Original Article

Highly Conserve Sequences in Envelope, Nucleoprotein and RNA-Dependent RNA Polymerase of SARS-CoV-2 in Nasopharyngeal Samples of the COVID-19 Patients; a Diagnostic Target for Further Studies

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Abstract

Background: The etiological agent of coronavirus diseases 2019 (COVID-19) is severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Conventional molecular methods are used to detect viruses in COVID-19 infected patients. This study aimed to investigate escape mutations from molecular detection on SARS-CoV-2 targeted genes, which indicates the importance of mutations in false-negative PCR test results in the detection of virus in clinical specimens of patients with COVID-19.

Materials and Methods: The 20 nasopharyngeal swabs samples collected from COVID-19 confirmed patients. The SARS-CoV-2 E, nsp12, and N genetic regions are amplified by RT-PCR assay. PCR products were sequenced using the Sanger sequencing method and Multiple sequence alignment (MSA) to assess the polymorphism and mutations performed using MEGA X software and the Maximum likelihood method for the phylogenetic evaluation.

Results: Among all COVID-19 cases, 60% and 40% were male and female, respectively. The MSA showed high conservation between all evaluated samples and VOCs in all N, E, and nsp12 genes. Also, the phylogenetic evaluation by the Maximum likelihood method reported high similarity between all SARS-CoV-2 sequenced samples, VOCs, and Wuhan reference sequences in the evaluated region.

Conclusion: Our study results approved the relatively conserved suitability of the E, N, and RdRp-gene regions with no diversity, therefore, making them perfect candidates for first-line screening.

Keywords: SARS-CoV-2, COVID-19, Mutation, Phylogenetic

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Introduction

In the recent 20 years, coronaviruses have caused several outbreaks in the human population. In 2003, the severe acute respiratory syndrome coronavirus (SARS-CoV) detected in an animal reservoir infected over 8000 individuals with a 10% mortality rate. Since 2012, another coronavirus, Middle East respiratory syndrome (MERS-CoV), has been transmitted to over 1,700 people with a 36% mortality rate (1). At the end of 2019, a novel coronavirus, SARS-CoV-2, was recognized that caused coronavirus disease 2019 (COVID-19). SARS-CoV-2 has been a global health priority for the past years(2). SARS-CoV-2 is causing over four million deaths.

Due to a mechanism named copy choice, this virus had several mutations in its genome (3). Therefore, many studies have been performed on this virus. This event allowed scientists to document more variants of SARS-CoV-2, which are associated with clinical symptoms and epidemiological data (4). In the middle of this vast data collection, there is a perspective on what may occur in the future. To diagnose COVID-19, there are different methods, including CT (Chest computed tomography) scan and confirmatory molecular nucleic acid base methods (5). The PCR is the gold standard assay for viruses with a high sensitivity and specificity rate (6). The real-time reverse transcriptase-PCR (RT-PCR) is the most exciting test for following coronaviruses because of its advantages, such as easy handling and specificity as a qualitative assay (7, 8). Although, various commercial RT-PCR diagnostic kits have different sensitivity for detecting the SARS-CoV-2 genome(9). One of the essential aspects of the real-time RT-PCR method is the possibility of provoking false positive and negative results (10). Many factors, including mutation, could be related to the instability of real-time RT-PCR (11). This work aimed to determine the presence of escape mutations from molecular detection on target genes of SARS-CoV-2, which indicates the importance of mutations in false-negative PCR test results in the detection of virus in clinical specimens of COVID-19 infected patients.

Methods

Patient's selection and sample preparation: The nasopharyngeal swabs samples collected from COVID-19 confirmed cases. Inclusion criteria included a positive molecular test to confirm COVID-19 and written ethical consent (ethical code: IR.KHOMEIN.REC.1399.005). Patients were randomly included regardless of age, gender, or other demographical parameters.

RNA extraction and cDNA synthesis: The viral RNA genome was extracted by the RNJia Virus Kit (Roje-Technologies, Yazd, Iran) according to manufacturer guidelines from the nasopharyngeal sample of the confirmed COVID-19 patients. Extracted RNA was checked using a Nanodrop spectrophotometer (Thermo fisher scientific, inc). The cDNA synthesis was performed by a commercial kit (Sinna clon First Strand cDNA Synthesis Kit) based on the manufacturer's protocols. The cDNAs are stored at -20°C for the next steps.

Genome amplification and sequencing: The SARS-

Table 1: Primers list for E, nsp12, and N genes.

Gene	Forward sequence	Reverse Sequence	product base pairs (base pair)
E	ACAGGTACGTTAATAGTTAATAGCGT	ATATTGCAGCAGTACGCACACA	113
nsp12	TCTGTGATGCCATGCGAAAT	ACTACCTGGCGTGGTTTGTA	113
N	CAATGCTGCAATCGTGCTAC	GTTGCGACTACGTGATGAGG	118

Table 2: Demographic detail of enrolled COVID-19 patients.

Clinical features	No. (%)	Background disorder	No. (%)
Fever	20 (100)	Hypertension	19 (95)
Diarrhea	6 (30)	Diabetes	14 (70)
Shortness of breath	5 (25)	Cardiovascular Disease	4 (20)
Headache	5 (25)		
Myalgia	18 (90)	Asthma	3 (15)
Cough	18 (90)		
Chills	7 (35)		

CoV-2 genome detection in respiratory samples was performed by RT-PCR assay according to the WHO protocol (12), and E, nsp12, and N genetic regions of SARS-CoV-2 were amplified (Table 1). PCR products were analyzed by 1.5% agarose gel electrophoresis and sequenced by the Sanger sequencing technique. PCR was conducted by the 2X Super PCR Master mix (Yekta Tajhiz Azma Co., Iran) for all three genes and 20µL as the final volume. 1 µL of 10 µM concentration of each reverse and forward primer was used for each reaction. PCRs are performed at 95°C for 10 minutes as primary denaturation and followed by 40 cycles of 95˚C (30 seconds), aneling temperature (30 seconds), extension to 72°C (30 seconds), and a final extension to 72°C (10 minutes). The aneling temperature for E, nsp12, and N genes was 57°C, 56°C, and 51°C, respectively.

MSA and phylogenetic Evaluation: The multiple sequence alignment (MSA) was performed to assess polymorphism and mutations. The sequences for all specific regions were aligned and compared with the Wuhan reference sequence and all of the current variants of concern (VOC) by CLC Workbench. The MEGA X and Maximum likelihood method were used for the phylogenetic assessment.

Results

Demographic data of patients: Of all cases, 12 (60%) and 8 (40%) were male and female, respectively. The mean age of patients was 55 years. Clinical features and background conditions of patients are presented in Table 2.

Sequencing and phylogenetic: The Sanger sequencing results for E, nsp12 and N genes between 20 positive samples were registered in NCBI GenBank (MW623369, MW623370, MW623371, MW623372, MW623373, MW623374, MW623375, MW623376, MW623377, MW623378, MW623379, MW623380, MW623381, MW623382, MW623383, MW623384, MW623385, MW623386, MW623387, MW623388, MW623389, MW623390, MW623391, MW623392, MW623393, MW623394, MW623395, MW623396, MW623397, MW623398, MW623399, MW623400, MW623401, MW623402, MW623403, MW623404, MW623405, MW623406, MW623407, MW623408, MW623409, MW623410, MW623411, MW623412, MW623413, MW623414, MW623415, MW623416, MW623417 , MW623418, MW623419, MW623420, MW623421, MW623422, MW623423, MW623424, MW623425, MW623428). The MSA represented high conservation between all evaluated samples and VOCs in all three genes (N, E, and nsp12). This conservation is represented in all of the evaluated sequences, but a partial figure for this conservation is illustrated in Figure 1. Furthermore, the phylogenetic evaluation by the Maximum likelihood method represented high similarity between all SARS-CoV-2 sequenced samples, VOCs, and Wuhan reference sequences in the evaluated region (Figure 2).

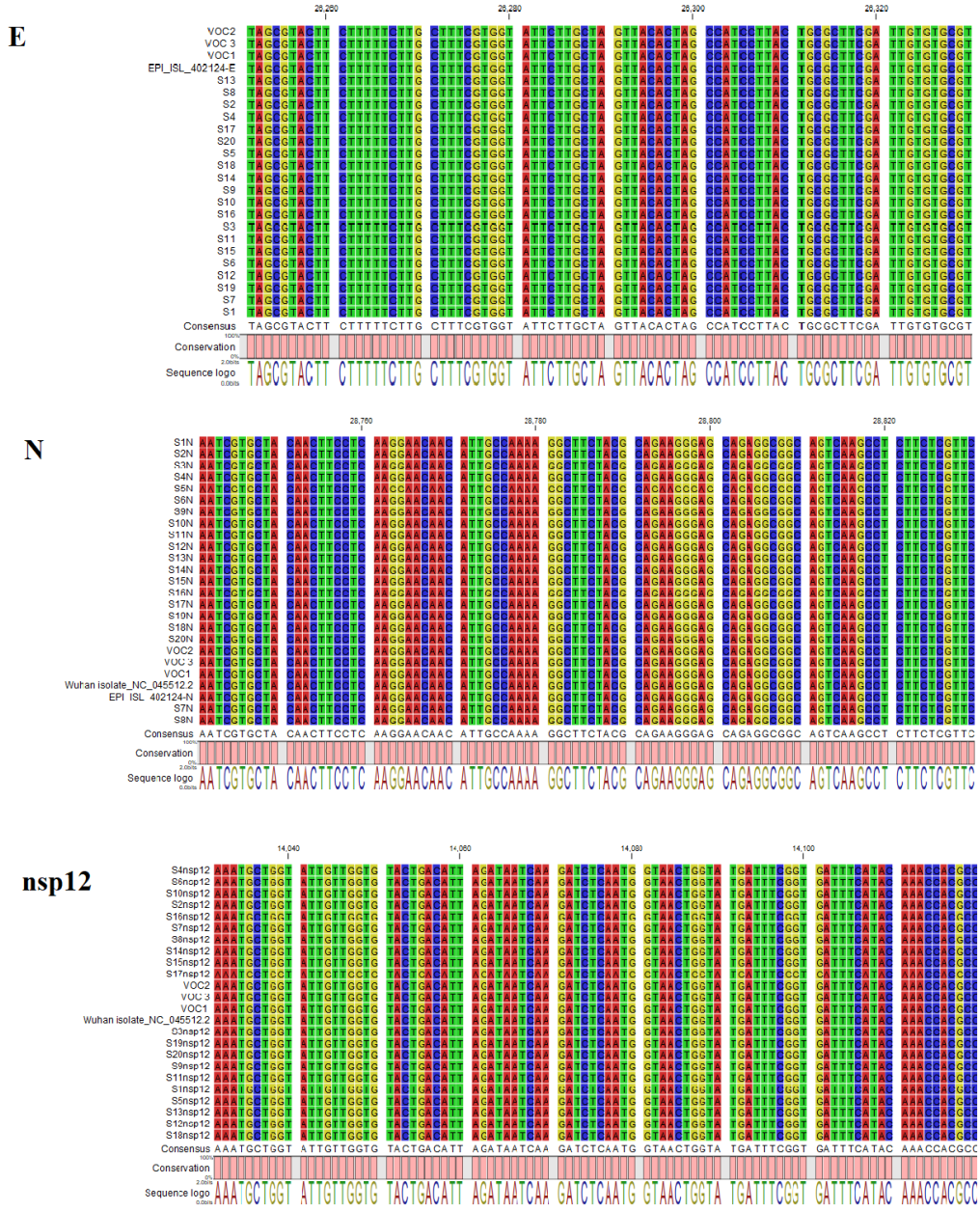

Figure 1. The MSA analysis of the E, N, and nsp12 sequences in the CLC workbench.

Discussion

Regarding lacking an appropriate drug for COVID-19 and mutations in the SARS-CoV-2 genome, studying this virus genome is very important to clarify the molecular mechanism of pathogenesis and progress in describing variances in geographic-related fatality rates and host susceptibility to SARS-CoV-2 (13). Also, mutations in the virus genome can interfere with primer and probe routine functions designed to target the Envelope, Nucleocapsid, and RdRp genes, which are globally utilized for SARS-CoV-2 detection (14). The present study demonstrated that all viruses had conserved sequences in E, N, and RNA-dependent RNA polymerase segments. This data clarified mentioned gene capacity as the first step to COVID-19 diagnosis in our region.

In contrast to other RNA viruses, coronaviruses display a different mutation rate due to their polymerase proofreading ability and more replication

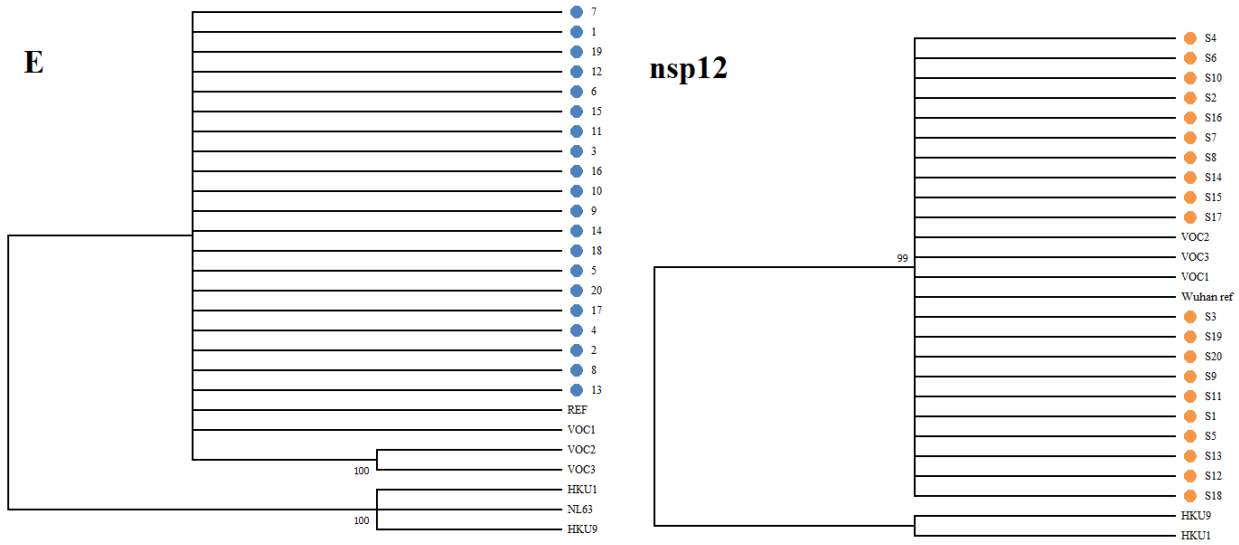

Figure 2. The phylogenetic analysis by maximum likelihood method using 1000 replicates for the bootstrap method. The cut-off was set at less than 70.

fidelity (15). According to data on global SARS-CoV-2 genome variations, the estimated mutation amount of this virus has been reported (2-6 × 10^4 nucleotides/genome/year) (16). These mutations are mainly described as silent mutations (17). There are various mutation types, such as mismatches and deletions, in different translations of the untranslated area of the SARS-CoV-2 genomes (18). The geography and climate probably could affect the distribution and occurrence of particular mutations (18). However, other variables are introduced related to countries and cities (19).

As a recommendation, the Pan-American Health Organization (PAHO), its new guidelines for diagnostic issue of COVID-19, mentioned a double target detection method following different genetic markers such as E, N, or RdRp genes is advised (20). Among all suggested target genes, the PAHO recommendation is the usage of E or RdRP genes for detection, ordering the E gene for single gene targeting, regarding its definite higher sensitivity (21). Considering this guideline and attention to lacking SNVs within the E gene from 20 Iranian genomes analyzed, the E gene remains the most engaged hopeful and first experiment tool for SARS-CoV2 diagnosis in the region.

RT-PCR efficiently amplifies partial genomic analysis as the gold standard method for detecting SARS-CoV-2. However, there are other diagnostics methods for detecting COVID-19 (22). Besides RT-PCR, serological techniques are considerably used for screening COVID-19 cases. This process does not detect the virus but can recognize antibodies during past or current diseases. However, this method's accuracy results remain challenging (23, 24). While the biosensors, nanotechnology-based approaches, and metagenomic sequencing are even predicted to be additionally generated as large-scale detection strategies (22). Using an Enzyme-linked immunosorbent assay (ELISA) test, the antibody Binds against the SARS-CoV-2 antigen, which is antigen-coated in ELISA plates. Also, in molecular approaches, RNA of SARS-CoV-2 via transcriptase enzyme converts to cDNA and is amplified by real-time PCR. The RT-PCR procedure is the gold standard test that has reliable Sensitive for following COVUD-19 cases but is more expensive than the ELISA test (22).

Conclusion

This preliminary experiment tried to find genomic variation in SARS-CoV-2 from Iranian samples and revealed the incidence of mutations in selected targeted

genes. Current study outcomes approve the relatively conserved suitability of the E, N, and RdRp genes regions in which no diversity was detected, therefore candidate them for first-line screening.

Acknowledgment

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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