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ORIGINAL ARTICLE

Partial sequence conservation of SARS-CoV-2 NSP-2, NSP-12, and Spike in stool samples from Abadan, Iran

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Abstract

Since the onset of the coronavirus disease 2019 (COVID-19) pandemic, the clinical manifestations of the virus have undergone many changes. Recently, there have been many reports on gastrointestinal symptoms in COVID-19 patients. This study is aimed to perform a detailed phylogenetic study and assessment of different SNVs in the RNA genome of viruses isolated from fecal samples of patients with COVID-19 who have gastrointestinal symptoms, which can help better understand viral pathogenesis. In the present study, 20 fecal samples were collected by written consent from COVID-19 patients. According to the manufacturer's protocol, virus nucleic acid was extracted from stool samples and the SARS-CoV-2 genome presence in stool samples was confirmed by RT–PCR assay. Three viral genes, S, nsp12, and nsp2, were amplified using the reverse transcription polymerase chain reaction (RT–PCR) method and specific primers. Multiple sequencing alignment (MSA) was performed in the CLC word bench, and a phylogenetic tree was generated by MEGA X based on the neighbor-joining method. Of all cases, 11 (55%) were males. The mean age of the patients was 33.6 years. Diabetes (70%) and blood pressure (55%) were the most prevalent comorbidities. All 20 patients were positive for SARS-CoV-2 infection in respiratory samples. Molecular analysis investigation among 20 stool samples revealed that the SARS-CoV-2 genome was found among 10 stool samples; only three samples were used for sequencing. The polymorphism and phylogenetic analysis in SARS-CoV-2 showed great similarity among all of the evaluated genes with the Wuhan reference sequence and all of the current variants of concern (VOCs). The current study represents a great similarity in polymorphism and phylogenetic analysis of the SARS-CoV-2 isolates with the Wuhan reference sequence and all of the current VOC in the particular evaluated partial sequences of S, nsp12, and nsp2.

KEYWORDS
COVID 19, Iran, phylogenetic relationship, SARS coronavirus 2, spike glycoprotein, viral non-structural proteins

1 | INTRODUCTION

An ongoing pandemic of the coronavirus disease 2019 (COVID-19) caused by a novel β-coronavirus has been designated as the severe acute respiratory syndrome 2 (SARS-CoV-2).[1] Since the onset of the COVID-19 pandemic, the clinical manifestations of the virus have undergone many changes.[2,3] Apart from respiratory symptoms and pneumonia, gastrointestinal manifestations are also common in patients with Middle East respiratory syndrome (MERS), severe acute respiratory syndrome (SARS), and COVID-19.[4] The number of patients suffering from diarrhea during SARS was reported to be 16%–73%.[5] Additionally, the presence of viral genomic RNA in the feces of COVID-19 patients were reported between 28.8%-70.3%.[6] Recently, there have been many reports on gastrointestinal symptoms in COVID-19 patients. According to the expression of angiotensin converting enzyme-2 (ACE-2) receptor on the surface of gastrointestinal epithelial cells, it is hypothesized that the virus can attach to the surfaces of epithelial cells and change its structure.[7] SARS-CoV-2 leads to the death of a large section of the human populations in different geo-climatological regions of the world. The greatest number of infected cases is seen in Western Pacific, European, and Americas regions, but European countries tend to have higher numbers of both cases and deaths.[8] SARS-CoV-2 is similar to other RNA viruses characterized by a high mutation rate.[9] The existence of quasispecies (subpopulations) within the same host infected with SARS-CoV-2 affords internal or external selective constraints, host range, and cell tropism.[10] Scientists have identified thousands of mutations in the SARS-CoV-2 genome, some of which are completely new in the coronavirus family.[11] All variants of SARS-CoV-2 carry multiple mutations, including on the Spike protein alone, deletion, insertion, and amino acid substitution. Mutations may alter the virus' biological characteristics, including immune escape and increasing transmissibility.[12] The results of these studies showed that the frequency of single nucleotide variations (SNVs) in the SARS-CoV-2 genome is high.[13] In addition, there is evidence that SARS-CoV-2 mutations can significantly alter its pathogenicity.[14] Accurate estimation of the extent, type, and location of genetic mutations in the virus genome is crucial to understand the evolution of the virus and how to control it, especially when faced with an epidemic as well as a virus with unknown characteristics.[13,15] This study is aimed to perform a detailed phylogenetic study and assessment of different SNVs on the RNA genome of viruses isolated from fecal samples of patients with COVID-19 who have gastrointestinal symptoms, which can help better understand the viral pathogenesis and effective ways to deal with it soon.

2 | MATERIALS AND METHODS

2.1 | Patient selection

In the present study, 20 fecal samples were collected from Abadan City, Iran, by written consent from COVID-19 patients whose disease was confirmed by real-time reverse transcription–polymerase chain reaction (RT–PCR) (ethical code: IR.ABADANUMS.REC.1399.093). A questionnaire was used for demographical data and background disorders.

2.2 | Genomic extraction and SARS-CoV-2 detection

According to the manufacturer's protocol, virus nucleic acid was extracted from stool samples using a viral RNA kit (QIAGEN, QIAamp Viral RNA Mini Kit). For confirmation of the SARS-CoV-2 genome detection in stool samples, RT–PCR assays were performed according to the protocol established by the World Health Organization (WHO).[16]

2.3 | cDNA synthesis and specific SARS-CoV-2 genome amplification

cDNA synthesis was performed using a commercial cDNA synthesis kit (Sinnaclon First Strand cDNA Synthesis Kit) based on the manufacturer's protocols. Three viral genes, S, nsp12, and nsp2, were amplified using the RT–PCR method and specific primers (Table 1), and finally, the products were electrophoresed on a 1.5% agarose gel. The PCR products were used for the sequencing by the Sanger sequencing method. The 2X Super PCR Mastermix (Yekta Tajhiz Azma Co., Iran) was used for all three gene PCRs in a 20-μl final volume. Every reaction included 10 μl of the

2X Super PCR Mastermix, 1 μl of each reverse and forward primer, and 2-3 μl of template , and was round out to 20 μL with PCR grade water. PCR was performed at 95°C for 10 minutes (primary denaturation) and 40 cycles of 95°C for 30 seconds, annealing temperature for 30 seconds (the temperatures were 57°C, 55°C, and 58°C for S, nsp12, and nsp2), 72°C for 30 seconds, and 72°C for 10 minutes (final extension).

2.4 | NSP-2, NSP-12, and Spike polymorphism and phylogenetic tree

The PCR products were used for the sequencing by the Sanger sequencing method. The sequences were aligned by using the BLAST algorithm by NCBI. Multiple sequencing alignment (MSA) was performed in the CLC Workbench. The MSA is performed by the muscle algorithm. All of the genomic segments and the similar BLAST results were used in the analysis. The phylogenetic tree generated by MEGA X is based on the neighbor-joining method by using the bootstrapping method for statistical evaluation. The same methodology was used for all of the genomic segments.

3 | RESULTS

3.1 | Patient's demographic, clinical, and laboratory evaluations

Of all cases, 11 (55%) were males. The mean age of the patients was 33.6 years. Enrolled patients had no history of travel to other countries. In terms of clinical symptoms, 65% of patients experienced fever, 50% headache, 35% chills, 30% dry cough, 15% lack of both smell and taste, 50% diarrhea, and 40% vomiting. Comorbidity assessment revealed that diabetes (70%) and hypertension (55%) were the most prevalent conditions (Table 2). All 20 patients were positive for SARS-CoV-2 infection in respiratory samples. Molecular analysis investigation among 20 stool samples revealed that the SARS-CoV-2 genome was found among 10 stool samples. However, only three samples were used for sequencing due to the poor quality of the PCR product and Sanger sequencing reads.

Research Highlights

- There are several reports about gastrointestinal manifestations in COVID-19 patients.
- SARS-CoV-2 RNA was detected in stool samples of COVID-19 patients with gastrointestinal manifestations.
- The polymorphism and phylogenetic analysis were used for follow-up mutation.
- Data showed similarity in polymorphism and phylogenetic analysis of the SARS-CoV-2 isolates with the Wuhan reference sequence.

3.2 | Polymorphism and phylogenetic tree

The Sanger sequencing results for S, nsp12, and nsp2 genes between three positive stool samples were registered in NCBI GenBank (MW626149, MW626150, MW626151, MW626152, MW626153, MW626154, MW626155, MW626156, MW626157). The polymorphism analysis in SARS-CoV-2 showed great similarity among all of the evaluated genes (partial nsp2, nsp12, and S) with the Wuhan reference sequence and all of the current variants of concern (VOCs). The MSA analysis for all three genes is illustrated in Figure 1. The phylogenetic analysis revealed great similarity among all of the SARS-CoV-2-evaluated genomic segments (Figures 2–4).

4 | DISCUSSION

In addition to the respiratory system, SARS-CoV-2 disrupts the function of other body organs such as the heart, liver, kidneys, and gastrointestinal system during infection.[17] This virus can be detected in the body's secretions, fluids, and excretions using molecular techniques. PCR testing of upper respiratory specimens is now the first step in diagnosing acute SARS-CoV-2 infection; however, several interfering factors such as poor sample collection, virus recovery at low viral load, and absence or intermittent shedding contribute to an increase in

TABLE 1 Primers used for S, nsp12, and nsp2 gene amplification

Gene	Forward sequence (5′ to 3′)	Reverse sequence (5′ to 3′)	PCR product Length
S	AGGAAGAGAATCAGCAACTGTGT	CCTGGAGCGATTTGTCTGACT	176
nsp12	CATGTGTGGCGGTTCACTAT	TGCATTAACATTGGCCGTGA	118
nsp2	CCTATTGGGTTCCACGTGCT	GTACAACACGAGCAGCCTCT	372

TABLE 2 Comorbidity, clinical symptoms, and demographic characteristics of COVID-19

Clinical features			No.
Comorbidity	Hypertension	No	9
		Yes	11
	Diabetes	No	6
		Yes	14
	Chronic renal disease	No	19
		Yes	1
	Hypothyroidism	No	17
		Yes	3
	History of allergy	No	19
		Yes	1
Clinical symptoms	Fever	No	7
		Yes	13
	Nausea	No	16
		Yes	4
	Diarrhea	No	10
		Yes	10
	Vomiting	No	12
		Yes	8
	Headache	No	10
		Yes	10
	Myalgia	No	19
		Yes	1
	Cough	No	14
		Yes	6
	Ageusia	No	17
		Yes	3
	Anosmia	No	17
		Yes	3
	Chills	No	13
		Yes	7

false-negative results.[18,19] In most patients, the ability to diagnose SARS-CoV-2 in the respiratory tract disappears 2-3 weeks after the onset of symptoms. SARS-CoV-2, on the other hand, is detectable in the stool of some patients for more than 4 weeks, implying that stool may be an additional source of virus diagnosis.[15] Szymczak et al. compared the results of nasopharyngeal and fecal samples from COVID-19-infected patients and found that when SARS-CoV-2 is undetectable in the upper respiratory tract, stool specimen PCR can be used for virus identification.[20] Based on the foregoing, it is possible to argue that SARS-CoV-2 can proliferate independently in intestinal cells and be excreted in feces. As a result, if COVID-19 is suspected despite a negative pharyngeal PCR test, stool examination can be used to make a diagnosis.

SARS-CoV-2 contains single-stranded RNA, which can be found in the feces of patients with and without gastrointestinal symptoms.[21,22] Moreover, the RNA genome of SARS-CoV-2 was reported in the duodenum and rectum, stomach, and esophagus of patients.[23] During infection, the viral balance and fecal microbiome of the gastrointestinal (GI) tract could be disordered in COVID-19 patients, which could further impact the homeostasis of the gut flora. Detection of live virus and viral RNA in the feces of COVID-19 patients supported potential oral–fecal transmission of COVID-19.[24]

All these reports indicated the possibility of oral–fecal transmission of SARS-CoV-2.[24] In patients with COVID-19, viral protein and nucleocapsid of SARS-CoV-2 were detected in the feces in approximately 50%.[24]

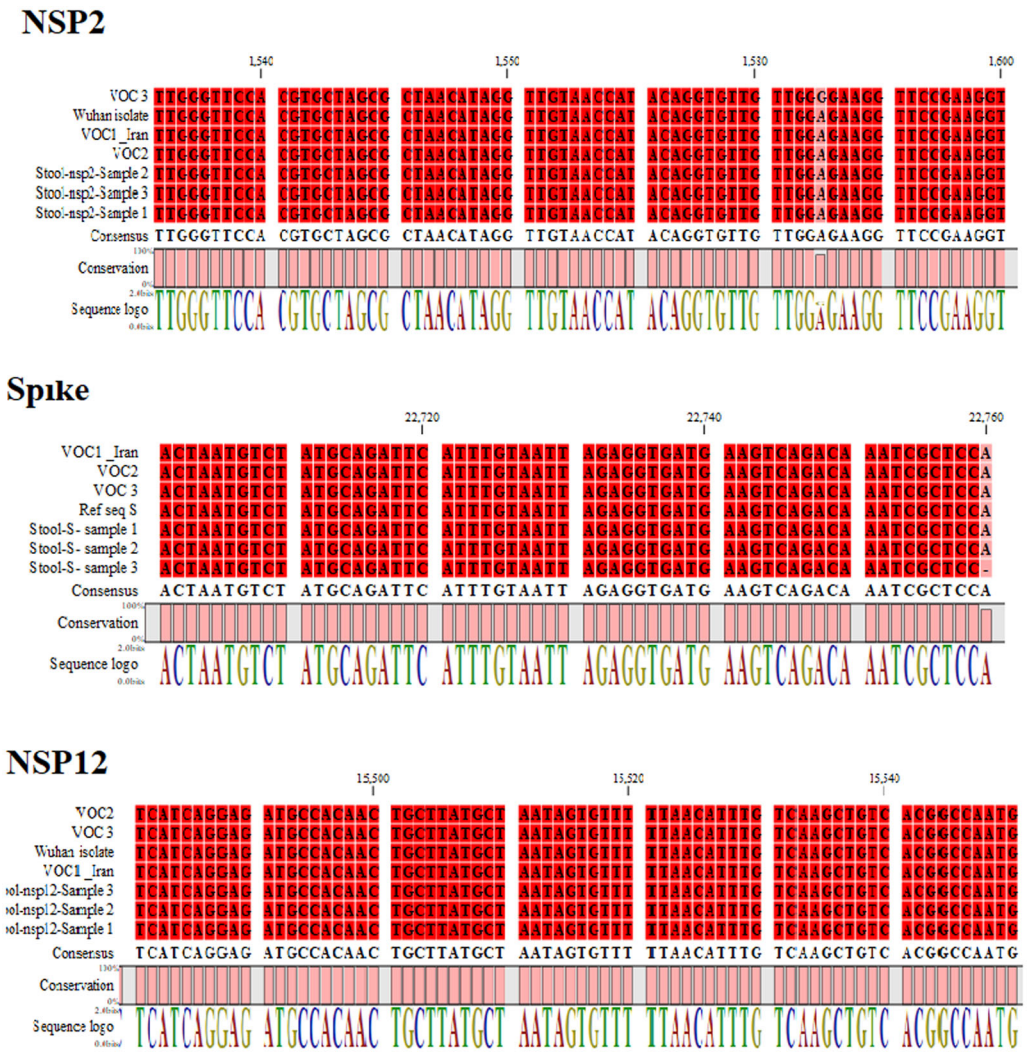

FIG 1 The NSP-2, NSP-12, and Spike multiple sequencing alignment (MSA) analyses in the CLC workbench represent a great similarity among the evaluated samples, variants of concern (VOCs), and reference genome

Different SNVs in genomes have been found simultaneously in samples from COVID-19 patients. SNVs may vary significantly in other countries and different regions over time.[9] In this study, we evaluated RNA SNVs in SARS-CoV-2 isolated from the feces of COVID-19 patients with gastrointestinal symptoms and three viral genes, S, nsp12, and nsp2, in positive samples using nested RT–PCR to expand the diagnostic capability. The PCR testing of stool confirmed COVID-19 in three patients in our study.

We found that the phylogenetic analysis in SARS-CoV-2 showed great similarity among all of the evaluated genes and other strains and VOCs (Figures 1–4).

It has been reported that viral nucleic acid is positive in fecal samples and have potential for fecal–oral transmission.[25] According to Zhang et al., COVID-19 patients can be positive for fecal RNA but negative for pharyngeal PCR.[26] This is in regard to Min Kim et al.'s results, who identified SARS-CoV-2 in the feces of 15 of 74 patients.[27]

The presence or absence of SARS-CoV-2 RNA in the feces of COVID-19 patients could be due to genetic differences as well as differences in the expression of ACE2, the SARS-CoV-2 receptor on the cell surface.[21] Several risk factors associated with increasing severity of COVID-19, including diabetes mellitus (DM), chronic obstructive pulmonary disease (COPD), hypertension, hyperlipidemia, smoking, and old age, are comorbidities for COVID-19. A number of these factors maybe cooperate to determine ACE2 expression.[28] Infected patients who do not have respiratory complications may exhibit other symptoms such as gastrointestinal symptoms or vice versa. This disparity in the occurrence of COVID-19 symptoms could be caused by differences in ACE2 expression or genetic differences between individuals.

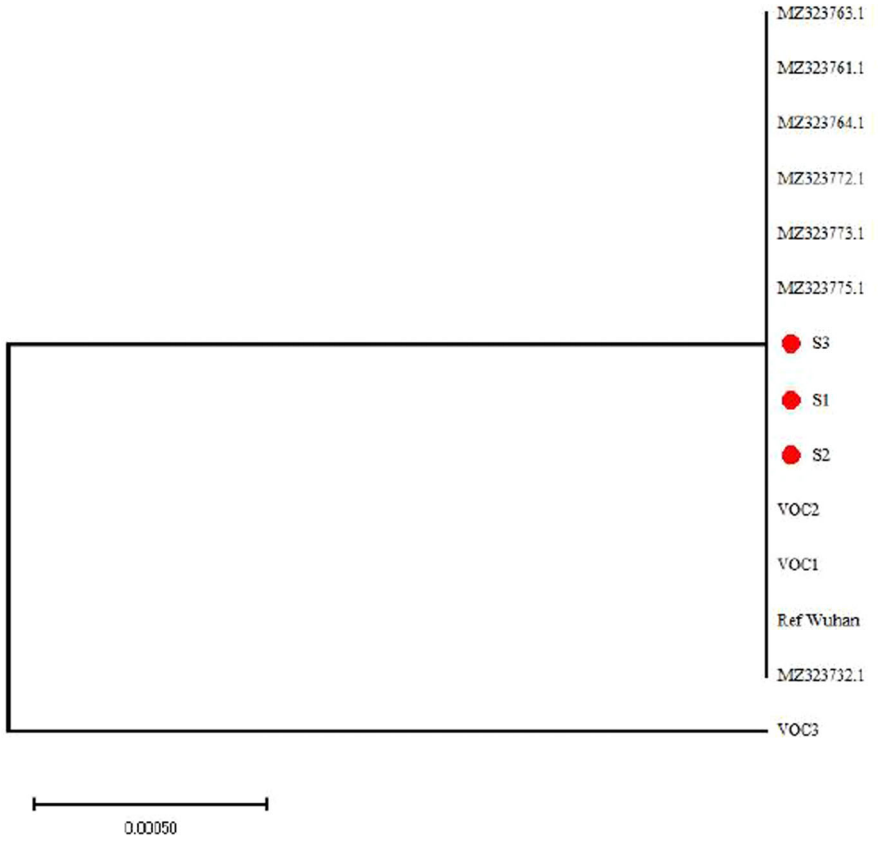

FIG 2 The NSP-2 phylogenetic analysis in MEGA X represents a great similarity among evaluated samples, variants of concern (VOCs) and the reference genome (by the 70 replicates cut off in 100 bootstraps)

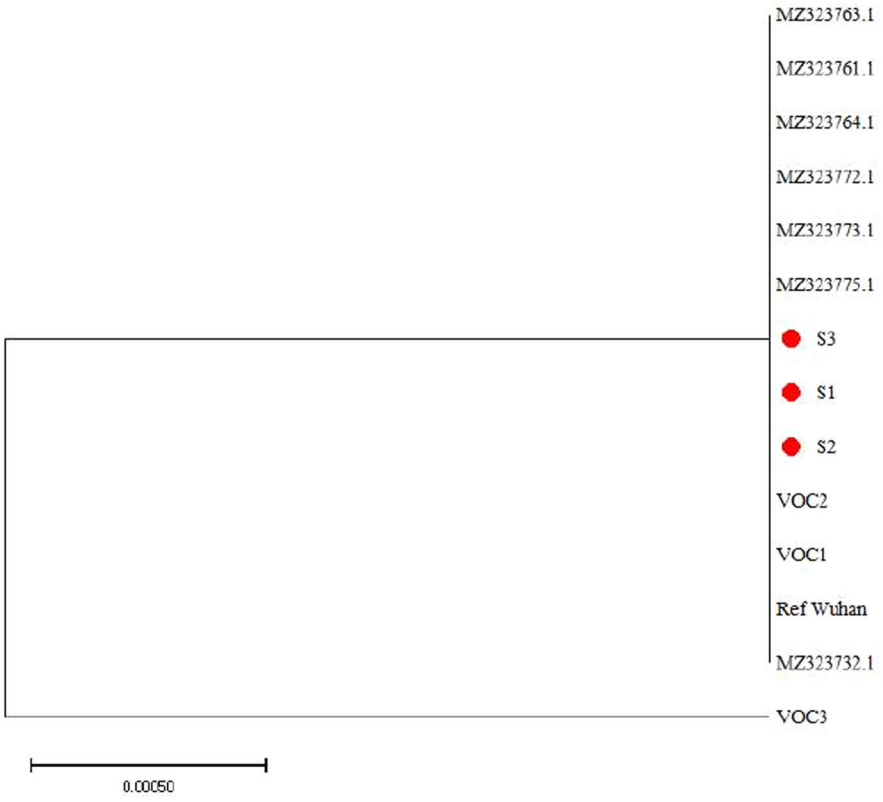

FIG 3 The NSP-12 phylogenetic analysis in MEGA X represents a great similarity among evaluated samples, variants of concern (VOCs), and the reference genome (by the 70 replicates cut off in 100 bootstraps)

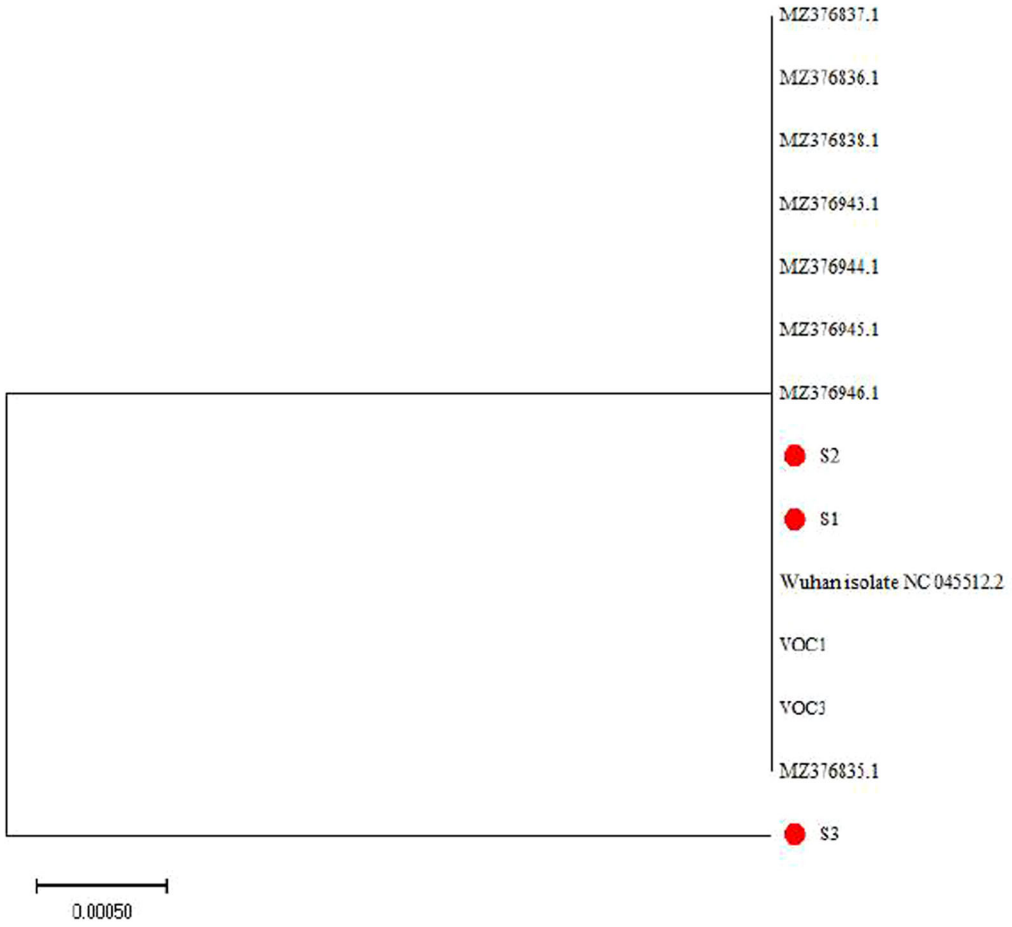

FIG 4 The Spike phylogenetic analysis in MEGA X represents a great similarity among evaluated samples, variants of concern (VOCs), and the reference genome (by the 70 replicates cut off in 100 bootstraps)

Stool PCR may be useful for patients who present late in the disease's course or who have negative upper respiratory PCR results, as well as for children.[22,29,30] Because studies have revealed that only a small percentage of children are positive for upper respiratory PCR and because COVID-19 patients lose viral RNA four weeks after the onset of symptoms, the ability to detect viral RNA through the upper respiratory tract is lost at this point. On the other hand, the long period (126 days)[31] that viral RNA can be recovered from feces can be extremely useful in the diagnosis process, particularly in children. It is important to note that the excreted virus is not infectious in recovering patients, but hand and toilet disinfection is essential in preventing transmission due to the prolonged excretion of SARS-CoV-2 in the feces.[31] SARS-CoV-2 can be transmitted through close contact with mild enteric symptoms and asymptomatic carriers via the fecal–oral route.[24] We recommend using a stool test only to rule out disease when upper respiratory PCR is not possible because not all patients shed viral RNA in the stool, and the detection of viral RNA and other pathogens in fecal and sewage samples has raised concerns about fecal transmission.[32,33]

One of the major limitations of the current study was the small number of sequenced nucleotides in all three genomic segments. The phylogenetic high similarity could be due to the short genomic segments in all three evaluated genes. Furthermore, this region appears to be conserved across all SARS-CoV-2 strains. This conservation may provide excellent targets for diagnostic PCR evaluation.

5 | CONCLUSION

The current study represents a great similarity in polymorphism and phylogenetic analysis of the SARS-CoV-2 isolates with the Wuhan reference sequence and all of the current VOCs in the particular evaluated partial sequences of S, nsp12, and nsp2. In general, examination of body excretions (not just pharyngeal secretions) through genetic-molecular tests for the diagnosis of COVID-19 can reduce various diagnostic limitations and speed up the process of assessing the patient's clinical condition.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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