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False-negative molecular diagnosis of SARS-CoV-2 in samples with amplification inhibitors

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Abstract

Although rRT-PCR is the gold standard method for SARS-CoV-2 detection, some factors, such as amplification inhibitors presence, lead to false-negative results. Here we describe differences between rRT-PCR results for SARS-CoV-2 infection in normal and diluted samples, simulating the need for dilution due to amplification inhibitors presence. Viral RNA extraction of nasopharyngeal swabs samples from 20 patients previously detected as 'Negative' and 21 patients detected as 'Positive' for SARS-CoV-2 was realized with the EasyExtract DNA-RNA (Interprise®). rRT-PCR was realized with OneStep/COVID-19 (IBMP) kit with normal and diluted (80µl of H_2O RNAse free) samples, totaling 82 tests. The results indicate that there is an average variation ($\alpha < 0.05$) delaying Cq between the amplification results of internal control (IC), N Gene (NG), and ORF-1ab (OF) of 1.811 Cq, 3.840 Cq, and 3.842 Cq, respectively. The extraction kit does not completely purify the inhibitor compounds, therefore non-amplification by inhibitors may occur. In this study, we obtained a 19.04% false-negative diagnosis after sample dilution, and this process reduces the efficiency of rRT-PCR to 29.80% for detecting SARS-CoV-2. Knowing the rRT-PCR standards of diluted samples can help in the identification of false-negative cases, and consequently avoid a wrong diagnosis.

Keywords: COVID-19; rRT-PCR; dilution; viral diagnosis; RNA extraction.

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1. Introduction

The first confirmed case of Coronavirus disease 2019 (COVID-19) in Latin America occurred in Brazil, on February 25, 2020[1]. Since then, until August 2020, Brazil has recorded about 4.1 million cases and about 126 thousand deaths due to COVID-19[2].

Early detection of infected individuals with large-scale testing, immediate isolation of cases with tracking and preventive self-isolation of close contacts, and prompt treatment of severe cases are essential measures to reduce the spread of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)[3].

Therefore, to quickly diagnose infections and mitigate transmission of SARS-CoV-2, the Real-Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) is being used as the primary method in research and hospital laboratories to identify the virus in respiratory samples such as sputum or nasal, throat, nasopharyngeal swabs[4].

rRT-PCR tests typically take 4 to 6 hours to complete, with RNA extraction, amplification, and detection[5]. Considering the limited supply of extraction reagents and test kits worldwide, extraction kits without RNA purification aim to solve this limitation and short the extraction time, thereby shortening the response time[4,5].

However, amplification inhibitors, organic and inorganic substances, can be present in original samples or be introduced in samples transportation, processing, or RNA extraction, causing partial amplification inhibition, leading to a decrease of PCR sensitivity or total inhibition and consequently false-negative results[6].

Extraction kits without RNA purification may need to optimized the rRT-PCR by sample dilution when there are problems with rRT-PCR amplification, thus minimizing the presence of amplification inhibitors[7], allowing amplification even if there are inhibitors or some sample degradation, avoiding the need to request a new sample from the patient, however, it is necessary to know the diluted amplification patterns, avoiding false-negative diagnosis.

Due to the severity of the pandemic, test kits were and are being developed and approved quickly, to meet the worldwide demand for large-scale tests, creating the need for information on real data on the use of these kits in diagnostic laboratories[8]. Here we describe differences between rRT-PCR results for SARS-CoV-2 infection in normal and diluted samples, simulating the need for dilution due to amplification inhibitors presence.

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2. Materials and Methods

2.1. *Nasopharyngeal swab samples of RNA extraction*

Nasopharyngeal swabs samples from 41 patients admitted to the Ministro Costa Cavalcanti Hospital in Foz do Iguaçu, Paraná state - Brazil, were selected. Twenty of these patients were previously detected as 'Negative' and 21 patients were detected as 'Positive' for SARS-CoV-2 infection in rRT-PCR diagnosis. The swabs were stored in tubes with 1x Phosphate-Buffered Saline (PBS 1x), at -20 ºC, until extraction.

The EasyExtract DNA-RNA kit (Interprise®), lot ITBR0720, was validated by comparing the results found using the Applied Biosystems™ MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit results (Thermo Fisher Scientific®), lot 200312, at 1% significance level (N = 96). For the viral RNA extraction, 20µl of reagent EasyExtract DNA-RNA (Interprise®), was mixed with 20µl of PBS 1x from the swab samples in 1,5mL Eppendorf tubes. The tubes have been shaken in a vortex mixer (Kasvi, K45-2810) at 1.050 rpm for 15 seconds, incubated at 95 ºC for 5 minutes, and refrigerated at -20 ºC for RNA stabilization[7].

2.2. *Samples dilution and rRT-PCR for SARS-CoV-2*

The samples were diluted in 80µL of UltraPure® H_2O RNAse free (1:2), totaling 82 tests (41 normal samples and 41 diluted samples).

The rRT-PCR assay was performed using the Biomol OneStep/COVID-19™ Kit (IBMP), lot 200399Z074, 15µL of the Reaction rRT-PCR Mix and 5µL of purified sample RNA (from RNA extraction) or purified Negative Control, were mixed by pipetting up and down, and for positive control, 15µL of the Reaction Mix were mixed with 5µL of Positive Control[9].

The analysis was performed using the QuantStudio™ 5 Real-Time PCR Systems equipment (Thermo Fisher Scientific®), under the conditions: Hold Stage: 50 ºC for 15 minutes (1 cycle), 95 ºC for 3 minutes (1 cycle); PCR Stage: 95 ºC for 15 seconds e 55 ºC for 40 seconds (40 cycles) and Hold Stage: 25 ºC for 10 seconds (1 cycle). The threshold values of the Internal Control (ROX), ORF1ab (FAM),

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and GENE N (HEX / VIC) were 20.000, 30.000, and 40.000 respectively, with a baseline from 5 to 15, according to the IBMP protocol[9].

The results were evaluated by the rRT-PCR amplification standards, amplification values, and submitted to descriptive analysis, normality test and variance analysis (ANOVA), to detect differences between the results before and after dilution.

2.3. *Efficiency of the rRT-PCR*

The analytical efficiencies for detecting SARS-CoV-2 from the normal methodology and after dilution were performed by serial dilution in the following proportions: 1, 1:2, 1:4, 1:8, and 1:10. The results were evaluated by scatter plots and the efficiency values calculated from the R^2 of the linear regression.

3. Results

The results are described in Table 1. Comparisons between diluted and undiluted sample results indicate that there is an average variation ($\alpha < 0.05$) delaying Cq between the amplification results of the internal control (IC), N Gene (NG), and ORF-1ab (OF) of 1.811 Cq, 3.840 Cq, and 3.842 Cq, respectively.

The Cq means of the Internal Control were 29.423 for original samples and 31.280 for diluted samples; for N Gene, the mean Cq of the original value was 25.816 and 29.848 for diluted samples; for ORF-1ab, the average Cq results for samples without dilution were 27.104 against 31.138 for diluted samples.

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Diagnose	Sample ID	IC	IC*	NG	NG*	OF	OF*	ΔIC	ΔNG	ΔOF
Negative	1	26.224	28.376	-	-	-	-	-2.152	-	-
	2	28.941	31.024	-	-	-	-	-2.083	-	-
	3	29.585	31.871	-	-	-	-	-2.286	-	-
	4	29.436	30.428	-	-	-	-	-0.992	-	-
	5	32.186	34.128	-	-	-	-	-1.942	-	-
	6	30.660	32.673	-	-	-	-	-2.013	-	-
	7	27.193	29.721	-	-	-	-	-2.528	-	-
	8	29.451	31.890	-	-	-	-	-2.439	-	-
	9	29.092	31.230	-	-	-	-	-2.138	-	-
	10	25.364	27.492	-	-	-	-	-2.128	-	-
	11	30.608	32.700	-	-	-	-	-2.092	-	-
	12	29.246	31.522	-	-	-	-	-2.276	-	-
	13	30.690	32.037	-	-	-	-	-1.347	-	-
	14	28.590	30.194	-	-	-	-	-1.604	-	-
	15	27.598	28.500	-	-	-	-	-0.902	-	-
	16	27.651	29.382	-	-	-	-	-1.731	-	-
	17	28.691	30.320	-	-	-	-	-1.629	-	-
	18	25.980	27.693	-	-	-	-	-1.713	-	-
	19	28.382	31.124	-	-	-	-	-2.742	-	-
	20	27.813	29.600	-	-	-	-	-1.787	-	-
Positive	21	28.941	30.974	21.011	24.046	19.121	21.320	-2.033	-3.035	-2.199
	22	27.536	29.633	19.276	22.270	24.767	27.180	-2.097	-2.994	-2.413
	23	29.052	31.038	22.909	24.030	27.013	29.706	-1.986	-1.121	-2.693
	24	29.668	31.944	17.969	23.490	25.689	28.211	-2.276	-5.521	-2.522
	25	**29.488**	**32.191**	**21.520**	**35.000**	**28.990**	**40.000**	**-2.703**	**-13.480**	**-11.010**
	26	**30.437**	**32.970**	**20.175**	**32.377**	**28.299**	**40.000**	**-2.533**	**-12.202**	**-11.701**
	27	28.067	29.983	23.868	28.955	24.136	26.759	-1.916	-5.087	-2.623
	28	22.793	23.674	24.278	25.226	25.345	26.093	-0.881	-0.948	-0.748

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29	23.423	25.454	21.491	24.937	21.280	25.490	-2.031	-3.446	-4.210
30	26.926	28.030	17.773	21.417	17.873	21.780	-1.104	-3.644	-3.907
31	30.772	30.907	31.018	31.312	29.833	31.767	-0.135	-0.294	-1.934
32	28.332	30.406	24.273	27.285	23.178	25.684	-2.074	-3.012	-2.506
33	27.453	28.774	28.912	30.416	27.939	29.197	-1.321	-1.504	-1.258
34	29.475	30.981	24.052	25.287	23.684	24.918	-1.506	-1.235	-1.234
35	32.237	32.427	27.702	31.440	27.225	30.644	-0.190	-3.738	-3.419
36	26.155	28.695	27.684	31.167	27.783	30.702	-2.540	-3.483	-2.919
37	29.219	31.453	27.088	30.406	26.308	29.036	-2.234	-3.318	-2.728
38	28.364	30.639	24.162	28.913	24.462	27.117	-2.275	-4.751	-2.655
39	**31.006**	**32.548**	**32.500**	**35.380**	**32.914**	**40.000**	**-1.542**	**-2.880**	**-7.086**
40	**31.776**	**32.410**	**34.074**	**35.756**	**32.333**	**40.000**	**-0.634**	**-1.682**	**-7.667**
41	32.434	34.160	24.577	27.840	23.906	27.165	-1.726	-3.263	-3.259

Table 1. Results of the rRT-PCR ΔCq amplification of normal and diluted samples. The variations were calculated considering the values of the original samples as the true Cq. *: samples diluted in 80µL, IC: Internal Control Cq, NG: N Gene Cq, OF: ORF-1ab Cq, Δ: Cq variation.

Before dilution, samples 25, 26, 39, and 40 showed lower values than the cut-off stipulated for ORF-1ab, being considered positive. After dilution, they all shifted the ORF-1ab Cq values to the right, and are then considered negative due to non-amplification within the cut-off parameters (Figure 1).

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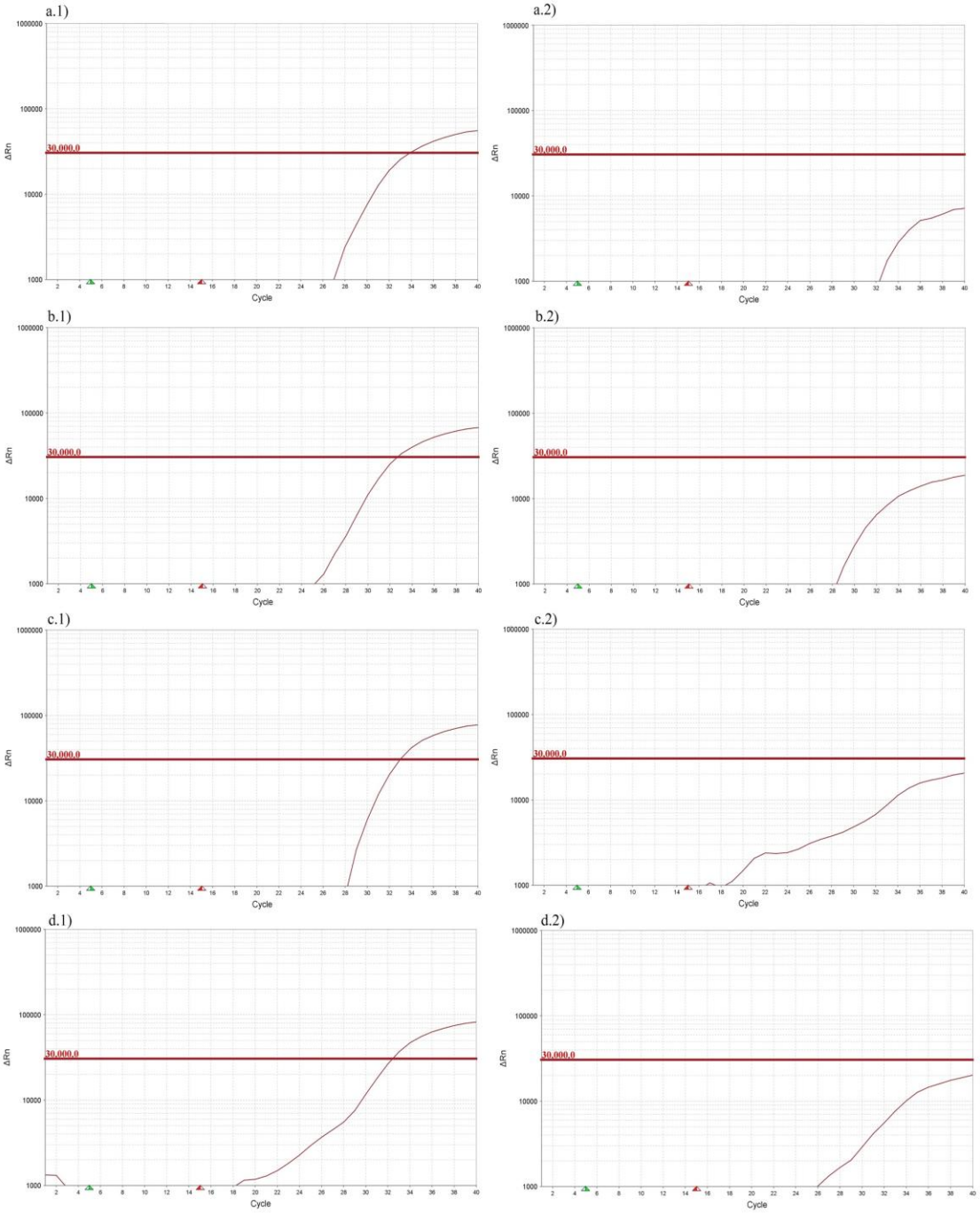

Figure 1. Amplification of ORF-1ab from samples 25 (a), 26 (b), 39 (c) and 40 (d) before (.1) and after (.2) dilution.

The normality test considered normal values for Internal Control and N Gene ($\alpha > 0.05$), however, for ORF-1ab the values were considered out of normal ($\alpha < 0.05$). When removing outliers,

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the data returns to normal, indicating that the amplification values of samples 25, 26, 39, and 40 are not within the expected range, indicating a great variation with the other samples ($\alpha > 0.05$).

The amplification efficiency for the normal sample was 99.79% for CI, 99.51% for NG, and 97.09% for OF. For the diluted sample, the amplification efficiency was 98.88% for CI, 78.33% for NG, and 67.29% for OF, indicating a decrease of 21.18% for NG detection and 29.8% for OF detection.

4. Discussion

The positive control showed amplification for the 3 targets evaluated in all tests ($Cq \leq 35$) and the negative control did not show any amplification for the three evaluated targets, according to the mix manufacturer's protocol, validating the results.

The tests performed to demonstrate 1:2 dilutions were interesting to obtain a reliable amplification in samples with inhibitors, as shown in Figure 2. However, it is important to pay attention to the result curve patterns after dilution.

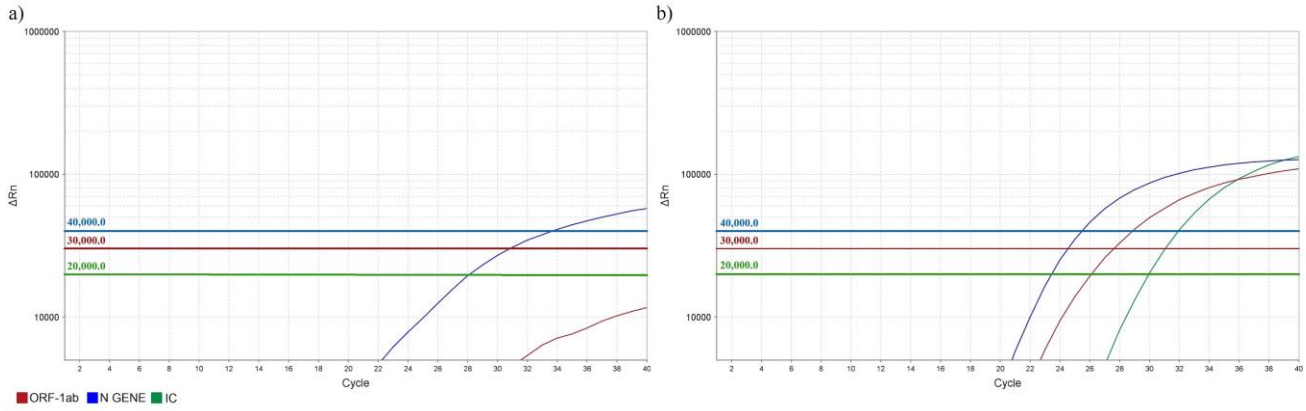

Figure 2. Differences in amplification patterns of a sample with inhibitors before (a) and after (b) dilution.

In the example shown in figure 2.a, the sample without dilution did not obtain amplification of the internal control (Cq = undetermined value) and OF patterns (Cq = undetermined value), and NG (Cq = 29.995) not defined and not showing a perfect exponential curvature. After dilution (b), the perfect amplification of the three markers is perceived, indicating a superior sample quality and the absence of

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inhibitors. The Cq values of the amplifications were 29.951 for the IC, 25.444 for the NG, and 27.579 for OF.

In cases like in Figure 2, only dilution is sufficient to diagnose the sample as positive, avoiding new patient stress when redoing the collection, and new exposure by the health professional, sending the infected patient to the correct area for treatment in a short time, with a low period between collection and diagnosis. However, in cases where the result after the dilution is negative, a series of precautions should be taken when releasing the diagnosis, such as curve characteristics, evaluating the graph completely and not just the values that exceeded the Cq.

According to the Thermo Fisher® manual, considering a process efficiency of 100%, there is a known variation in Cq when the sample is diluted, being variable according to the proportion of the dilution. This dilution variation can be ΔCq = 1 from 1:2, ΔCq = 2 from 1:4, ΔCq = 3 to 1:8 and ΔCq = 3,3 from 1:10. However, these values vary according to the efficiency of the process and presence of inhibitors[10], which can result in false-negative diagnosis in low viral load samples, depending on the value used as a parameter to distinguish between positive and negative.

Positive samples tested that had divergent results after dilution obtained ΔCq values between 28 and 33, which should not make them negative after dilution even with low efficiency in the amplification process, since there was a variation between 8 and 12 Cq (Figure 1). Considering the progression of ΔCq according to greater dilutions, the dilution proposal by the manufacturer of the viral RNA extraction kit (1:10) would not be interesting, as theoretically 1:10 would cause the Cq values to be even later. Larger tests involving smaller dilutions can be performed, verifying in what proportion there would be no significant differences in the Cq values and effectiveness in the dilution of rRT-PCR inhibitors.

The importance of performing rRT-PCR in kits that provide internal control marking has already been reported by Kim et al.[10], generating conclusive results about the extraction process, avoiding the release of false-negative results in samples that were not amplified with precision since the interpretation of results is not always straightforward. The sensitivity of the rRT-PCR is negatively impacted by compounds present in the clinical sample that may partially or completely inhibit rRT-PCR chemistries[11-15].

Protocols with purification steps can avoid the presence of amplification inhibitors, removing potential endogenous rRT-PCR inhibitors such as detergents, chelating compounds, and guanidinium HCl[11,13,16-19]. The efficiency of removing inhibitors in patient samples may be related to the intrinsic properties of the method used to extract the RNA[20], which does not happen in the kit used in this study.

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The Easy Extract™ kit does not completely purify the inhibitor compounds, which significantly reduces the extraction time, however, non-amplification by inhibitors may occur.

A diagnostic error can lead infected patients to non-COVID-19 areas with the subsequent risk of infection for others; or patients which are negative SARS-CoV-2 sent to COVID-19 areas[21], generating possible contamination to uninfected patients and also the spread of viruses in the disinfected areas, which can lead to viral spread within hospitals and treatment centers, and contaminate the health workers. Knowing the rRT-PCR standards of diluted samples can help in the identification of false-negative cases, and consequently avoid a wrong diagnosis.

5. Conclusion

The 1:2 dilution of the sample with inhibitors in UltraPure® H_2O RNA*se free* generated amplification in 100% of the tested cases, being an alternative to avoid new sample collection in the patient. However, we emphasize that in this study we obtained 19.04% false-negative diagnosis after sample dilution, and this process reduces the efficiency of rRT-PCR to 29.8% for detecting SARS-CoV-2. It is possible to infer that the dilution helps in cases where a new sample collection is not feasible, but caution is needed in the evaluation of the result of the rRT-PCR.

It is important to assess the pattern of the amplification curves after dilution to avoid inaccurate diagnosis. If the sample with inhibitors is positive with a high viral load, the result will be reliable if Internal Control and Gene N amplification occur up to Cq 30 and ORF-1ab up to Cq 35. In the case of non-amplification of the N Gene and ORF-1ab curve after dilution, we recommend assessing the need for a new sample and new analysis.

The authors declare no conflict of interest.

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