Stability of SARS-CoV-2 RNA in Samples Stored in Ultrapure Water at –80 °C for up to 24 Months

Estabilidade do RNA do SARS-CoV-2 em amostras armazenadas em água ultrapura a –80 °C por até 24 meses

Matheus Victor de Souza Laurentino[1,2], Sérgio Dias Costa-Júnior[3], Cristiane Oliveira Santos[1], Fabrine Felipe Hilário[1], Francisco Allysson Assis Ferreira Gadelha[2], Marcia Regina Piuvezam[2], Sandrelli Meridiana de Fátima Ramos dos Santos Medeiros[1], Eduardo Sérgio Soares Sousa[1,4], Eloiza Helena Campana[1,5], Naiara Naiana Dejani[1,3]

[1] Universidade Federal da Paraíba, Laboratório de Biologia Molecular. João Pessoa, PB, Brazil.

[2] Universidade Federal da Paraíba, Programa de Pós-graduação em Produtos Naturais e Sintéticos Bioativos. João Pessoa, PB, Brazil.

[3] Universidade Federal da Paraíba, Departamento de Ciências Biomédicas. João Pessoa, PB, Brazil.

[4] Universidade Federal da Paraíba, Departamento de Obstetrícia e Ginecologia. João Pessoa, PB, Brazil.

[5] Universidade Federal da Paraíba, Departamento de Ciências Farmacêuticas. João Pessoa, PB, Brazil.

Abstract

During the COVID-19 pandemic, the detection of SARS-CoV-2 infection by nucleic acid amplification assays was a crucial tool for epidemiological surveillance and control strategies. Prospective and retrospective analyses of viral genomes may reveal important information about viral adaptation and evolution across different epidemiological periods, and the quality of stored viral genome samples is critical for such analyses. In this study, the stability of SARS-CoV-2 RNA samples stored after extraction from human nasopharyngeal swab specimens was evaluated over a two-year period. SARS-CoV-2 RNA samples were randomly selected to assess the detection of the ORF1ab and N genes after long-term storage in DNase/RNase-free water at –80 °C. A multiplex RT-qPCR assay was performed to determine Ct values for the N and ORF1ab genes after different storage periods (six, 12, 18, or 24 months) and compared with the Ct values obtained before storage (time zero). Ct values did not differ after six and 12 months compared with time zero for all targets. The N gene showed increased Ct values at 18 and 24 months compared with time zero, whereas the ORF1ab gene was the most stable target, showing no significant differences in Ct values even after 24 months of storage. Collectively, our results suggest a slight reduction in SARS-CoV-2 RNA stability after 12 months of storage and provide useful insights into the stability of viral RNA samples stored long-term for retrospective genomic surveillance.

Keywords: COVID-19; pandemic; RNA virus; RT-qPCR.

Resumo

Durante a pandemia de COVID-19, a detecção da infecção por SARS-CoV-2 por meio de ensaios de amplificação de ácidos nucleicos foi uma ferramenta crucial para a vigilância epidemiológica e para as estratégias de controle. Análises prospectivas e retrospectivas do genoma viral podem revelar informações importantes sobre a adaptação e a evolução do vírus em diferentes períodos epidemiológicos, e a qualidade das amostras virais armazenadas é fundamental para esse tipo de análise. Neste estudo, a estabilidade de amostras de RNA do SARS-CoV-2 armazenadas após extração de swabs nasofaríngeos humanos foi avaliada ao longo de um período de dois anos. Amostras de RNA do SARS-CoV-2 foram selecionadas aleatoriamente para avaliar a detecção dos genes ORF1ab e N após longos períodos de armazenamento em água livre de DNase/RNase a –80 °C. Foi realizado um ensaio de RT-qPCR multiplex para determinar os valores de Ct dos genes N e ORF1ab após diferentes períodos de armazenamento (seis, 12, 18 ou 24 meses) e compará-los com o valor de Ct obtido antes do armazenamento (tempo zero). Os valores de Ct não diferiram após seis e 12 meses em comparação com o tempo zero para todos os alvos. O gene N apresentou aumento nos valores de Ct aos 18 e 24 meses quando comparado ao tempo zero, enquanto o gene ORF1ab foi o alvo mais estável, não apresentando diferenças significativas nos valores de Ct mesmo após 24 meses de armazenamento. Em conjunto, os resultados sugerem uma redução na estabilidade do RNA do SARS-CoV-2 após 12 meses de armazenamento e fornecem informações relevantes sobre a estabilidade de amostras de RNA viral armazenadas por longos períodos para vigilância genômica retrospectiva.

Palavras-chave: COVID-19; pandemia; vírus de RNA; RT-qPCR.

Correspondence
Sérgio Dias Costa-Júnior
E-mail: sergio.costa@ufpe.br

Received on 03/31/2025 | Approved on 08/22/2025 | DOI: 10.21877/2448-3877.202600229.en

INTRODUCTION

Since 2020, the world has faced the Coronavirus disease (COVID-19) pandemic, a severe respiratory disease caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). SARS-CoV-2, a virus belonging to the species *Betacoronavirus pandemicum* and the genus *Betacoronavirus*, was isolated for the first time in Wuhan, China.[1] There has been significant mobilization to control and treat COVID-19, and the prompt diagnosis of viral infection has been crucial for an effective emergency response.[2]

Laboratory diagnosis of viral infections can be performed using several different methods, including detection of the viral genome through nucleic acid amplification tests (NAATs), detection of viral antigens or the presence of patient-specific antibodies through immunological assays, and less common approaches such as visualization of viruses by electron microscopy and detection by cell culture methods.[3,4] NAATs are more accurate and sensitive than immunological assays and are faster and less complex than cell culture or electron microscopy methods.[5,6]

SARS-CoV-2 has a positive-sense single-stranded RNA genome of approximately 29.9 kb, encoding 13 open reading frames (ORFs).[7] Viral RNA detection in patient specimens by RT-qPCR, a NAAT methodology, has been universally used as the gold standard method to confirm viral infection. A cornerstone of the RT-qPCR method is the initial step of extracting and purifying viral nucleic acid from patient specimens. Several respiratory tract specimens (saliva, nasopharyngeal swabs, sputum, and bronchial fluid) can be used for RNA extraction.[2,8] Since the viral genome is an RNA molecule, detection involves the use of a reverse transcriptase (RT) step to synthesize complementary DNA (cDNA) from the RNA genome, followed by real-time polymerase chain reaction to amplify specific cDNA sequences using gene-specific primers and fluorescent probes.[9]

The main limitations of RT-qPCR are related to issues in sample collection, sample storage, and the initial steps of RNA extraction and purification, which may lead to RNA degradation and contamination with PCR inhibitors. Additionally, genomic mutations may result in reduced sensitivity.[10] Furthermore, the quality and stability of viral genomic RNA are important variables for retrospective genomic surveillance studies after long periods of storage.[11-18] The stability of biological products during ultra-low-temperature freezing can be influenced by several factors, such as sample volume, which directly affects the freezing rate and freezing time. In addition, the effects of cryoconcentration and mechanical stress related to the freezing process play a significant role in product stability.[12]

Considering the importance of maintaining sample integrity for retrospective genomic studies, this study aimed to evaluate the stability of SARS-CoV-2 RNA stored in ultrapure water at −80 °C for up to 24 months using RT-qPCR.

MATERIAL AND METHODS

Genomic RNA of SARS-CoV-2 was extracted and purified from nasopharyngeal swab samples using the automated Maxwell Rapid Sample Concentrator Instrument (Promega®, USA) and the RSC Viral Total Nucleic Acid Purification Kit (Promega®, USA), resulting in a final eluate of 75 µL of DNase/RNase-free water (ultrapure water), according to the manufacturer's instructions. After extraction and purification, the samples were immediately subjected to viral RNA detection for diagnostic purposes (time zero) and then stored at −80 °C in an ultra-low temperature (ULT) freezer (Indrel Scientific®, Brazil).

For this study, samples containing SARS-CoV-2 RNA detected by RT-qPCR from symptomatic patients, with no previous freeze–thaw cycles and with a cycle threshold (Ct) < 35, and stored for six, 12, 18, or 24 months at −80 °C were randomly selected (n = 20 per period). Detection of the N and ORF1ab targets of SARS-CoV-2 was performed by RT-qPCR using the OneStep/COVID-19-IBMP Kit (Instituto Brasileiro de Biologia Molecular do Paraná, Brazil), according to the manufacturer's instructions, in a QuantStudio 3 thermocycler (Thermo Fisher Scientific®, USA). The probes used were FAM for ORF1ab, HEX/VIC for the N gene, and ROX for the RNA extraction internal control (IC). Positive and negative controls for the evaluated gene targets were included in all RT-qPCR assays to ensure the reliability of the results. The Ct values were subjected to the Anderson–Darling normality test, followed by a paired t-test. The coefficient of variation of Ct values was evaluated for the SARS-CoV-2 targets (N and ORF1ab) and IC at time zero and after six, 12, 18, or 24 months of storage. In addition, samples were analyzed in duplicate on two independent RT-qPCR plates.

A significance level of $p < 0.05$ was considered statistically significant. Statistical analysis was performed using GraphPad

Prism software, version 9.0.0 (GraphPad Software Inc., San Diego, CA, USA). This study was approved by the Research Ethics Committee of the Universidade Federal da Paraíba (UFPB) (approval number 4,026,905).

RESULTS

As shown in Table 1, there was no significant difference in the mean Ct value of the human internal control target (IC) at time zero compared with six, 12, and 18 months of storage. However, after 24 months of storage (28.00 ± 0.33 to 29.22 ± 0.70), there was a significant increase in the mean Ct value for IC.

In the analysis of SARS-CoV-2 targets, an increase in the Ct value was observed for the N gene after 18 (22.35 ± 4.40 to 23.45 ± 4.23) and 24 (24.20 ± 3.7 to 25.75 ± 3.38) months compared with time zero. Detection of the ORF1ab gene did not differ over two years; there was no significant difference in the mean Ct value for ORF1ab after 24 months compared with time zero.

All SARS-CoV-2 targets showed an increase in the mean coefficient of variation (CV). For the SARS-CoV-2 targets, the lowest mean CV was 2.64% (N gene after six months), and the highest was 4.19% (ORF1ab after six months). The IC also showed increased mean CV values over time, ranging from 3.46% (after 18 months) to 6.16% (after six months).

Table 1

Analysis of SARS-CoV-2 RNA stability at different storage periods.

Target	Storage periods (Mean ± SD) (n=20 per period)		*P** Value	Coefficient of variation (Mean ± SD)
	Time zero	06 Months		
Internal control	25.40 ± 6.80	27.40 ± 4.24	0.317	6.16% ± 8.40%
N	22.90 ± 3.43	22.40 ± 3.89	0.056	2.64% ± 3.11%
ORF1ab	22.65 ± 4.73	21.58 ± 3.76	0.250	4.19% ± 3.32%
	Time zero	12 Months		
Internal control	28.35 ± 3.23	26.22 ± 2.21	0.050	4.47% ± 7.48%
N	23.10 ± 4.49	23.00 ± 3.92	0.803	3.15% ± 3.32%
ORF1ab	22.10 ± 3.71	22.30 ± 3.72	0.463	3.23% ± 2.10%
	Time zero	18 Months		
Internal control	27.10 ± 2.42	26.95 ± 6.92	0.491	3.46% ± 3.45%
N	22.35 ± 4.40	23.45 ± 4.23	0.012*	4.06% ± 4.96%
ORF1ab	22.80 ± 4.51	22.55 ± 4.45	0.675	4.37% ± 6.62%
	Time zero	24 Months		
Internal control	28.00 ± 0.33	29.22 ± 0.70	0.0479*	4.92% ± 1.24%
N	24.20 ± 3.70	25.75 ± 3.38	0.004**	3.83% ± 3.02%
ORF1ab	23.40 ± 4.07	24.05 ± 3.79	0.389	3.74% ± 5.46%

SD: Standard variation.

DISCUSSION

Previous studies have demonstrated that respiratory samples positive for SARS-CoV-2 RNA can be maintained for up to six months at temperatures equal to or below −70 °C without compromising sample integrity.[19,20] In the present study, the evaluation of extracted and purified SARS-CoV-2 RNA samples stored for different periods demonstrated that after 12 months of storage at −80 °C, RNA detection remained without significant differences in RT-qPCR results, corroborating the findings of De Oliveira et al. (2022)[11] regarding SARS-CoV-2 RNA quality. However, our study showed that after 18 and 24 months there were significant increases in the mean Ct value of the N target.

In this study, the Ct value of the N target increased by 1.1 after 18 months and by 1.55 after 24 months. One possible explanation for this increase is that freeze–thaw cycles can reduce RNA quality and consequently increase Ct values. It has been reported that multiple freeze–thaw cycles increase Ct values of SARS-CoV-2 targets by approximately 0.08 per cycle.[18] Additionally, Dzung et al. (2020),[12] in their evaluation of the detectability of SARS-CoV-2 RNA from swab samples subjected to freeze–thaw cycles, observed increases in Ct values of 0.10 to 0.19 per cycle for three SARS-CoV-2 targets (S, N, and ORF1ab). In another study, Li et al. (2020) [13] reported that freeze–thaw cycles affected the detection of SARS-CoV-2 RNA extracted from nasopharyngeal swab samples, resulting in an increase of 0.41 in the Ct value of the ORF1ab gene after one freeze–thaw cycle and 0.82 after two cycles.[13] In contrast, in the present study, freeze–thaw cycles were not considered a variable, since the RNA samples in ultrapure water were tested for SARS-CoV-2 and stored at −80 °C, being thawed only once at the end of the storage period to evaluate RNA stability.

In this study, the samples showed CV values between 2.6% and 4% for the SARS-CoV-2 targets. Previous research has demonstrated that samples with higher Ct values tend to present higher CVs when compared with a second RT-qPCR assay.[11,20] Samples with an initial Ct around 36 showed an average CV of approximately 10%, whereas samples with an initial Ct around 27 showed CV values between 2% and 3%.[20] Here, an increase in the coefficient of variation for N, ORF1ab, and IC was observed in samples with mean Ct values at all evaluated time points compared with time zero, as previously reported for the N1 and N2 genes of SARS-CoV-2 after 12 months of storage.[11]

In all periods, the Ct values of SARS-CoV-2 gene targets remained below 30 cycles, indicating that even with reduced stability of the N target after 18 and 24 months, the Ct values remained suitable for sequencing purposes, since SARS-CoV-2 sequencing studies commonly consider Ct < 30 for viral targets as an inclusion criterion to obtain higher sequencing yield.[21,22] Thus, even after long periods of storage at −80 °C in water, the extracted and purified samples remained viable for retrospective SARS-CoV-2 sequencing and genomic surveillance studies, especially when the initial Ct values were low. SARS-CoV-2 genome sequencing studies are extremely important to provide insights into viral evolutionary and epidemiological dynamics.[23,24] Furthermore, genomic surveillance is necessary to track virus evolution and spread, and to optimize molecular diagnostics and vaccine production.[25]

This study has limitations. The analysis relied exclusively on RT-qPCR Ct values, without complementary assessments of RNA integrity. Additionally, the sample size per group was modest, and the storage conditions may not reflect more variable real-world conditions such as transport or repeated thawing. Future studies incorporating different storage buffers, quantitative integrity metrics, and stratification by viral load may provide broader validation of these findings.

CONCLUSION

This study evaluated the long-term stability of SARS-CoV-2 RNA samples stored in ultrapure water at −80 °C and demonstrated that the samples remained suitable for analysis for RT-qPCR analysis for up to 24 months, with minor increases in Ct values after 18 months. These findings support the use of long-term stored samples in retrospective genomic surveillance studies and reinforce the importance of ultra-low-temperature storage for viral RNA biobanking.

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