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Evaluation of Integrated SARS-CoV-2 Genome Presence in PBMC, Oropharyngeal, and Nasopharyngeal Samples of COVID-19 Patients

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Abstract

Background: Persistent detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA in individuals who have recovered from coronavirus disease 2019 (COVID-19) remains an unexplained phenomenon warranting further study. Recent research suggests that this RNA could be the result of transcription from an integrated SARS-CoV-2 genome.

Objectives: This study aimed to investigate the presence of the DNA form of the SARS-CoV-2 genome in oropharyngeal, nasopharyngeal, and peripheral blood mononuclear cell (PBMC) samples from COVID-19 patients with prolonged viral detection.

Methods: We examined the presence of the reverse-transcribed viral genome in samples from eighty COVID-19 patients, including 40 outpatients (group 1), 40 hospitalized patients (group 2), and 40 healthy individuals (group 3), using a TaqMan® based real-time RT-PCR assay.

Results: The mean ages of groups 1, 2, and 3 were 36.1 ± 11.0, 61.6 ± 18.4, and 39.0 ± 8.7, respectively. The molecular tests did not detect viral DNA forms, which may be produced during the SARS-CoV-2 life cycle, in the examined samples.

Conclusions: Although no evidence of integrated viral DNA was found in this study, further research is essential to confirm these findings and explore the underlying mechanisms of prolonged SARS-CoV-2 RNA presence in recovered COVID-19 patients.

Keywords: SARS-CoV-2, Integration, Reverse Transcription, COVID-19

1. Background

As of November 27, 2022, coronavirus disease (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in over 645 million confirmed cases and more than 6.6 million deaths (1). Severe acute respiratory syndrome coronavirus 2 infection can lead to a range of clinical outcomes, from asymptomatic carriers to patients with severe acute respiratory disease (ARD) (2-4). Typically, PCR tests turn negative within three weeks for patients recovering from COVID-19; however, there are reports of prolonged positive PCR tests in recovered individuals, persisting for months post-recovery despite non-infectivity (5). While re-infection cases have been documented, cohort studies indicate that some instances of PCR re-positivity months after recovery are not due to re-infection, as these individuals remained in quarantine (6). The phenomenon of prolonged or recurrent viral RNA shedding remains poorly understood due to the novel nature of the disease and warrants further study (6). Studies have detected SARS-CoV-2 RNA in peripheral blood mononuclear cell (PBMC) samples by analyzing sequence data from various studies (7).

Research shows that SARS-CoV-2 is a positive-sense single-stranded RNA virus (8, 9). Recent studies, however, suggest that SARS-CoV-2 DNA may be produced via reverse transcription from the viral RNA genome. This DNA could potentially integrate into the host

genome, leading to the production of viral RNA through host-dependent transcription pathways (10). Two mechanisms are proposed: Some viruses, like human immunodeficiency virus (HIV), can encode enzymes such as reverse transcriptase and integrase to synthesize complementary ssDNA and integrate it into the host genome. Another mechanism involves the viral genome recombining with the host genome through components of endogenous transposons like intracisternal A-particle (IAP) and long interspersed element-1 (LINE-1) (11)

Integrating the viral genome into host chromosomal DNA can lead to various consequences, including gene disruption, premature cell death, and oncogene activation, and may contribute to species evolution through inherited genomic inclusions. While integration is a necessary stage for some viruses, such as retroviruses, it may occur incidentally in others (12). Establishing the capability of the SARS-CoV-2 genome to integrate into the host genome opens new avenues for future studies to understand the pathogenic mechanisms of SARS-CoV-2. Such studies could explore the viral integration sites within the host genome, the stage of the viral life cycle at which integration occurs, and the cellular and viral factors involved in this process. They could also aim to develop biomarkers for the persistent presence of the viral genome in recovered COVID-19 cases and determine whether the virus can integrate in all individuals infected with SARS-CoV-2.

2. Objectives

This study aims to investigate the presence of the DNA form of the SARS-CoV-2 genome in oropharyngeal, nasopharyngeal, and PBMC samples from individuals who have recovered from COVID-19 as well as from a healthy control group.

3. Methods

3.1. Study Population

From January 2022 to October 2022, this cross-sectional study enrolled eighty individuals diagnosed with SARS-CoV-2, referred to clinics or hospitals affiliated with Iran University of Medical Sciences (IUMS) in Tehran, Iran. The study consisted of forty outpatient respondents with no specific complications (group 1) and another forty patients who were hospitalized due to significant clinical manifestations and remained molecularly positive for COVID-19 45 days post-onset of the illness (group 2). Additionally, forty healthy individuals served as controls (group 3). Notably, none of the COVID-19 patients or healthy control participants had co-infections with human cytomegalovirus (HCMV), Mycobacterium tuberculosis, hepatitis B virus (HBV), hepatitis C virus (HCV), or HIV.

3.2. Sample Collection and Processing

To assess the presence of the integrated SARS-CoV-2 genome in the participants' specimens, oropharyngeal and nasopharyngeal samples were collected and stored in viral transport media (VTM). Additionally, 5 mL of peripheral blood was drawn from each participant and placed into sterile vacutainer tubes containing Ethylenediaminetetraacetic acid (EDTA). PBMCs were then isolated from the blood samples using Ficoll-Hypaque (Lympholyte H, Cedarlane, Hornby, Canada) density gradient centrifugation. The resulting PBMC pellet was resuspended in 350 µL of RNALater solution (Ambion, Inc., Austin, TX) and stored at -80°C for subsequent analysis.

3.3. Genomic DNA Isolation

Total DNA was extracted from the oropharyngeal, nasopharyngeal samples, and pellets of $4 - 5 \times 10^6$ PBMCs using the QIAamp® DNA Mini kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocols. The quality and quantity of the isolated DNA were assessed using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, MA).

3.4. Amplification of SARS-CoV-2 Genomic DNA Via Real-Time PCR

To determine the presence of the SARS-CoV-2 genome in the samples, cDNA synthesis was omitted, and DNA sequences of the virus were directly tested. Real-time PCR was conducted using specific TaqMan probes and primers targeting the genomic DNA of SARS-CoV-2 in the isolated DNA. The PCR reactions were performed on a Rotor-Gene Q system (QIAGEN, Germany), targeting conserved regions of the N (Nucleocapsid) (13), E (Envelope), and RdRp (RNA-dependent RNA polymerase) (14) genes, with RNase P serving as an internal control (13, 15) (Table 1). The reaction mixture for RT-PCR included 10 pmol of each primer and 5 pmol of each TaqMan probe for the N, E, RdRp, and RNase P genes, 12.5 µL Premix Ex Taq™ (Probe qPCR, TaKaRa Bio Inc., Shiga, Japan), and 5 µL of total DNA as the template. The thermal profile included an initial step at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Table 1. Primers and Probes for Detection of N, E, and RdRp Genes of COVID-19, and RNaseP as an Internal Control Using Real-Time PCR

Assay Use and Polarity	Name	Sequences
RNase P		
Forward primer	RP2-F	AGA TTT GGA CCT GCG AGC G
Reverse primer	RP2-R	GAG CGG CTG TCT CCA CAA GT
Probe	RP2-probe	ROX- TTC TGA CCT GAA GGC TCT GCG CG -BBQ
E		
Forward primer	E-Sarbeco	ACA GGT ACG TTA ATA GTT AAT AGC GT
Reverse primer	E-Sarbeco	ATA TTG CAG CAG TAC GCA CAC A
Probe	E-Sarbeco	FAM- ACA CTA GCC ATC CTT ACT GCG CTT CG -BBQ
N		
Forward primer	N-Pearson	CCA GAA TGG AGA ACG CAG T
Reverse primer	N-Pearson	TGA GAG CGG TGA ACC AAG A
Probe	N-Pearson	Cy5- GCG ATC AAA ACA CGT CG GCC CC -BBQ
RdRp		
Forward primer	RdRP-SARSr	GTG ARA TGG TCA TGT GTG GCG G
Reverse primer	RdRP-SARSr	CAR ATG TTA AAS ACA CTA TTA GCA TA
Probe	RdRP-SARSr	VIC- CAG GTG GAA CCT CAT CAG GAG ATG C -BBQ

Abbreviations: RNase P, ribonuclease P; E, envelope; N, nucleocapsid; RdRp, RNA-dependent RNA polymerase.

3.5. Statistical Analysis

Data were analyzed using SPSS version 20 (SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was used to assess data normality. Categorical variables were compared using Fisher's exact test or the chi-square test, as appropriate. A P-value of less than 0.05 was considered statistically significant.

4. Results

As previously stated, 80 patients with COVID-19 were referred to hospitals associated with IUMS in Tehran, Iran. In addition, 40 healthy individuals (group 3) were recruited for this cross-sectional study. Among the COVID-19 cases, forty were treated on an outpatient basis (group 1) and forty were hospitalized due to the severity of their symptoms (group 2). The mean age of the non-hospitalized COVID-19 patients (group 1) was 36.1 ± 11.0 years (range 22 - 63 years), 61.6 ± 18.4 years (range 13 - 92 years) for hospitalized COVID-19 patients (group 2), and 39.0 ± 8.7 years (range 25 - 51 years) for the healthy participants (group 3). In groups 1 and 2 of patients with COVID-19, and in the healthy controls, 17 (42.5%), 27 (67.5%), and 17 (42.5%) were males, respectively, as detailed in Table 2.

The laboratory and clinical characteristics of the participants, as well as those of the healthy controls, are presented in Tables 3 and 4. The laboratory results for the three groups are summarized in Table 5. After conducting the real-time TaqMan® RT-PCR assay with an internal amplification control to detect the presence of the integrated SARS-CoV-2 genome in the host cell genome, no integration of the virus genome into the host genome was detected. It is important to note that three conserved regions of the virus genome were tested —genes N, E, and RdRp—and all tests returned negative results. Consequently, it can be concluded that the virus genome does not convert into DNA within the host cells, nor does it integrate into the host genome.

5. Discussion

When COVID-19 escalated into a pandemic, several reports documented instances where individuals, including recovered and asymptomatic patients, continued to test positive for the virus weeks later, stirring considerable debate (16, 17). These reports raised the possibility of SARS-CoV-2 genome integration into human cellular DNA, akin to what is observed with retroviruses (18). Our study sought to detect the DNA form of SARS-CoV-2 as an indicator of viral integration in the PBMC, oropharyngeal, and nasopharyngeal samples of COVID-19 patients using a TaqMan® RT-PCR assay. Our results did not yield any positive detections in these samples. However, the absence of detected integration does not conclusively disprove our hypothesis about the potential integration of the SARS-CoV-2 genome.

Unlike retroviruses, where genome integration is a crucial part of the viral lifecycle (19), such integration is

Table 2. Demographic Parameters of Studied Participants

Parameters	Male	Female	Total	P-Value [a]
Non-hospitalized patients with COVID-19 (group 1)				
No. (%)	17 (42.5)	23 (57.5)	40 (100.0)	-
Age; mean (range)	37.4 ± 8.3 (29 - 53)	35.2 ± 12.8 (22 - 63)	36.1 ± 11.0 (22 - 63)	0.055
Hospitalized patients with COVID-19 (group 2)				
No. (%)	27 (67.5)	13 (32.5)	40 (100.0)	-
Age; mean (range)	60.3 ± 19.2 (13 - 85)	64.5 ± 17.3 (28 - 92)	61.6 ± 18.4 (13 - 92)	0.942
Healthy controls (group 3)				
No. (%)	17 (42.5)	23 (57.5)	40 (100.0)	-
Age; mean (range)	34.4 ± 5.7 (25 - 40)	42.4 ± 9.1 (25 - 51)	39.0 ± 8.7 (25 - 51)	< 0.001 [b]

[a] Mann-Whitney U test.

[b] Statistically significant.

Table 3. Clinical profiles of Studied Participants [a]

Parameters	Group 11	Group 22	Group 33	P-Value [b, c]
Male/female ratio	17/23	27/13	17/23	0.036
Fever	33 (82.5)	34 (85.0)	0 (0.0)	< 0.001
Chills	24 (60.0)	25 (62.5)	0 (0.0)	< 0.001
Headache	24 (60.0)	27 (67.5)	0 (0.0)	< 0.001
Weakness	7 (17.5)	16 (40.0)	0 (0.0)	0.008
Skeletal pain	26 (65.0)	29 (72.5)	0 (0.0)	< 0.001
Chest pain	15 (37.5)	19 (47.5)	0 (0.0)	< 0.001
Shortness of breath	11 (27.5)	17 (42.5)	0 (0.0)	0.002
Dry cough	24 (60.0)	25 (62.5)	0 (0.0)	< 0.001
Sputum cough	3 (7.5)	6 (15.0)	0 (0.0)	0.001
Deceased smell	6 (15.0)	5 (12.5)	0 (0.0)	< 0.001
Deceased taste	9 (22.5)	10 (25.0)	0 (0.0)	< 0.001
Runny nose	24 (60.0)	6 (15.0)	0 (0.0)	< 0.001
Cape of nose	25 (62.5)	12 (30.0)	0 (0.0)	< 0.001
Diabetes	0 (0.0)	13 (32.5)	0 (0.0)	< 0.001
Bleeding stomach	4 (10.0)	2 (5.0)	0 (0.0)	0.025
Gastrointestinal symptom	22 (55.0)	8 (20.0)	0 (0.0)	< 0.001

[a] Values are expressed as No. (%).

[b] Chi-square test.

[c] P-values are significant at 0.05.

rare in other viruses. Yet, there are exceptions, such as lymphocytic choriomeningitis virus (LCMV) and bornavirus, which may integrate into the host genome under certain conditions facilitated by host factors like IAP and LINEs, respectively (20-22). Long interspersed element-1, a non-long terminal repeat retrotransposon that constitutes about a fifth of the human genome, includes two open reading frames that encode an endonuclease/reverse transcriptase and a nucleic acid-binding protein (23).

It is hypothesized that SARS-CoV-2 RNA could be reverse transcribed and integrated into the host genome by the endogenous reverse transcriptase protein encoded by LINE-1. Due to this protein's high affinity for RNA, it may bind to viral RNAs and facilitate their retro-integration. Zhang et al. (6) proposed two mechanisms for the integration of SARS-CoV-2, noting that LINE-1 expression is significantly increased in SARS-CoV-2-infected or cytokine-exposed cells. Consequently, the retro-integration of viral RNAs may activate the

Table 4. Clinical Profiles of Non-hospitalized and Hospitalized Patients with COVID-19 [a]

Parameters	Group 1 [b]	Group 2 [c]	P-Value [d, e]
Male/female ratio	17/23	27/13	0.021
Positive result of PCR for SARS-CoV-2, day			0.659
≤ 15	35 (87.5)	32 (80.0)	
16 - 30	3 (7.5)	5 (12.5)	
31 - 45	2 (5.0)	3 (7.5)	
Fever	33 (82.5)	34 (85.0)	< 0.001
Chills	24 (60.0)	25 (62.5)	0.090
Headache	24 (60.0)	27 (67.5)	0.012
Weakness	7 (17.5)	16 (40.0)	0.390
Skeletal pain	26 (65.0)	29 (72.5)	0.022
Chest pain	15 (37.5)	19 (47.5)	0.039
Shortness of breath	11 (27.5)	17 (42.5)	0.500
Dry cough	24 (60.0)	25 (62.5)	0.036
Sputum cough	3 (7.5)	6 (15.0)	0.033
Deceased smell	6 (15.0)	5 (12.5)	0.057
Deceased taste	9 (22.5)	10 (25.0)	0.006
Runny nose	24 (60.0)	6 (15.0)	< 0.001
Nasal congestion	25 (62.5)	12 (30.0)	< 0.001
Diabetes	0 (0.0)	13 (32.5)	< 0.001
Bleeding stomach	4 (10.0)	2 (5.0)	0.259
Gastrointestinal symptoms	22 (55.0)	8 (20.0)	< 0.001

[a] Values are expressed as No. (%).

[b] Non-hospitalized patients with COVID-19.

[c] Hospitalized patients with COVID-19.

[d] Fisher's exact test except for positive result of PCR for SARS-CoV-2 (chi-square test).

[e] P-values are significant at 0.05.

host's immune system, potentially triggering severe immune responses and cytokine storms (6). In this study, we also investigated the integration of the SARS-CoV-2 genome in long-term hospitalized patients with symptoms and inflammatory conditions, and as mentioned, no positive samples were detected in this group.

Several publications have discussed the integration of the SARS-CoV-2 genome into infected cells under both in vitro conditions and in clinical samples. In these studies, the integrated viral genome was not detected. The negative results might be attributed to increased virus-induced cell death in culture mediums before sample collection and the relatively low and rare occurrence of this phenomenon, which should be investigated with larger sample sizes. In one study, the small sample size could have contributed to the lack of findings (10, 24). Furthermore, given the random nature of the integration process, the likelihood of integration occurring at the same genomic locus across different cases and/or tissues is low (11).

Smits et al. were unable to find any evidence of SARS-CoV-2 integration when investigating with long-read DNA sequencing, aligning with the results of this study (24). It should be noted that the negative results in both the current and previous studies might be influenced by the therapeutic drugs used to treat COVID-19 (6, 25). However, these negative results do not conclusively dismiss the hypothesis of viral genome integration, suggesting that further and more detailed studies are necessary to understand the mechanisms and effects of virus integration into the genomes of infected host cells.

In some viruses, such as human papillomavirus (HPV), only 14 of over 200 HPV types, known as high-risk HPV types, are capable of integrating into the host genome (26, 27). No studies have yet investigated the potential for integration among various SARS-CoV-2 variants. We collected all samples while the delta variant was dominant, yet no positive samples were detected in this study. The small sample size and focus on cases infected with the delta variant are limitations of this

Table 5. The Laboratory Data of the Studied Participants (3 Groups) [a]

Parameters	Group 1 [b]	Group 2 [c]	Group 3 [d]	P-Value [e, f]
Male/female ratio	17/23	27/13	17/23	0.036
WBC	7.9 ± 1.0 (5.7 - 9.6)	7.4 ± 5.3 (2.0 - 32.4)	7.6 ± 1.4 (4.1 - 9.7)	0.051
RBC	4.4 ± 0.4 (3.4 - 5.1)	4.3 ± 1.1 (1.0 - 7.0)	4.4 ± 0.4 (3.4 - 5.4)	0.665
Hb	13.6 ± 1.2 (11.8 - 15.4)	12.7 ± 3.6 (1.6 - 20.6)	13.7 ± 1.4 (10.6 - 16.5)	0.054
Hct	41.4 ± 3.8 (35 - 48)	37.5 ± 9.4 (7.6 - 59.2)	41.9 ± 4.4 (32 - 49)	0.001
Platelet	240 ± 108 (105 - 437)	184.1 ± 110.1 (20 - 571)	242.8 ± 113.2 (115 - 465)	0.004
INR	1.0 ± 0.1 (0.9 - 1.3)	1.3 ± 0.7 (1.0 - 5.3)	1.0 ± 0.1 (0.8 - 1.2)	0.001
PTT	30.0 ± 3.2 (26 - 38)	36.7 ± 14.4 (24 - 84)	30.1 ± 4.0 (24 - 38)	0.021
FBS	86.3 ± 9.3 (77 - 110)	175.4 ± 121.6 (77 - 512)	81.8 ± 8.3 (69 - 102)	< 0.001
Urea	20.5 ± 4.2 (14 - 31)	25.2 ± 14.3 (7 - 77)	19.8 ± 4.0 (14 - 29)	0.186
Cr	0.93 ± 0.2 (0.5 - 1.2)	2.3 ± 4.2 (0.6 - 20)	0.96 ± 0.2 (0.5 - 1.2)	0.009
AST	17.6 ± 9.4 (9 - 33)	56.0 ± 32.0 (19 - 154)	14.7 ± 5.0 (9 - 24)	< 0.001
ALT	19.5 ± 10.1 (10 - 39)	47.0 ± 30.6 (10 - 145)	16.4 ± 5.2 (10 - 27)	< 0.001
LDH	262 ± 90.0 (120 - 439)	658.0 ± 332.0 (121 - 1479)	222.7 ± 89.4 (109 - 430)	< 0.001
CPK	61.5 ± 36.3 (24 - 143)	201.9 ± 503.4 (19 - 3200)	58.2 ± 31.8 (22 - 140)	1.000
ALP	92.4 ± 43.2 (41 - 178)	301.2 ± 529.5 (45 - 3431)	91.1 ± 34.3 (40 - 135)	< 0.001
Na	140 ± 2.8 (136 - 145)	136 ± 4.6 (115 - 146)	140 ± 2.7 (134 - 145)	< 0.001
K	4.0 ± 0.5 (3.3 - 5.0)	4.1 ± 0.8 (2.0 - 5.7)	4.0 ± 0.4 (3.3 - 5.4)	0.126
Ca	10.0 ± 0.6 (9.0 - 11.2)	8.7 ± 0.7 (6.5 - 10.2)	9.9 ± 0.7 (8.9 - 11.0)	< 0.001
Ph	4.0 ± 0.6 (2.8 - 4.9)	2.8 ± 0.9 (1.6 - 5.8)	4.0 ± 0.4 (3.0 - 4.8)	< 0.001
CRP	7.2 ± 3.2 (2.0 - 12.0)	26.0 ± 13.0 (4 - 48)	1.7 ± 0.7 (1.0 - 3.0)	< 0.001
Vitamin D	23.4 ± 10.5 (11 - 44)	25.5 ± 12.7 (6 - 47)	33.3 ± 16.0 (11 - 65)	0.006

Abbreviations: WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; Hct, hematocrit; FBS, fast blood sugar; PTT, partial thromboplastin time; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CPK, creatine phosphokinase; ALP, alkaline phosphatase; Cr, creatinine; LDH, lactate dehydrogenase; Na, sodium; K, potassium; Ca, calcium; CRP, C-reactive protein; Ph, phosphorus.

[a] Values are expressed as mean ± SD.

[b] Non-hospitalized patients with COVID-19.

[c] Hospitalized patients with COVID-19.

[d] Healthy controls individuals.

[e] Mann-Whitney U except for male/female ratio (chi-square test).

[f] P-values are significant at 0.05.

study. These factors may impact our findings, so our interpretations should be approached with caution.

5.1. Conclusions

The potential integration of SARS-CoV-2 RNA into host cells remains uncertain, as this study found no evidence of virus-related DNA sequences. Nonetheless, further research is required to explain the phenomenon of long-term PCR positivity in recovered COVID-19 patients.

Footnotes

Authors' Contribution: Kh.Kh., T.D., J.S.N., and F.B.S. designed the study and wrote the paper; A.Kh. and M.R. collected the samples and S.H.M., S.J.K. and A.T. analyzed the results obtained from the research.

Conflict of Interests: Hereby, it is declared that the authors do not have any conflict of interests and the resulting paper has been endorsed by them.

Ethical Approval: The local ethics committee of the IUMS in Tehran, Iran confirmed this survey (ethical code: IR.IUMS.FMD.REC.1401.273), which was conducted according to Helsinki's Declaration.

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