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Short communication

Evaluation of long-term stability of SARS-CoV-2 nucleic acid extracted from human nasopharyngeal samples

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ABSTRACT

The maintenance of SARS-Cov-2 RNA samples poses a new challenge for laboratories and researchers. In addition, it is a requirement in order to identify what strain of the new coronavirus is predominant in a region, for instance. Therefore, it is a must to keep the quality and viability of stored RNA to respond to this and other valid questions. In other to test the quality of our samples and storing protocols, we randomly checked RNA samples four different times over one year using a second RT-PCR assay after the first test. The virus genes, N1 and N2, showed no significant increase in the media of the CT value between the first assay and subsequent times with $p > 0.05$. However, the human RP gene showed differences in the first three times analyzed, but within the acceptable sample cut-off, according to the test manufacturer. After one year, the RNA extracted from human nasopharyngeal specimens are viable to detect the virus SARS-CoV-2 genes with minor changes.

1. Introduction

Diagnostic tests for COVID-19 have stood out as important tools in screening for the spread of the new coronavirus. More than a year after the beginning of the pandemic, laboratories keep diagnosing SARS-CoV-2 by Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR). This technique is still the gold standard for virus detection even with the advent of alternative methodologies (Brault et al., 2021; Corman et al., 2020). In Brazil, local health institutions recommend the retention of primary naso/oropharyngeal swab samples indefinitely; however, the storage of the extracted RNA sample is at the discretion of each laboratory (Pernambuco, 2020). Many laboratories faced great challenges on different fronts, including a great increase in the number of samples. This sample overflow led to delays in processing, extracting RNA and specially in executing the RT-qPCR, and it has resulted in failures due to viral RNA degradation.

Thus, as strategy to minimize delays and optimize the workflow, some diagnostic centers are extracting the RNA from the nasopharyngeal samples and keeping them at low temperatures before testing by RT-qPCR. As a known fact, the conditions under which the RNA was handled certainly define the quality of samples (Perumal et al., 2020). Furthermore, the extracted RNA from positive samples that was properly stored can be sent for screening of variants by sequencing in Reference Centers. As a result, the epidemiological monitoring of the investigated areas becomes clearer (Freitas et al., 2021). Nevertheless, it is very important to verify more precisely the RNA stability under low temperatures, especially at − 80 °C, and at different storage periods. Therefore, to evaluate our biorepository of positive SARS-CoV-2 RNA samples, we carried out a long-term viability test on a representative amount of them.

Abbreviations: Ct, cycle threshold; SD, Standard deviation.

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2. Material and methods

The viability test consisted of replicating the RT-qPCR on a time series after the first assay. For this, SARS-CoV-2 positive RNA samples collected from the nasopharynx were randomly selected for the study. The molecular detection of SARS-CoV-2 consisted of three sequential distinct steps: first, the tubes containing Viral Transport Media (VTM) solution were freshly aliquoted into a 2.0 mL sterile tube. Then, there was an automated extraction of the RNA using the Maxwell® RSC Viral TNA Kit (AS1330) in the Maxwell RSC 48 Instrument (Promega, Wisconsin, USA). Moreover, the RT-qPCR was carried out in a one single step using GoTaq® Probe 1-Step RT-qPCR System (Promega, Wisconsin, USA) and primers/probes 2019-nCoV_N1/N2/RP CDC Kit (Atlanta, USA). According to the manufacturer's recommendation, the detection of an amplification in a cycle with values lower than 40 in both N1 and N2 targets characterizes the presence of the virus. The RNA samples were stored at − 80 °C and thawed only once to perform the test. There were four rounds of tests, done in four different times. Each viability test cycle had 20 different samples per set. No sample was reused on a different cycle. Each cycle was done 3, 6, 9 and 12 months after the first RT-qPCR. We analyzed the coefficient of variation between the Cycle threshold (C*t*) values in two-time points for the two genes used for SARS-CoV-2 identification, as well for the human endogenous control. The PRISM software 6.0 version (San Diego, USA) analyzed data using the paired t test, after D'Agostino-Pearson omnibus normality test confirmed Gaussian distribution.

3. Results

As shown in Table 1, there was no significant difference between the mean CT value of viral genes N1 and N2 at time zero when compared to the subsequent time interval of 3, 6 and 9 months in all sample groups. However, the gene that encodes the RP presented a significant difference after of the first assay in the three analyzed periods. Only the analysis period after 1 year had no significant difference between Ct values for the RP genes. Both N1 and N2 genes had the coefficient of variation increased in all tests evaluated, with N2 being the most unstable target, showing the higher coefficient of variation at 6, 9 and 12 months.

Table 1
Stability of SARS-CoV-2 RNA after different periods of storage.

Genes	Storage time/ CT values Mean ± SD (n = 20 per series)		p value[#]	Coefficient of variation Mean ± SD (%)
	Day 0	**03 months**		
N1	30.13 ± 4.1	30.82 ± 4.5	0.07	3.3 ± 2.2
N2	32.15 ± 5.1	32.02 ± 4.7	0.6430	2.3 ± 1.8
RP	27.17 ± 1.9	28.26 ± 1.9	< 0.001*	2.8 ± 1.9
	Day 0	**06 months**		
N1	23.51 ± 6.2	23.55 ± 6.5	0.9106	3.2 ± 2.7
N2	24.52 ± 6.3	25.07 ± 6.8	0.2684	4.1 ± 3.3
RP	26.30 ± 1.8	27.26 ± 1.9	0.002*	3.1 ± 2.7
	Day 0	**09 months**		
N1	24.65 ± 5.8	24.99 ± 5.7	0.8134	8.64 ± 14.1
N2	25.87 ± 5.6	25.50 ± 6.46	0.7497	10.15 ± 14.6
RP	27.73 ± 2.3	30.18 ± 2.2	< 0.001*	6.31 ± 5.7
	Day 0	**12 months**		
N1	25.17 ± 5.0	26.48 ± 4.4	0.3543	13.19 ± 11
N2	26.10 ± 5.46	23.47 ± 3.9	0.0832	14.78 ± 12.2
RP	27.79 ± 1.8	27.91 ± 1.7	0.4886	1.43 ± 1.4

Ct: cycle threshold; SD: Standard deviation. # Comparison between two evaluated times.

4. Discussion

We evaluated SARS-CoV-2 RNA samples to ensure the viability and quality of our biorepository for further investigations. The results achieved over one year showed that the nucleic acids were detectable after one thawing with minor changes.

Li and colleagues evaluated the processing conditions, transport and storage of throat swab samples immediately after collection. They reported that after freezing and thawing twice, almost all samples increased in up to 0.8 the ORF1ab gene Cts detection (Lin et al., 2020). Here, we also demonstrate an increase in CT detection of N1 and N2 progressively over the time in some samples, despite this increase was not significant between the times.

CT value is inversely proportional to the amount of nucleic acid at the collecting time of the sample. This could be a useful parameter to direct samples for genomic sequencing, for instance. Pillay and colleagues demonstrated a clear relationship between Ct value and genome coverage. They stated a cutoff threshold with CT below 27.0 cycles to obtain optimal full-length genomes (Pillay et al., 2020). As a result, we observed that after one year, most of the samples that had CT below 27.0 in the first testing remained at this C*t* value range. Aligning this reporting with our results, it is expected that samples that had a CT value below 27.0 in the first testing will be suitable for sequencing even after one year, if stored at − 80 °C and not thawed more than one time.

Additionally, the behavior of the N2 gene was consistent with what Vogel and colleagues previously described about the N1 being more sensible than N2 (Vogels et al., 2020). They also used the set of primers-probes N1 and N2 for N gene recommended by the Center for Disease Control (CDC) from the Unites States of America (CDC, 2020). Furthermore, the method of extraction used in this investigation ensures refined samples than manual extraction methods for instance, and, thereby, translating into longer-lasting stability (Lewis, 2021).

Interestingly, the human Rnase gene showed a significant difference between the first 3 times evaluated. This gene is used to assess specimen quality, thus indicating the presence of sufficient nucleic acid from human sample. For this gene, our samples had acceptable CT's detection after second assay, once most of specimens crossed up the threshold before 35 cycles[8]. Furthermore, virus RNA may contain stabilizing elements that evade as decay pathways in host cells. Therefore, this may explain why RNAse P, molecular of human origin, showed greater instability than viral genes (Krishna and Shinji, 2013).

While our study gives some insights on the viability and quality of stored samples, it still has some limitations. The greatest ones were the sample size and different sample groups per series. The physical storage space was the limiting factor, which made us store only one aliquot of freshly transported media for all received cases and the RNA from just positive ones. Therefore, we did not have RNA aliquots from the same sample to be used in our time series analyses. Moreover, as mentioned above, reusing the same sample multiple times would add thawing bias.

Assuming optimal conditions of storage and manipulation, we evidenced the viability of SARS-CoV-2 RNA after 12 months of extraction. We believe that laboratories that have a biobank of SARS-CoV-2 RNA, under ideal conditions, would be able to reproduce this analysis as a quality control tool.

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CRediT authorship contribution statement

Priscilla Stela Santana de Oliveira: Formal analysis, Investigation, Writing original draft. **Barbara Oliveira da Silva:** Execution**,** Writing original draft. **Rômulo Pessoa e Silva:** Formal analysis and writing original draft. **Lília Vieira Galdino:** Execution and writing original draft. **Vanessa Mylenna Florêncio de Carvalho and Anderson Almeida:** Execution; **Moacyr Rêgo and Michelle Rosa** Writing-Reviewing and Editing. **Maira Pitta:** Funding acquisition, Project administration, Writing- reviewing **Michelly Pereira**: Conceptualization, Project administration Writing- reviewing and editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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