81
61A	30.04	30.72	0.68	31.13	31.48	0.35	1.09	0.76
62A	18.69	18.19	0.5	19.04	18.63	0.41	0.35	0.44
63A	19.23	19.73	0.5	20.41	20.07	0.34	1.18	0.34
64A	29.01	29.31	0.3	30.09	30.11	0.02	1.08	0.8
65A	28.71	28.78	0.07	29.81	29.23	0.58	1.1	0.45
66A	17.3	16.2	1.1	18.77	18.17	0.6	1.47	1.97
67A	27.6	26.68	0.92	28.71	28.62	0.09	1.11	1.94
68A	20.4	19.5	0.9	20.79	20.65	0.17	0.39	1.15
69A	36.8	36.1	0.7	38.14	38.48	0.31	1.34	2.38
70A	26.4	25.9	0.5	27.97	27.31	0.66	1.57	1.41
71A	33.6	34.2	0.6	37.83	35.32	2.51	1.72	1.12
72A	36.97	37.9	0.93	*ndt*	*ndt*	-	-	-
73A	19.72	20.27	0.55	21.1	21.34	0.24	1.38	1.07
74A	25.1	24.4	0.7	26.06	25.87	0.19	0.96	1.47
75A	36.3	37.8	1.2	37.44	*ndt*	-	1.14	-
76A	16.9	15.7	1.2	17.58	17.24	0.34	0.68	1.54

77A	14.9	14.59	0.31	15.86	16.18	0.32	0.96	1.59
78A	34.42	34.09	0.33	37.5	34.95	2.55	3.08	0.86
79A	20.48	20.65	0.17	22.14	21.29	0.85	1.66	0.64
80A	18.35	19.37	1.02	20.83	19.85	0.98	2.48	0.48
81A	22.31	21.84	0.47	22.93	21.62	1.31	0.62	-0.22
82A	21.99	23.53	1.54	23	21.36	1.64	1.01	-2.17
83A	20.53	19.9	0.63	22.01	20.92	1.09	1.48	1.02
84A	18.4	17.74	0.66	19.34	18.48	0.86	0.94	0.74
85A	21.94	21.76	0.18	24.35	23.16	1.19	2.41	1.4
86A	34.13	35.64	1.51	37.27	37.91	0.64	3.14	2.27
87A	20.77	20.28	0.49	22.35	21.27	1.08	1.58	0.99
88A	19.04	20.02	0.98	21.84	20.89	0.95	2.8	0.87
89A	14.69	15.31	0.62	17.13	16.09	1.04	2.44	0.77
90A	29.92	32.67	2.75	32.34	30.97	1.37	2.42	-1.7
91A	15.86	23	7.14	17.93	17.07	0.3	2.07	5.93
92A	*ndt*	38.64	-	*ndt*	*ndt*	-	-	-
93A	*ndt*	38.42	-	*ndt*	*ndt*	-	-	-
94A	*ndt*	37.21	-	*ndt*	*ndt*	-	-	-
95A	36.44	38.32	-	*ndt*	38.03	-	-	-0.29
96A	36.9	*ndt*	-	*ndt*	*ndt*	-	-	-
97A	*ndt*	37.54	-	38.2	*ndt*	-	-	-
98A	35.35	*ndt*	-	38.98	*ndt*	-	3.63	-
99A	*ndt*	36.5	-	*ndt*	*ndt*	-	-	-
100A	*ndt*	37.8	-	*ndt*	*ndt*	-	-	-
101A	*ndt*	35.6	-	*ndt*	*ndt*	-	-	-
102A	*ndt*	38.3	-	38.11	*ndt*	-	-	-
103A	*ndt*	37.51	-	*ndt*	*ndt*	-	-	-
104A	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
105A	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
106A	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
107A	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
108A	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
109A	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
110A	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
111A	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
112A	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
113A	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-

Elaborated by the authors (2023).

"Protocol 2": From the 107 samples tested, 23 presented positive results for *SARS-CoV-2* by the multiplex and singleplex RT-qPCR techniques (Table 3). Eighty-two samples presented negative results by both assays. Two inconclusive results by the singleplex RT-qPCR presented discrepancy results in the multiplex RT-qPCR: one was *SARS-CoV-2*-positive (Ct above 36 for both N1 and N2) and the other was *SARS-CoV-2*-negative by the multiplex technique. The agreement between singleplex and multiplex RT-qPCR assays was 98.1%. The average increase in Ct values was 1.18 ± 0.59 and 0.56 ± 0.67 for the N1 and N2 targets, respectively.

Table 3 - Protocol 2 results: Comparison of Ct values of singleplex RT-qPCR and the multiplex RT-qPCR.

	Singleplex			Multiplex				
Sample	**N1**	**N2**	**Variation N1-N2**	**N1**	**N2**	**Variation N1-N2**	**Variation N1**	**Variation N2**
1B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
2B	18.9	18.82	0.08	19.53	18.11	1.42	0.63	0.71
3B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
4B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
5B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
6B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
7B	24.71	25.12	0.41	25.99	25.22	0.77	1.28	0.1
8B	32.76	33.1	0.34	34.11	32.67	1.44	1.35	0.43
9B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
10B	26.16	25.98	0.18	27.06	25.75	1.31	0.9	0.23
11B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
12B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
13B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
14B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
15B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
16B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
17B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
18B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
19B	20.54	21.5	0.96	22.04	20.66	1.38	1.5	0.84
20B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
21B	27.22	26.47	0.75	27.62	26.61	1.01	0.4	0.14
22B	24.49	24.13	0.36	25.45	24.37	1.08	0.96	0.24
23B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
24B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
25B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
26B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
27B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
28B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
29B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
30B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
31B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
32B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
33B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
34B	35.12	33.63	1.49	36.91	34.23	2.68	1.79	0.27
35B	14.21	13.14	1.07	13.95	13.4	0.55	0.26	0.26
36B	20.93	20.17	0.76	21.18	20.38	0.8	0.25	0.21
37B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
38B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
39B	13.99	13.23	0.76	13.84	13.23	0.61	0.15	0
40B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
41B	*ndt*	37.52	-	*ndt*	*ndt*	-	-	-
42B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
43B	15.89	16.19	0.3	16.91	16.3	0.61	1.02	0.11
44B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
45B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
46B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-

47B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
48B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
49B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
50B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
51B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
52B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
53B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
54B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
55B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
56B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
57B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
58B	16.04	16.55	0.51	17.63	16.29	1.34	1.59	0.26
59B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
60B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
61B	26.13	26.58	0.45	27.28	25.72	1.56	1.15	0.86
62B	31.9	32.9	1	34.3	32.6	1.7	2.4	0.3
63B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
64B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
65B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
66B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
67B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
68B	17.02	16.97	0.05	18.62	17.16	1.46	1.6	0.19
69B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
70B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
71B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
72B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
73B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
74B	21.16	21.24	0.08	22.01	22.82	0.81	0.85	1.58
75B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
76B	32.83	32.97	0.14	34.96	32.48	2.48	2.13	0.49
77B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
78B	18.96	19.43	0.47	20.67	18.64	2.03	1.71	0.79
79B	16.35	16.94	0.59	17.8	16.88	0.92	1.45	0.06
80B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
81B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
82B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
83B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
84B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
85B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
86B	29.19	28.85	0.34	30.3	29.28	1.02	1.11	0.43
87B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
88B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
89B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
90B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
91B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
92B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
93B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
94B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
95B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
96B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-

97B	17.01	17.44	0.43	18.09	16.97	1.12	1.08	0.47
98B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
99B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
100B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
101B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
102B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
103B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
104B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
105B	*ndt*	*ndt*	-	*ndt*	*ndt*	-	-	-
106B	26.51	27.99	1.48	26.19	26.72	0.53	1.48	1.27
107B	*ndt*	39.16	-	36.81	37.04	0.23	-	3.12

Elaborated by the authors (2023).

The RT-qPCR efficiencies regarding the multiplex RT-qPCR were 95.63% and 103.96% for N1 and N2 targets, respectively. For the singleplex RT-qPCR reaction, the efficiency values were 104.3% (N1) and 102.3% (N2). The R2 for each target was found to be higher than 0.95 for both reactions. Serial dilutions of the nucleocapsid RNA transcripts were tested to assess the detection limits and dynamic range of the RT-qPCR assays. The LoD was 20 copies/µl in the singleplex RT-qPCR and 10 copies/µl in the multiplex RT-qPCR (Table 4).

Table 4 - Lower limit of detection of *SARS-CoV-2* in singleplex and multiplex RT-qPCR.

Copies/µL	Ct: average (SD)				Detected/tested		
	Singleplex		Multiplex		Singleplex		Multiplex
	N1	N2	N1	N2	N1	N2	N1/N2
100000	24.98 (0.08)	25.18 (0.03)	23.97 (0.78)	23.53 (0.14)	20/20	20/20	20/20
10000	28.48 (0.4)	28.56 (0.13)	27.24 (0.3)	27.11 (0.16)	20/20	20/20	20/20
1000	32.16 (0.33)	31.77 (0.42)	31.02 (0.09)	30.57 (0.03)	20/20	20/20	20/20
100	35.62 (0.74)	34.98 (0.24)	34.24 (0.24)	33.77 (0.26)	20/20	20/20	20/20
50	35.95 (0.55)	35.51 (0.97)	35.33 (0.37)	34.74 (0.32)	20/20	20/20	20/20
40	36.23 (1.06)	36.09 (1.23)	35.46 (0.19)	34.8 (0.19)	20/20	20/20	20/20
30	36.47 (0.75)	36.81 (0.23)	35.53 (0.52)	35.53 (0.64)	20/20	20/20	20/20
20	36.98 (0.92)	37.18 (1.6)	36.86 (1.26)	35.96 (0.62)	20/20	20/20	20/20
10	38.27 (1.21)	37.81 (0.76)	37.22 (1.01)	36.44 (1.06)	16/20	18/20	20/20

Ct: cycle threshold; SD: standard deviation.

Elaborated by the authors (2023).

DISCUSSION

The COVID-19 pandemic became the accelerator for the development of new detection methods for SARS-CoV-2 to better support the clinicians and front-line healthcare professionals. Rapid and accurate detection of the SARS-CoV-2 is essential for the successful control of COVID-19 (MANNONEN *et al*., 2021). While vaccines have been developed, the use of high-quality diagnostic methods remains essential. The present study aimed to validate a multiplex RT-qPCR assay to detect SARS-CoV-2 and compare it with singleplex RT-qPCR. The efficiencies of singleplex and multiplex

RT-qPCR were similar and suitable for a diagnostic method. LoD (Table 4) was lower in multiplex RT-qPCR (10 copies/µl) compared to multiplex RT-qPCR (20 copies/µl), demonstrating that both were able to detect a minimal number of RNA copies. To compare singleplex and multiplex RT-qPCR assays, two protocols were used.

In the protocol 1, the agreement between singleplex and multiplex was 88.5%. Of the 92 *SARS-CoV-2*-positive, only 6 (6.52%) presented negative or inconclusive results by the multiplex RT-qPCR technique. Noteworthy, these six samples presented Ct values of N1 and N2 above 36. It is worth mentioning that Ct values very close to the threshold limit of the rRT-PCR (Ct = 40.0) may not be reproducible, either due to the technique sensitivity or low viral load in the samples. In the protocol 2, the agreement between singleplex and multiplex RT-qPCR assays was 98.1%. Two inconclusive results by the singleplex RT-qPCR presented discrepancy results in the multiplex RT-qPCR: one was *SARS-CoV-2*-positive (Ct above 36 for both N1 and N2) and the other was *SARS-CoV-2*-negative by the multiplex technique. This result emphasizes the fact that the inconclusive results or results with Ct close to the 40th cycle threshold may not be reproducible.

The variation between the Ct of N1 and N2 targets in the singleplex and multiplex assays, in both the first and second protocols, demonstrates that there is no great variation between protocols, and that multiplex RT-qPCR could be an alternative for COVID-19 diagnosis. Noteworthy, after the validation of the multiplex RT-qPCR assay, this methodology was applied in the routine of the diagnostic laboratory and 2,015 samples were analyzed in the first month of the multiplex use. In this period, we found that the multiplex assay proved to be a practical approach which provided reliable results (Ct variation between N1 and N2 targets was 0.82 ± 0.68) as only 7 samples (0.35%) presented results which required confirmation (data not shown).

RT-qPCR assays for the detection of *SARS-CoV-2* are generally based on targets for the nucleocapsid (N), viral envelope (E), RNA-dependent RNA polymerase (RdRp), ORF1 and spike (S) glycoprotein (BROEDERS *et al.*, 2014; CORMAN *et al.*, 2020; LU *et al.*, 2020; SHIRATO *et al.*, 2020; US CDC, 2020; WAGGONER *et al.*, 2020; WHO, 2020). Among these targets, the N gene is abundantly expressed during infection and appears to be the most conserved, with the least mutations over time (WAGGONER *et al.*, 2020). Studies also demonstrate that the N gene appears to be more sensitive when compared to other targets (PERCHETTI *et al.*, 2020). The multiplex RT-qPCR allows testing larger number of samples per plate, reducing the number of reagents and amount of clinical sample, preparation time, cost, labor, and risk of contamination. In contrast, mixing multiple primer sets can affect the amplification process due to the competition of primer sets for the same reagents, such as dTNPs and enzymes (PARK *et al.*, 2020). We know that major diagnostic routines are subject to errors, and they should be recognized in order to obtain reliable results. Thus, each laboratory when implementing a new methodology must carry out tests that guarantee the correct standardization of the process.

CONCLUSION

In view of the results obtained in this study, the multiplex RT-qPCR using primers for the targets N1 and N2 is comparable to singleplex RT-qPCR and can be implemented by laboratories to reduce time, labor and costs for the molecular diagnosis of COVID-19.

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