Sustainability and Biodiversity Conservation

Genetic characterization of hepatitis B virus (S gene) among patients with/without SARS-CoV-2 in Iraq

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Abstract

Hepatitis B virus (HBV) continues to be a global public health problem, especially following the spread of SARS-CoV-2 and the prevalence of hepatic manifestations of COVID-19 in individuals with HBV. The current work aims to investigate HBV genetic sequences by analysis of S genes of selected patients for HBV alone and HBV with SARS-CoV-2. One hundred forty-one hospitalized cases were divided into patients infected with HBV with/ without SARS-CoV-2 diagnosed by automatic fluorescent immunoassay system COVID-19 Ab (IgM/IgG). Next, genetic diagnosis of the S gene for both HBV alone and HBV and SARS-CoV-2 was carried out at ASCO Learning Center in Baghdad using the Nested Polymerase Chain Reaction technology by HBV DNA extraction and amplification to determine the sequence of nucleotide and to compare them with the international strains in the NCBI GenBank. 115 (1, 80, and 34) of the 141 HBV patients showed positive COVID-19 results for IgM, IgG, and IgM with IgG, respectively. 10 out of 22 samples were selected for genetic study of the S gene and were amplified by the nested polymerase reaction technique for genome (S gene), and 9 samples were registered in the genebank with the accession number: LC705440, LC705441, LC705442, LC705443, LC705444, LC705445, LC705446, LC705447, LC705448. According to the results of this research, some of the samples in this study were recorded globally for the first time genetically in patients infected with HBV and SARS-CoV-2. These data suggest that the S gene is still the most critical gene in controlling nucleic acids.

Keywords: COVID-19 Ab, HBV, Nested PCR, SARS-CoV-2, S gene

Introduction

Hepatitis is a worldwide health problem that causes liver damage. Infection was associated with increased cardiovascular mortality (Babiker et al., 2017; Akkaif et al., 2021, 2022, 2023). Hepatitis is triggered mainly by viral infections like HBV and other non-viral illnesses. HBV is a DNA virus that belongs to the Hepadnaviridae family of the *Orthohepadnavirus* genus and is partially double-stranded. Hepatitis B infection can cause both acute and chronic hepatitis. Hepatocellular carcinoma (HCC) and liver cirrhosis resulting from progressive chronic HBV infection are life-threatening viruses worldwide with high fatality rates (Liu et al., 2019). Environmental variables, the virus itself (viral load and virus genotype), and immunological (deficiency of the immune response) ethnic distinctions all play a role in the virus's survival and cause heterogeneity in the pattern and clinical result of HBV infection (Abdul Amir, 2018; Akkaif & Daud, 2022).

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus has had broad effects worldwide and has been a substantial cause of morbidity and mortality. SARS-CoV-2-caused Coronavirus Disease 2019 (COVID-19) has exhibited a variety of clinical manifestations, the majority of which have been pulmonary indications (Kumar et al., 2021). Hepatic manifestations have been reported in up to 50% of infected subjects. The spectrum ranges from asymptomatic anomalies in hepatic biochemical tests to rare acute liver failure cases. The cause of the hepatic manifestations in this stage is unclear. It could be due to different reasons, including a symptom of a systemic disease, ischemic hepatic injury, immune-mediated hepatic injury, drug-induced hepatic injury, or a virus's direct cytopathic effect (Zhang et al., 2020; Xu et al., 2020). Concurrent infections, such as the human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV), are widespread in patients, and the influence of the pandemic and SARS-CoV-2 on these infections and associated liver illnesses is unknown. Furthermore, the consequences for individuals who inject drugs (PWIDs) may be distinct. Therefore, expectations and guidance on difficulties related to numerous viral infections are vital as observations about hepatic symptoms and complications with COVID-19 and the liver continue to evolve (Reddy, 2020; Akkaif et al., 2021, 2020).

Co-infection of hepatitis B patients may be associated with many diseases, including hepatitis C, D, E virus (HCV, HDV, HEV), human immunodeficiency virus (HIV), torque teno virus (TTV), human pestivirus HPgV (GBV-C/HGV), Epstein-Barr Virus (EBV), Cytomegalovirus (CMV),

human herpes virus-1 (HSV-1), adenovirus (ADV), and varicella-zoster virus (VZV), has already been reported. However, such co-infection prevalence, viral interactions, and clinical significance are not fully elucidated (McArdle et al., 2018; Al-Sadeq et al., 2019; Akkaif et al., 2022, 2021; Al-Kaif et al., 2023; Purdy, 2007), particularly in developing countries, including Iraq. This study aims to study the influence of HBV genetic mutations on the sensitivity of SARS-CoV-2 infection by analysis of S genes of selected patients for HBV alone and HBV with SARS-CoV-2.

Material and methods

Study design

In this case-control study, 141 blood specimens were obtained from confirmed HBV patients in the Hepatology and Gastroenterology Teaching Hospital in Baghdad Medical City from March to June 2021. All 141 hospitalized cases were divided into two groups: the first group included patients infected with HBV (non-infected with SARS-CoV-2), whereas the second group included patients with HBV (infected with SARS-CoV-2). Automatic fluorescent immunoassay system (AFIAS, Boditech Med Inc., Korea) COVID-19 Ab (IgM/IgG) tests were used to classify all studied subjects—measured parameters according to the instructions of the manufacturing company.

Ethical approval

The study was conducted following the ethical principles in the Declaration of Helsinki. Before the sample was taken, the patient gave verbal approval. The study protocol and patient consent forms were reviewed and approved by the Babylon and Baghdad Health Directorate and the committee on publication ethics at the College of Medicine, University of Babylon, Iraq, under reference no. BMS 0298 016.

Detection of S gene HBV by PCR (polymerase chain reaction)

Screening of HBV alone and HBV with SARS-CoV-2 genetically by Conventional PCR and Nested PCR, then a sequencing study was performed according to the following steps: HBV DNA extraction from plasma specimens, the achieved procedure according to the method recommended by the manufacturing company (Promega /USA) and Primer preparation for nested PCR, Nuclease-free water was used to dissolve all lyophilized primers, as shown in (Table 1). First, prepare the primer stock tube to add 300 microliters of nuclease-free water to give a final 100 picomole/microliter (stock solution) concentration. Then, I would prepare the working solution from the primer stock tube, according to the instruction provided by the primer

manufacturer (Macrogen, Korea), by adding 10 µl of primer stock solution (stored at freezer -20 C) to 90 µl of nuclease-free water to obtain a working primer solution of 10 pmol/µl.

Table 1. Selected primers for the HBV genome in this study

No.	Primer	sequence (5→3)	Size of amplified product (bp)	Reference
1	FHBS1	5-GAG TCT AGA CTC GTG GTG GAC TTC-3	positions 244 to 267	Ansari et al., 2015
	RHBS1	5-AAA TKG CAC TAG TAA ACT GAG CCA-3	positions 668 to 691	
2	FHBS2	5-CGT GGT GGA CTT CTC TCA ATT TTC-3	positions 255 to 278	
	RHBS2	5-GCC ARG AGA AAC GGR CTG AGG CCC-3	positions 648 to 671	

The amplification conditions for the two rounds of the nested PCR were as follows: initial denaturation at 94°C for 20 s, followed by 30 cycles of amplification at 94°C for 20 s, 56°C for the 20s, and 72°C for 30 s, followed by a final extension step at 72°C for 1 min in a PTC-200 Thermocycler (MJ Research, Watertown, Mass.)

Standard Sequencing

DNA sequencing was used to study the S gene's genetic changes analysis in a local HBV isolate compared with NCBI-GenBank HBV strains. The sequencing of the genes was done after amplification by the Nested PCR method. PCR products were purified from agarose gel using the EZ EZ-10 Spin Column DNA Gel Extraction Kit (Biobasic, Canada) as follows:

1. The specific PCR products were excised from the gel by a clean, sharp scalpel and then transferred into a 1.5 mL microcentrifuge tube.
2. 400µl Binding Buffer II was added to the gel fragment, incubated at 60 C for 10 min, and then shaken until the agarose gel was entirely dissolved.
3. The above mixture was added to the EZ-10 column and allowed to stand for 2 min, centrifuged at 10,000 rpm for 2 min, and discarded the flow-through in the tube.
4. 750µl Wash Solution was added to each tube and centrifuged at 10000 rpm for 1 min. Then, the solution was discarded.
5. Step 4 was repeated and centrifuged at 10000 rpm for another minute to eliminate residual wash Buffer.
6. The column was placed in a 1.5 ml microcentrifuge tube, and 30 µl of Elution Buffer was added to the center of the column and incubated at room temperature for 2 min. Then, the tube

was centrifuged at 10000 rpm for 2 min to elute the PCR product and stored at 20 C.

The purified nested PCR product samples were sent to Macrogen Company in Korea to Sanger sequencing using ABI3730XL, an automated DNA Sequencer. I received the results by email and then analyzed them using a mega (6) software program. The genetic changes, phylogenetic tree analysis, and multiple sequence alignment analysis were performed based on NCBI-Blast Alignment identification (Tamura et al., 2013). The sequences obtained in this study and the products were deposited in GenBank under accession numbers: LC705440, LC705441, LC705442, LC705443, LC705444, LC705445, LC705446, LC705447, and LC705448, along with the following reference strains for HBV of the S gene.

Results

Results of COVID-19 testing for SARS-CoV-2 antibodies among all patients with HBV infection. Only one HBV patient (0.7%) had COVID-19-IgM antibodies that were positive, 80 out of 141 HBV patients (56.7%) had COVID-19-IgG antibodies that were positive, and 34 out of 141 HBV patients (24.1%) had both COVID-19-IgM and IgG antibodies that were positive. In comparison, only 26 out of 141 (18.4%) of HBV-infected patients' sera were found to be negative for both COVID-19-IgM and IgG antibodies (HBV alone). Screening of HBV alone and HBV with SARS-CoV-2 genetically by conventional and Nested PCR, then a sequencing in this study . In this study, the S gene was investigated by PCR as a specific diagnostic tool for identifying HBV in plasma specimens by 22 subjects, divided into 12 (2,3,4,5,7,8,9,15, 16,17,20,21) subjects with HBV and SARS-CoV-2 while 10 (1,6,10,11,12,13, 14,18,19,22) subjects with HBV alone as a positive control. The PCR amplification product size for detecting the S gene was 447 bp for samples 2 and 16 with HBV&SARS-CoV-2. According to the first run, the sizes were detected using 100 bp of DNA Marker, as shown in Figure (1).

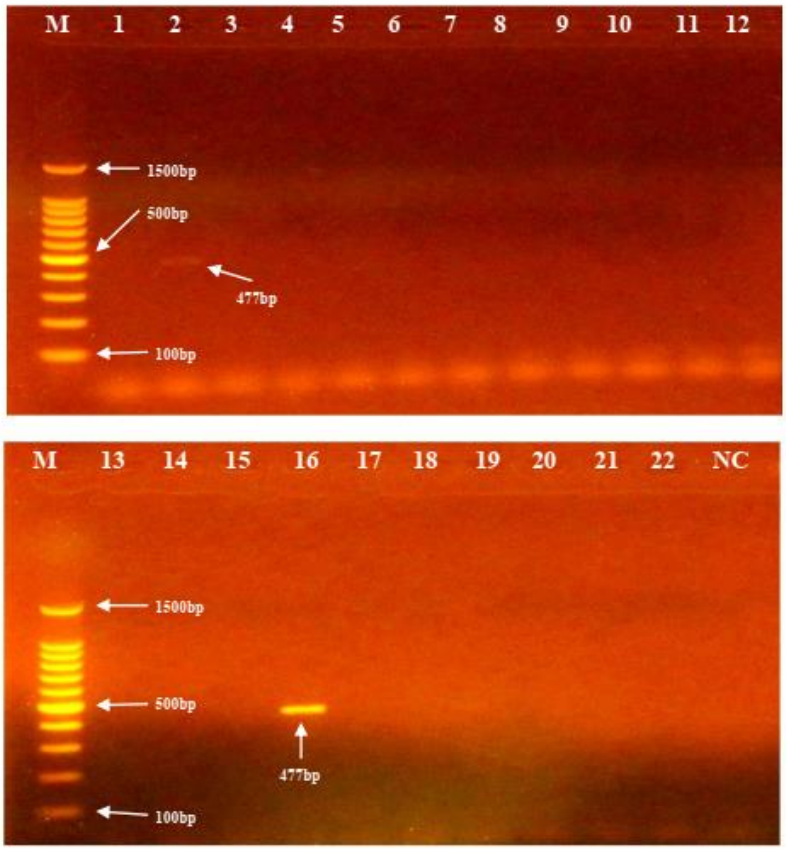


Figure 1. The amplification of the Hepatitis B Virus gene fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100-1500bp ladder marker. (2, 16) with positive results for the S gene, (1,3,4,5,6,7,8,9,10,11,12,13,14,15,17,18,19,20,21,22) with negative results for the S gene. On size 447 bp

After nested PCR, the amplification product size for detecting the S gene was 416 bp for sample numbers (2,3,4,5,16,17,20) with HBV&SARS-CoV-2 while sampling numbers (13,14,19) with HBV alone. According to the second run, the sizes were detected using 100 bp of DNA Marker. As shown in Figure (2).

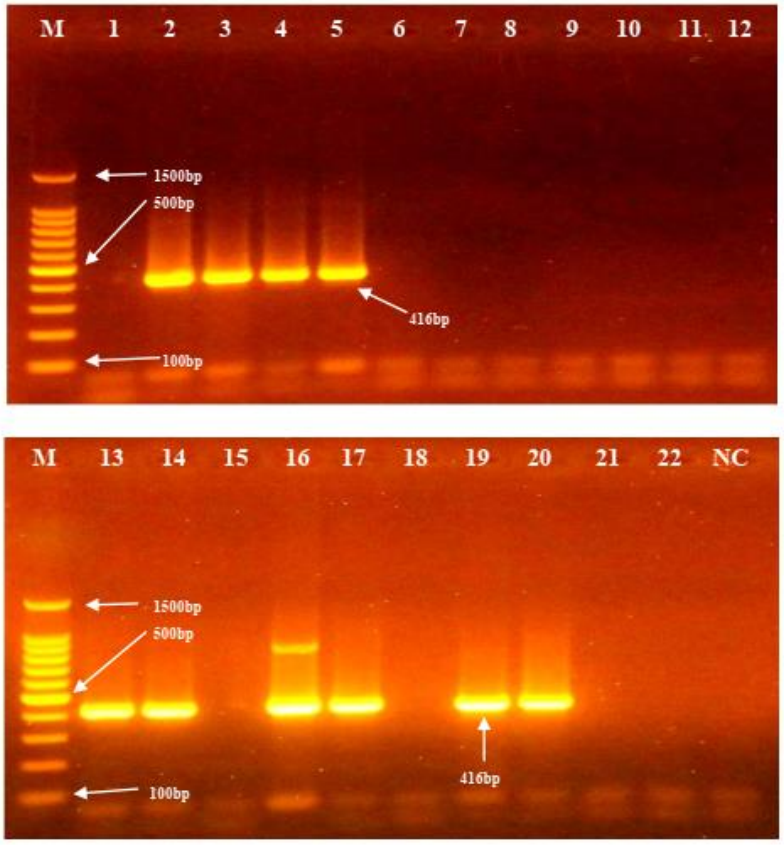


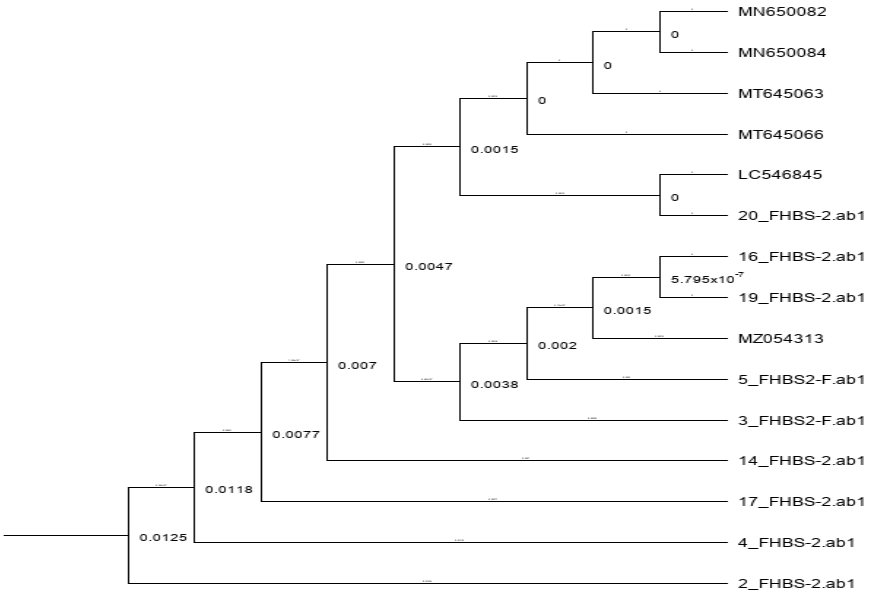
Figure 2. The amplification of the Hepatitis B Virus gene after nested PCR fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100-1500bp ladder marker. (2,3,4,5,13,14,16,17,19,20) with positive results for the S gene (1,6,7,8,9,10,11, 12, 15,18,21,22) and negative results for the S gene. On size 416 bp

To learn the sequences of nitrogen bases of the output of the nested PCR reaction for the S gene, 50ml of the output of nested PCR for each sample (with primers of the S gene) was sent to Macrogen South Korea. After obtaining the results, they were compared directly with the sequences of registered global strains from different parts of the world using a mega (6) software program. The results were compared with the original sequence of each gene. The results of the current study showed the sequence of nested PCR products for ten subjects suffering from HBV (Table: 2), where the results of the three samples (13, 14, 19) with HBV alone were 1 (rubbish), 1 (100%), and 1 (99.08%), respectively, while the results of HBV and SARS-CoV-2 for seven samples (2,3,4,5,16,17,20), so that 3 (5,16,20) conformity were 100% and 4 (2,3,4,17) conformity were 98.78%, 99.43%, 99.47%, 99.39%, respectively.

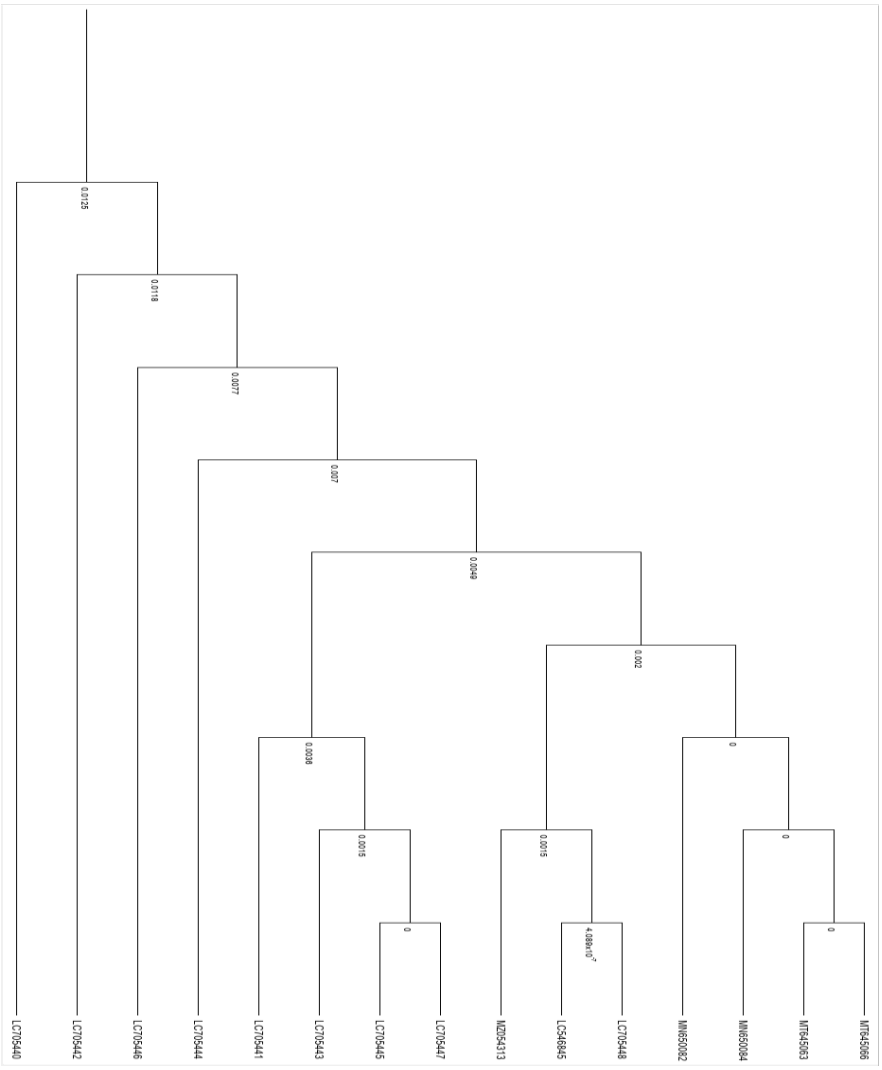
Table 2. Per. Identities and Accession of Sanger sequencing results for patients infected with HBV alone or HBV and SARS-COV-2.

Subject	No. specimen	Description	Per. Identities	Accession	Length	Frequency
HBV alone	13	Rubbish				
	14	Hepatitis B virus Al-Kaif,etal2022e genes for polymerase, S protein, partial cds	99.08%	LC705444.1	327	3
		HBV strain s 19 reverse transcriptase gene, partial cds		MK213873.1	1098	
	19	Hepatitis B virus Al-Kaif,etal2022h genes for polymerase, S protein, partial cds	100%	LC705447.1	327	-
		HBV ADS8374 S and P gene for S protein and polymerase, partial cds		LC546844.1	-	
HBV and SARS-CoV-2	2	Hepatitis B virus Al-Kaif,etal2022a genes for polymerase, S protein, partial cds	98.78%	LC705440.1	327	4
		HBV isolate 178 S protein (S) gene, complete cds		HM348691.1	681	
	3	Hepatitis B virus Al-Kaif,etal2022b genes for polymerase, S protein, partial cds	99.43%	LC705441.1	327	2
		HBV isolate Bushehr-DM-HBS64 (S) protein, partial cds		MF419216.1	387	
	4	Hepatitis B virus Al-Kaif,etal2022c genes for polymerase, S protein, partial cds	98.47%	LC705442.1	327	5
		HBV isolate GSK1901, complete cds		KY629632.1	3215	
	5	Hepatitis B virus Al-Kaif,etal2022d genes for polymerase, S protein, partial cds	100%	LC705443.1	327	-
		HBV isolate NOR560 polymerase (P) gene, partial cds		MK173170.1	-	
	16	Hepatitis B virus Al-Kaif,etal2022f genes for polymerase, S protein, partial cds	100%	LC705445.1	327	-
		HBV ADS8374 S and P gene for S protein and polymerase, partial cds		LC546844.1	-	
	17	Hepatitis B virus Al-Kaif,etal2022g genes for polymerase, S protein, partial cds	99.39%	LC705446.1	327	2
		HBV isolate NOR112 polymerase (P) gene, partial cds		MK173200.1	740	
	20	Hepatitis B virus Al-Kaif,etal2022i genes for polymerase, S protein, partial cds	100%	LC705448.1	327	-
		HBV ADS8376 S and P gene for S protein and polymerase, partial cds		LC546845.1	-	

In the phylogenetic analysis with nine strains retrieved from the Gen Bank, nine sequences were clustered into genotype C (Fig. 3).



A. Before samples in NCBI were confirmed



B. After samples in NCBI were confirmed

Figure 3. Phylogenetic Trees Based on a specific HBV-DNA nucleotide fragment (S gene) for viral isolates obtained from the patients infected with HBV alone or HBV and SARS_CoV-2

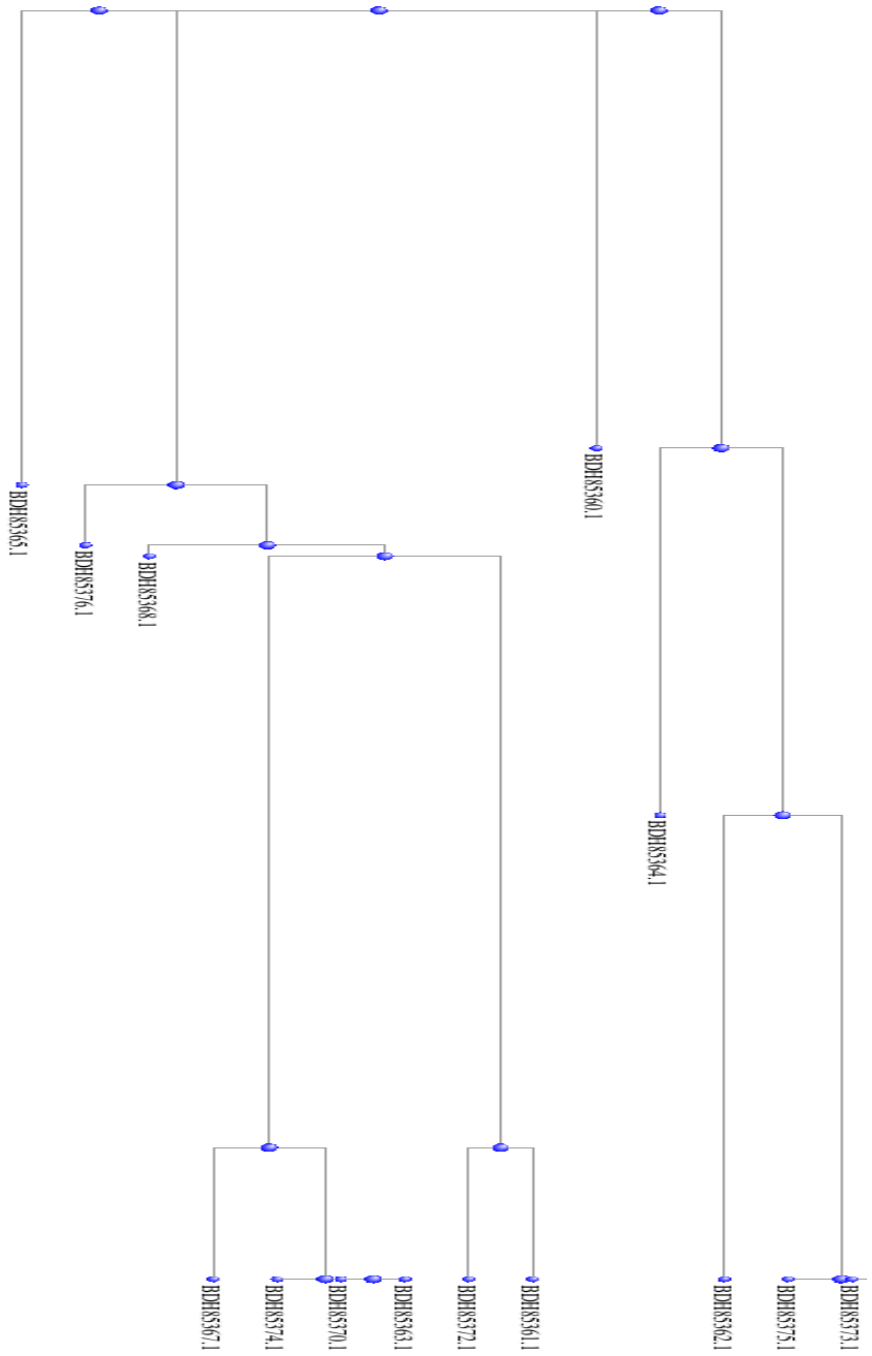


Figure 4. Phylogenetic Trees Based on an S protein for viral isolates obtained from the patients infected with HBV alone or HBV and SARS_CoV-2

Genomic sequencing has been used extensively to develop new diagnostic tests to identify pathogens, especially HBV. Moreover, genome sequencing helps identify new targets for diagnosis.

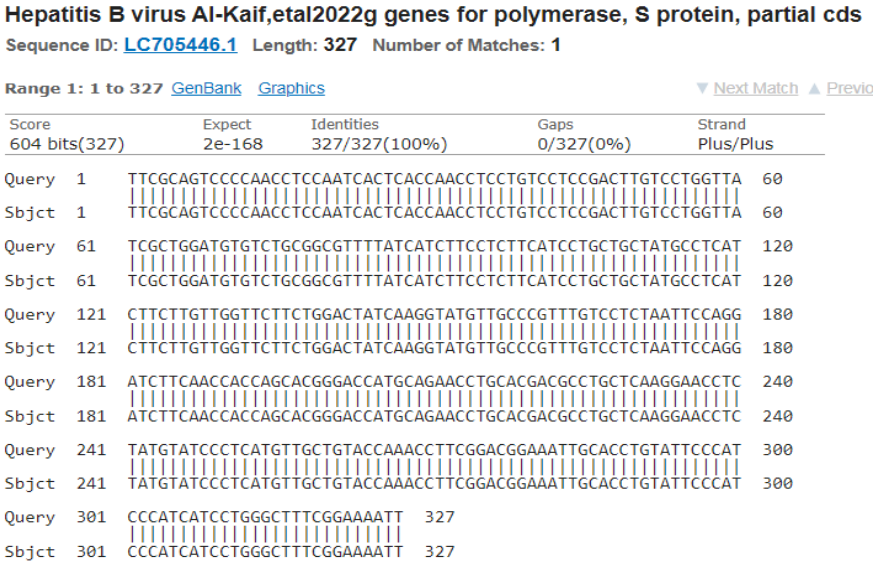
Therefore, the purpose of conducting the sequences in our study is to find the correlation of the isolates with the previous studies conducted in the countries of the world to show the extent of similarity and difference. In addition, knowing the specifications genetically will benefit us in the future, especially the Ministry of Health, by following the protocol through case management, treatment (follow-up), and vaccines. For example, suppose there is an affinity between genetically isolated individuals with hepatitis B virus in Iraq and a country such as China, Japan, Australia, America, England, or any other country. In that case, the Ministry of Health is recommended to follow the protocol used in the country's genetically related. Thus, it was a perfect program when examining the results of the current study, which was compared with other strains proven in the gene bank through the use of NCBI-BLAST-query nucleotide online. It gave the exact results of identifying percentages with other world strains, ranging from 98.47-100% for the HBV S gene.

Nine sequences of HBV alone and HBV with SARS-CoV-2 were identified from plasma human resources in Iraqi cities, and each sequence has a symbol code (No 2: Al-Kaif et al., 2022a; No 3: Al-Kaif et al., 2022b; No 4: Al-Kaif et al., 2022c; No 5: Al-Kaif et al., 2022d; No 14: Al-Kaif et al., 2022e; No 16: Al-Kaif et al., 2022f; No 17: Al-Kaif et al., 2022g; No 19: Al-Kaif et al., 2022h; No 20: Al-Kaif et al., 2022i).

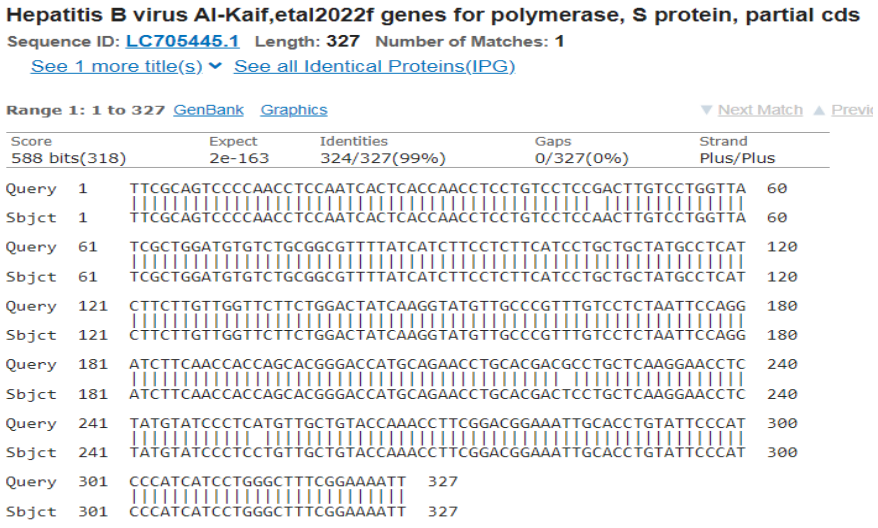
The S gene sequences were submitted to Genbank-bank under submission code No 14: Al-Kaif et al. 2022e and No 19: Al-Kaif et al. 2022 h for infected patients with HBV alone. These sequences' results were analyzed and examined by professional staff in the gene bank. As a result, all these sequences are accepted in the gene bank, and each sequence takes an accession number: LC705444.1 and LC705447.1, respectively. Regarding S protein for it as BDH85373.1and

The S gene sequences were submitted to Genbank-bank under submission code (No 2: Al-Kaif et al., 2022a; No 3: Al-Kaif et al., 2022b; No 4: Al-Kaif et al., 2022c; No 5: Al-Kaif et al., 2022d; No 14: Al-Kaif et al., 2022e; No 16: Al-Kaif et al., 2022f; No 17: Al-Kaif et al., 2022g; No 19: Al-Kaif et al., 2022h; No 20: Al-Kaif et al., 2022i) for infected patients with HBV with SARS-CoV-2. These sequences' results were analyzed and examined by professional staff in the gene bank. As a result, all these sequences are accepted in the gene bank, and each sequence takes an accession number: LC705440.1, LC705441.1, LC705442.1, LC705443.1, LC705445.1, LC705446.1, and LC705448.1, respectively.

Through the gene bank that confirmed the isolates of the current study, some isolates were recorded for the first time in the world. Also, the present study found a similarity in the type of genetically recorded isolate for the first time, LC705446.1(100%), with isolate LC705445.1(99.08%) from the same isolates of the current study for a patient infected with HBV and SARS-CoV-2 as shown in (Figure 5) compared to Norway isolate was MK173200.1 (99.39%).



(A)



(B)

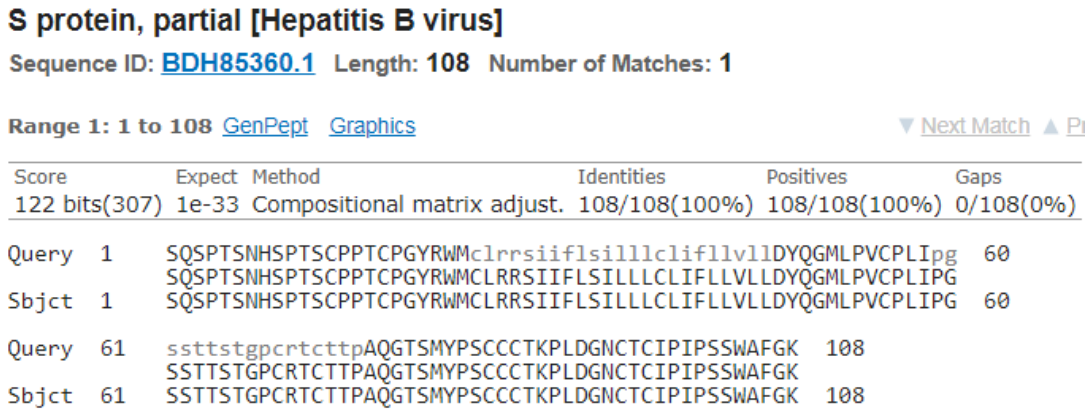
Figure 5. Local Basic Alignment of HBV S gene isolates No.16 (B) and 17(A) with similarity NCBI-BLAST HBV strain S gene isolate LC705445.1 for a patient infected with HBV and SARS-CoV-2

In our study also at the level of polymerase and S protein for the study isolates according to NCBI-BLAST identical protein, which showed a correlated level as Figure 4, and the table below:

Table 2. Accession of Sanger sequencing results for polymerase and S protein according to patients infected with HBV alone or HBV and SARS-COV-2.

Subject	No. specimen	Accession of Polymerase (109 aa)	Accession of S protein (108 aa)
HBV alone	13	Rubbish	
	14	BDH85368.1	-
	19	BDH85374.1	BDH85373.1
HBV and SARS-CoV-2	2	BDH85361.1	BDH85360.1
	3	BDH85363.1	BDH85362.1
	4	BDH85365.1	BDH85364.1
	5	BDH85367.1	BDH85366.1
	16	BDH85370.1	BDH85369.1
	17	BDH85372.1	BDH85371.1
	20	BDH85376.1	BDH85375.1

The gene bank confirmed the isolates of the current study according to (Table 4) and found a similarity in the type of genetically recorded isolate for the first time for S protein, BDH85360.1 (100%), compared to isolate BDH85366.1 (97.22%) from a patient infected with HBV and SARS-CoV-2 for the same isolates of the current study, as shown in (Figure 6).



S protein, partial [Hepatitis B virus]

Sequence ID: BDH85360.1 **Length:** 108 **Number of Matches:** 1

Range 1: 1 to 108 GenPept Graphics ▼ Next Match ▲ Pr

Score	Expect	Method	Identities	Positives	Gaps
122 bits(307)	1e-33	Compositional matrix adjust.	108/108(100%)	108/108(100%)	0/108(0%)

```
Query  1    SQSPTSNHSPTSCPPTCPGYRWMclrrsiiflsilllclifllvllDYQGMLPVCPLIpg  60
            SQSPTSNHSPTSCPPTCPGYRWMCLRRSIIFLSILLLCLIFLLVLLDYQGMLPVCPLIPG
Sbjct  1    SQSPTSNHSPTSCPPTCPGYRWMCLRRSIIFLSILLLCLIFLLVLLDYQGMLPVCPLIPG  60

Query  61   ssttstgpcrtcttpAQGTSMYPSCCCTKPLDGNCTCIPIPSSWAFGK  108
            SSTTSTGPCRTCTTPAQGTSMYPSCCCTKPLDGNCTCIPIPSSWAFGK
Sbjct  61   SSTTSTGPCRTCTTPAQGTSMYPSCCCTKPLDGNCTCIPIPSSWAFGK  108
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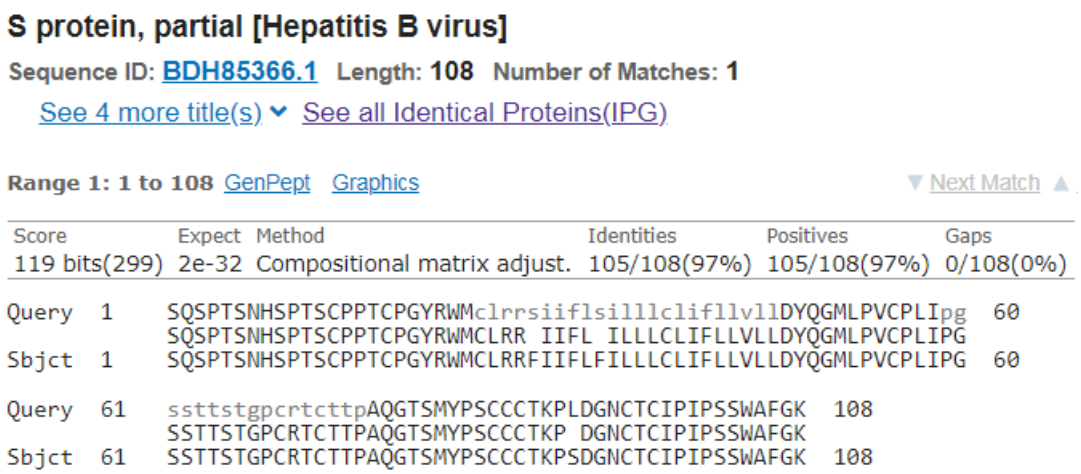

Figure 6. Local Basic Alignment of HBV isolates No.2, and 5 with similarity NCBI-blast (protein-protein BLAST) S protein isolate BDH85360.1 for a patient infected with HBV and SARS-CoV-2

However, these procedures also apply selective pressures on HBV in infected individuals, generating and accumulating mutations in the S gene. Most of these mutations occur in the S gene's major hydrophilic region (MHR). These mutations create public health concerns as they can be responsible for the reactivation of hepatitis B (Purdy, 2007), therefore, variations or mutations may result from SARS-CoV-2 infection for patients with HBV, whether at the nucleotide or protein level.

Conclusion

Some of the samples in this study were genetically recorded globally for the first time in patients infected with HBV and SARS-CoV-2. These data suggest that the S gene is still the most critical gene to control nucleic acids.

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